Molecular Regulation of the Cardiac-enriched Acetyl-CoA Carboxylase Isoform (ACCβ): A Novel Target for Therapeutic Interventions in Cardiovascular Disease

Siyanda S.S. Makaula

Submitted for the Degree of Doctor of Philosophy in Medicine

Hatter Institute for Cardiology Research
Faculty of Health Sciences
University of Cape Town
2006

Supervisor: Dr M. Faadiel Essop

ACKNOWLEDGEMENTS

This study was carried in the Hatter Institute for Cardiology Research of the Department of Medicine of the University of Cape Town. To my supervisor, my mentor Dr M. Faadiel Essop I wish to extend my sincere gratitude for your scientific guidance, intellectual input and encouragement. Furthermore, I thank you for initiating the 'metabolic switch', it has contributed tremendously to my scientific advancement and broader understanding of cardiac metabolism.

Professor L.H. Opie, a living cell has a plasma membrane that holds its intracellular contents together and allows for biochemical reactions to occur, it is however, the permeability of such a membrane that allows extracellular particles to diffuse through. The Hatter Institute is synonymous to such a living cell, I thank you for allowing me to 'diffuse-through' so as to advance my scientific career. To Sylvia Dennis, I thank you for distilled administration of my financial matters, scholarships and laboratory as a whole, keep it up. My studies were funded by the Medical Research Council (SA), the Guy Elliot Scholarship (SA) and the National Research Foundation (SA).

I also wish to thank the following science leaders for their generous contributions; Dr. K-S Kim, Yonsei University College of Medicine, Seoul, Korea (human ACCβ luciferase-promoter constructs), Dr. Tetsuya Kamataki, Hokkaido University, Japan (USF1-pUC-SRα); Dr. W. Wahli, University of Lausanne, Switzerland (pSG5rPPARα), Dr. Dan Kelly Washington University School of Medicine, MO (NRF1-pSG5 and MCAD cDNA probe).

To Tasneem Adam, words cannot explain my appreciation. In words, however, I wish to thank you for your technical expertise accompanied by passion and in-depth knowledge of molecular biology and cell culture. To Dr. K. Ngumbela, for she tolerantly tutored me through Northern and Western blotting techniques. I also wish to extend my gratitude to Noel Markgraaf and Jaap Visser for their technical involvement and assistance. To Mr Victor Claasen, for being there when needed most, technical assistance and for always reminding me to look at the 'fun-side' of research. To the tripartite buffer alliance: Seeyah!!, Naushaad 'Mr STAT3' Suleman and Peter Owira we had fun making buffer solutions and doing Westerns, all the best guys. To the metabolic group, transcriptionally driven by Dr M. Faadiel Essop, Dr Rebecca Felix, Dr Deon Pretorius, Joy McCarthy, Makhosi Zungu, Anna Chan and Tasneem Adam. I hope you undergo mitochondrial biogenesis for sustainable growth and advancement of cardiac metabolism and science in general. To the rest of the Mad Hatters, Dr Sandrine Lecour, Dr Graeme Deuchar, Lydia Lacerda, Sarin Somers and Patricia van der Walt, I say, "Once a Mad Hatter, always a Mad Hatter", keep up the good work.

Andiswa Nkonsto, for love and support throughout my studies, I thank you. My pals, Nceba Hene and Sakhumzi Ntayiya I thank you for your friendly advice, support and encouragement, socially and academically.

To my family = my parents: Prince ZS Makaula and Princess OL Makaula, when a child is born, you wish you had a crystal ball so you can see into their future, however, it is the absence of such a device that gives you (as a parent) strength to guide a child towards a brighter future, it is also up to a child to follow the correct path. You have achieved your goal, you patiently guided and encouraged me throughout my studies and today I see the light at the end of the tunnel, you have been an inspiration in so many different ways, I love you. To my brothers: the late Xola, Bagcine and Bonke, as my preferred musician (Andy Magazz) perfectly penned it "wherever you are, just call on me", together we stand, fall and stand tall. To my sons, Uzile and Qhawe, UQ can grow up to excel in your branches of learning, like father like son. Daddy will always be there for you, love you, boys!!

"Yesterday I was digging for gold and today I am wearing it"
Alive with possibility

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACS	acyl-CoA synthase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate protein kinase
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
AS	ATP synthase
ATP	adenosine triphosphate
AU	arbitrary units
β-GPA	β-guanadinopropionic acid
cDNA	complimentary DNA
ChoRE	carbohydrate response elements (ChoRE)
ChREBP	carbohydrate response element binding protein
CPT	carnitine palmitoyl transferase
COX	cytochrome c oxidase
CS	citrate synthase
DCA	dichloroacetate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
E-box	consensus sequence motif, CANNTG
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol-bis(2-amino-ethylether)-tetra-acetic acid
EMEM	Earle's Base Minimum Eagle's Medium
FA	fatty acids
FABP	fatty acid binding protein
FAO	fatty acid oxidation
FATP	fatty acid transport protein
FAT/CD36	fatty acid translocator
FAS	fatty acid synthase
FFA	free fatty acids
F-6-P	fructose-6-phosphate
GFAT	UDP-GlcNAc glutamine/F-6-P amidotransferase
GLUT	glucose transporter
G-6-P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
g	gram
HBP	hexosamine biosynthetic pathway
HEPES	N-2-Hydroxyethylpiperazine N'-2-ethanesulfonic acid
HK	hexokinase
HNF	hepatocyte nuclear factor
KCl	potassium chloride
LCAD	long-chain fatty acyl-CoA dehydrogenase
LPK	liver type pyruvate kinase
MCAD	medium chain acyl-CoA dehydrogenase
MCD	malonyl-CoA decarboxylase
MEF-2	myocyte enhancer factor 2

$MgSO_4$	magnesium sulphate
MyoD	muscle regulatory factor
mRNA	messenger RNA
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium dihydrogen orthophosphate
NEFA	nonesterified fatty acids
NRF	nuclear respiratory factor
PBS	phosphate buffered saline
PDC	pyruvate dehydrogenase complex
PDK	pyruvate dehydrogenase kinase
PDH	pyruvate dehydrogenase
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PGC	peroxisome proliferator-activated gamma coactivator
PKA	protein kinase A
PLB	Passive Lysis Buffer
PMSF	phenylmethylsulphonylfluride
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PPP	pentose phosphate pathway
PPRE	peroxisome proliferator response elements
pGL3-Basic	empty vector
pRL CMV	Renilla
pPIIβ-1317	1,317 bp human ACCβ promoter II –reporter luciferase construct
RHC	re-fed carbohydrate-enriched diet (following 48 hour fast)
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
R-5-P	ribulose 5-phosphate
rRNA	ribosomal RNA
SCAD	short-chain acyl-CoA dehydrogenase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Sp	stimulatory protein
SREBP	sterol regulatory element binding protein
sFCS	charcoal-stripped fetal calf serum
TFAM	mitochondrial transcription factor A
UCP	uncoupling protein
UDP-GlNAc	UDP-*N*-acetylglucosamine
USF	upstream stimulatory factor
X-5-P	xylulose-5-phosphate
x g	centrifugal force
2-DG	2-deoxyglucose

TABLE OF CONTENTS

Chapter 2

The human gene promoter of the cardiac isoform of acetyl-CoA carboxylase (ACCβ) is transactivated by upstream stimulatory factor1 (USF1) in a glucose-dependent manner

Chapter 3

Nuclear Respiratory Factor 1 (NRF1) inhibits transactivation of the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase (ACCβ)

Abstract

Metabolic remodeling is thought to be an important contributor towards the development of various cardiac pathophysiologic conditions. Therefore, studies attempting to delineate underlying mechanisms driving cardiac metabolic remodeling represent an important initiative toward the development of novel therapeutic interventions. To further investigate the role of metabolic substrate switches in the heart, we focused on a pivotal, rate-limiting step of cardiac fatty acid metabolism i.e. an upstream modulator of long-chain fatty acid importation into the mitochondrion. In the heart, long-chain fatty acids are transported into the mitochondrion by the rate-limiting enzyme, carnitine palmitoyl transferase 1 (CPT1). CPT1 is potently inhibited by malonyl-CoA, the product of the acetyl-CoA carboxylation reaction that is catalyzed by acetyl-CoA carboxylase (ACC).

Recent studies have demonstrated that metabolic fuels such as fatty acids and glucose can function as signaling ligands, directing transcriptional regulation of numerous metabolic genes. However, transcriptional mechanisms directing the gene expression of the cardiac isoform of acetyl-CoA carboxylase (ACCβ) are less well understood. Previously, four E-box (CANNTG) sequence motifs were identified on the human ACCβ promoter. Since E-boxes act as binding sites for upstream stimulatory factors (USF), putative glucose-responsive transcriptional modulators, we hypothesized that ACCβ is induced by USF1 in a glucose-dependent manner.

To investigate this, we began by acutely fasting and subsequently refeeding Balb/C mice with a carbohydrate-enriched diet. Here, high carbohydrate feeding resulted in elevated systemic glucose levels associated with increased cardiac ACCβ gene and protein expression. To further explore these interesting findings, we transiently cotransfected neonatal cardiomyocytes, H9C2 myoblasts, CV-1 fibroblasts and HepG2 hepatocytes with the full-length and deletion constructs of the human ACCβ gene promoter together with a putative activator and repressor

expression vector, respectively: a) USF1 (glucose-responsive transcription factor) – the rationale that it should elevate ACCβ gene promoter activity in accordance with the glucose-fatty acid cycle, and b) nuclear respiratory factor 1 (NRF1) – the hypothesis being that this mitochondrial biogenesis and β-oxidation enhancing modulator would be expected to attenuate ACCβ promoter activity in order to increase fatty acid oxidation capacity.

To assess whether USF1 plays a role in ACCβ gene induction in response to increased glucose supply, we performed cotransfection studies together with a USF1 expression vector under low or high glucose exposure. We found that USF1 overexpression markedly elevates ACCβ promoter activity in neonatal cardiomyocytes and CV-1 fibroblasts under low glucose culturing conditions. Moreover, high glucose levels significantly increased USF1-mediated ACCβ promoter activation compared to the low glucose exposure in CV-1 fibroblasts. These data suggest that glucose-responsive USF1 transactivation of the human ACCβ gene promoter occurs in a cell-type specific manner i.e. unlike the CV-1 fibroblasts the USF1 response in neonatal cardiac myocytes appears to be glucose-independent. We next performed transfection assays in the presence of 2-deoxyglucose, a glucose analog that is taken up into the cell and not further metabolized. In agreement with our earlier findings, 2-deoxyglucose did not inhibit the USF1-mediated transactivation of the human ACCβ gene promoter activity in neonatal cardiomyocytes. In contrast, 2-deoxyglucose administration robustly inhibited USF1-mediated human ACCβ promoter activity in CV-1 fibroblasts. We next tested whether phosphatase 2A (PP2A), a downstream target of the pentose phosphate metabolite xylulose-5-phosphate, plays a role in the USF1-mediated transcriptional activation of human ACCβ promoter. Here, PP2A inhibition attenuated USF1-mediated transactivation of the human ACCβ gene promoter in CV-1 fibroblasts. Surprisingly, PP2A inhibition also reduced USF1-mediated transactivation of the human ACCβ gene promoter in neonatal cardiomyocytes. Furthermore, endogenous USF1 transcriptional activity was also reduced following exposure to okadaic acid.

Since four E-box sequence elements were previously identified on the human ACCβ gene promoter, we next performed cotransfection studies with a USF1 overexpression vector to determine sequence elements responsible for the USF1-mediated transactivation of the ACCβ gene promoter. Employing deletion constructs, we found that the shortest ACCβ promoter deletion construct was able to induce ACCβ promoter activity in both cell lines. However, the induction was reduced compared to the full-length construct indicating that the observed USF1 induction may be mediated via E-box 4 interacting with other upstream regions of the promoter predominantly through E-box 4 located close to the transcription start site. In summary, these data show that glucose-mediated USF1 induction of the human ACCβ gene promoter occurs in a cell-specific manner. In CV-1 fibroblasts, USF1 transactivates the human ACCβ promoter in a glucose-dependent manner, in part via dephosphorylation of USF1 by PP2A. On the other hand, USF1-mediated induction of ACCβ also occurs through dephosphorylation of USF1 by PP2A, although unlike CV-1 fibroblasts, glucose metabolites do not appear to mediate this process.

We also found that NRF1 overexpression markedly attenuated ACCβ promoter activity in neonatal cardiomyocytes and cardiac-derived H9C2 myoblasts. However, NRF1 overexpression induced ACCβ promoter activity in both CV-1 fibroblasts and HepG2 hepatocytes. Interestingly, we found that USF1-mediated induction of ACCβ promoter activity was markedly attenuated in all cell lines tested. We therefore propose that NRF1 may interfere with USF1 binding of the ACCβ gene promoter binding, probably via E-box4 located close to the transcription start site. In summary, we suggest that under energy sparing conditions, the transcriptional modulator NRF1 not only induces genes required for mitochondrial biogenesis, but can also negatively regulate ACCβ gene expression. This in turn should result in reduced malonyl-CoA levels and elevated mitochondrial fatty acid oxidation

In conclusion, we have identified a novel transactivator (USF1) and repressor (NRF1) of the human ACCβ gene promoter in the heart. Our data may eventually result in the development of novel therapeutic interventions since we have identified unique modulators regulating ACCβ gene transcription, and by implication malonyl-CoA levels and mitochondrial fatty acid β-oxidation.

Chapter 1

Introduction

1.1. Metabolism and the heart

1.1.1. Glucose metabolism

The normal, adult mammalian heart relies mainly on fatty acids (FA) as a fuel substrate. However, glucose also serves as an important fuel substrate, for e.g. when cells are placed under stress conditions such as hypoxia or ischemia. Blood glucose is taken up by via glucose transporters situated on the plasma membrane of the cell. There are several glucose transporter isoforms and their expression is dependent on developmental stage and tissue type.[1-3] The fetal isoform is glucose transporter 1 (GLUT1) and the two well described adult isoforms, GLUT2 and GLUT4, are predominantly expressed in the liver and heart, respectively.[1-4] In the heart and skeletal muscle, insulin stimulates translocation of GLUT4 from intracellular vesicles to the plasma membrane in order to facilitate transportation of glucose into the cell[2, 3, 5] (Figure 1).

Inside the cell, glucose is rapidly converted into glucose-6-phosphate (G-6-P) by hexokinase, a rate-limiting and energy requiring step of glucose metabolism. At this stage, the fate of G-6-P is either glycolysis, the pentose phosphate pathway (PPP) or the hexosamine biosynthetic pathway (HBP), depending on a variety of conditions.[6, 7] Under normal circumstances, G-6-P undergoes glycolysis where it is converted into fructose-6-phosphate (F-6-P), which is then converted into fructose-1,6-bisphosphate by phosphofructokinase (PFK).

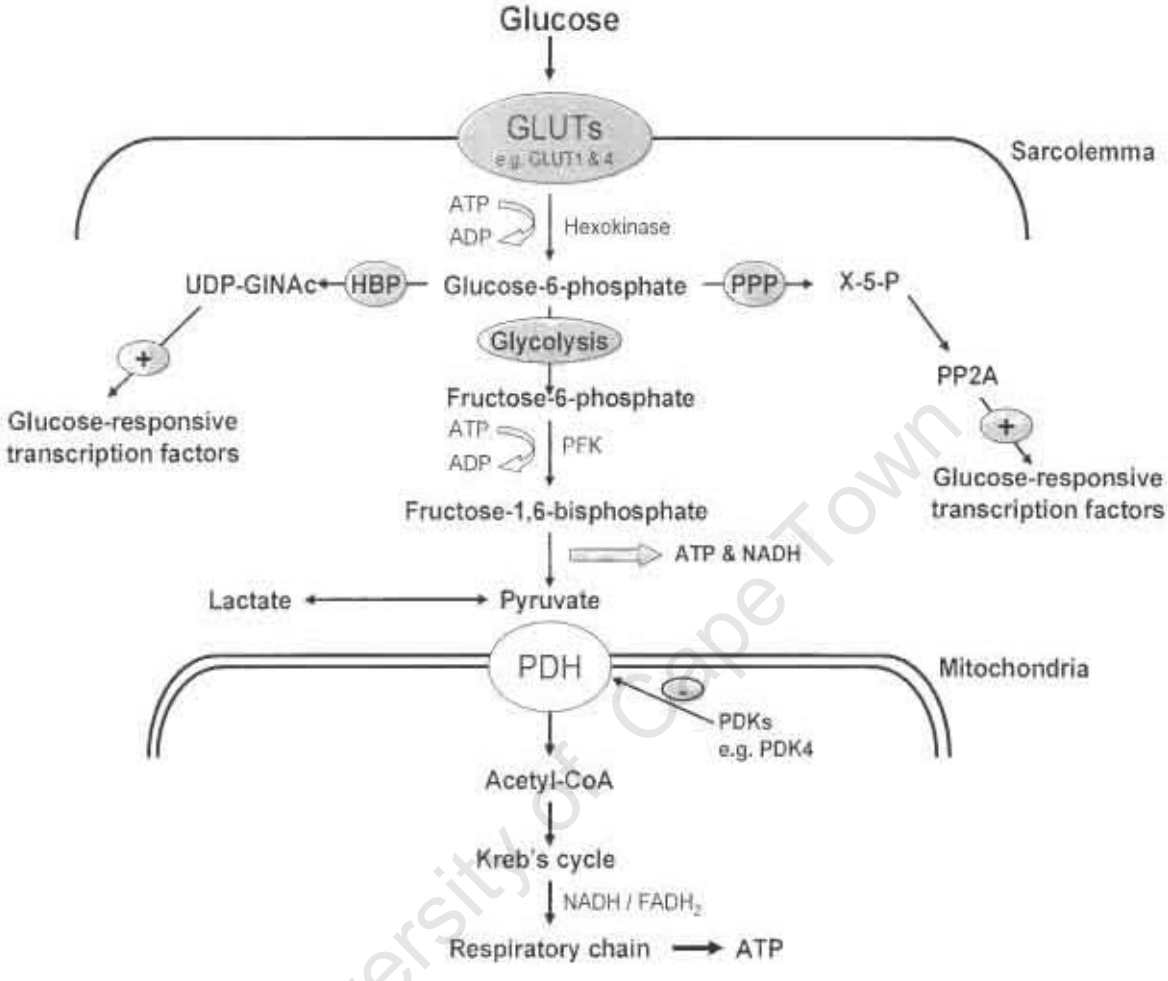


Figure 1. A simplified schematic representation of glucose metabolism
Abbreviations: Glucose transporter (GLUT), phosphofructokinase (PFK), xylulose-5-phophate (X-5-P), UDP-N-acetylglucosamine (UDP-GlNAc), protein phosphatase 2A (PP2A), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK).

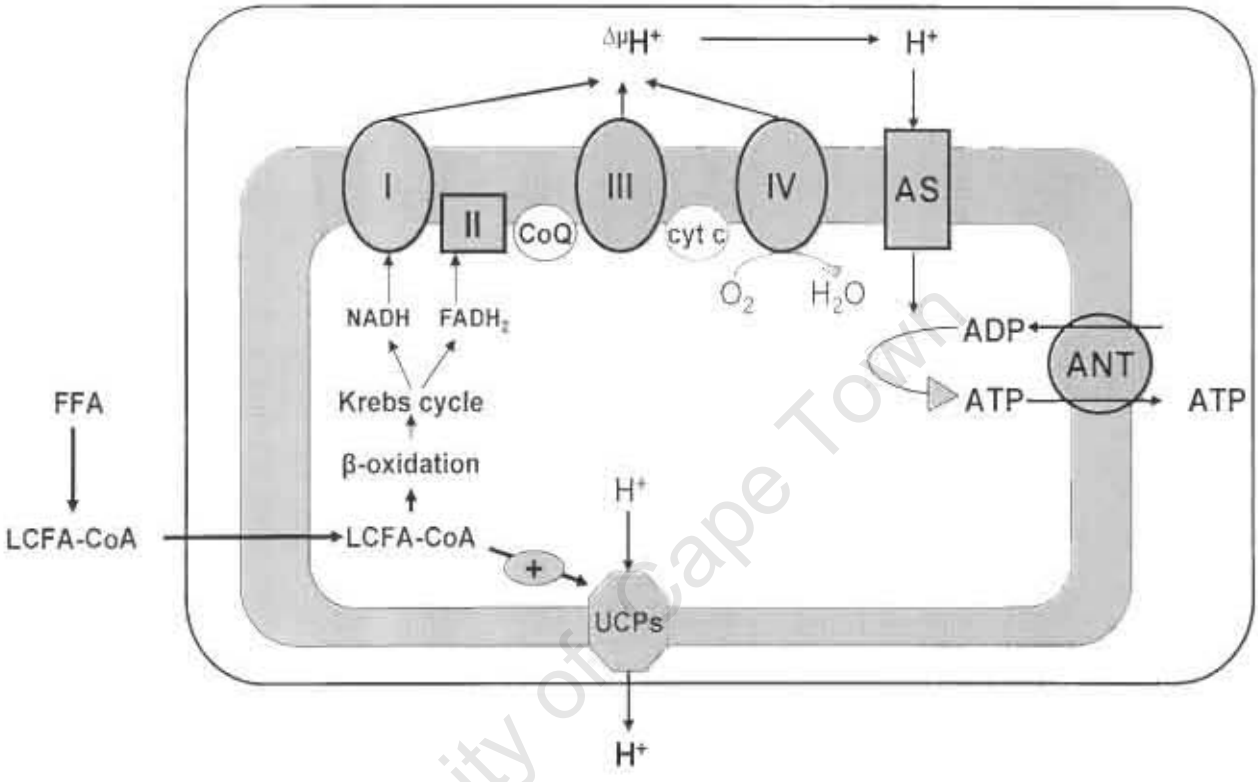


Figure 4. A simplified schematic representation of mitochondrial oxidative phosphorylation. The thin black line represents the outer mitochondrial membrane while the thicker blue band represents the inner mitochondrial membrane. Abbreviations: ATP synthase (AS), adenine nucleotide translocase (ANT), uncoupling protein (UCP).

1.1.3. The glucose-fatty acid cycle and its physiological relevance

The preferential utilization of glucose versus FAs (and *vice versa*) under varying physiological conditions forms the basis of the glucose-fatty acid cycle, initially described by Randle[44] (Figure 5). Randle et. al.[274] showed that FFAs inhibitory effect on glucose metabolism was mediated via inhibition of glucose transport, glycolysis and pyruvate oxidation. Others extended this hypothesis and revealed that glucose availability can have an inhibitory effect on FAO.[275] Interestingly, the mechanism for this inhibitory effect of glucose has been proposed to be through increased formation of malonyl-CoA (product of ACC) and subsequent inhibition of CPT1. In summary, the glucose-fatty acid cycle suggests that availability of free fatty acids promotes fatty acid oxidation and subsequently inhibits glucose oxidation. Moreover, the "reverse" Randle effect proposes that glucose availability promotes glucose oxidation, subsequently inhibiting fatty acid oxidation.

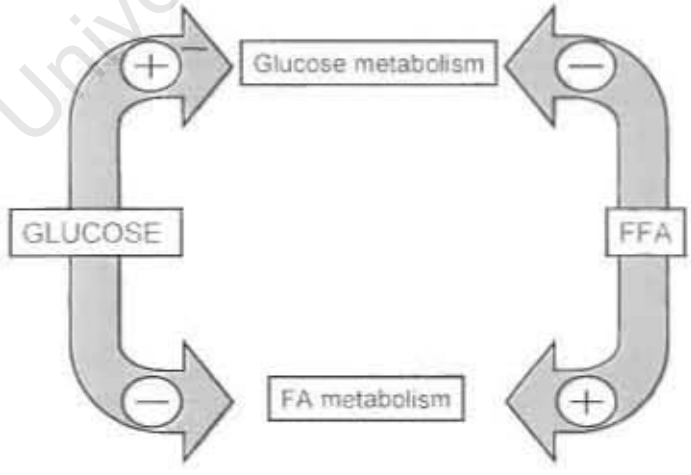


Figure 5. A simplified schematic representation of the glucose-fatty acid cycle. Abbreviation: free fatty acids (FFA).

Cardiac fuel substrate utilization can be altered in response to a variety of physiologic and pathophysiologic conditions.[33-38] For example, during the fetal to adult transition the major energy source of the heart switches from carbohydrates to FAs. During this period, plasma FFA levels are elevated and systemic glucose levels reduced in parallel. The heart responds to such whole-body metabolic remodeling by enhancing its capacity for FAO and concomitantly reducing glucose oxidation.[39] Conversely, previous studies have demonstrated reduced FA utilization and a corresponding increase in glucose utilization during hypoxia, cardiac hypertrophy and heart failure.[34, 35, 40-43] For example, glucose is proposed to inhibit CPT1[45] and therefore FAO possibly via activation of ACC and subsequent elevation of intracellular levels of malonyl-CoA, a potent inhibitor of CPT1.[46-48] Also, high carbohydrate feeding after an acute fast increased ACC expression in the liver[49, 50] and lowered LCFA-CoA levels.[47] Additionally, insulin inhibits the release of FFAs from the adipose tissue thereby leading to decreased NEFA levels and attenuated FAO.[51, 52] On the other hand, increased NEFA levels are associated with activation of PDK4, an inhibitor of the PDC complex, resulting in inhibition of glucose oxidation.[53] PDK4 is activated during high fat feeding and diabetes, conditions where glucose metabolism is impaired.[54-56] Saddick et al.[57] showed that administration of dichloroacetate (DCA), a PDC stimulator, to perfused hearts suppressed FAO via activation of the muscle- and cardiac-enriched ACC isoform (ACCβ), and the subsequent increase in malonyl-CoA levels. Moreover, accumulation of NEFA levels further impairs glucose tolerance and decrease muscle insulin sensitivity in high fat feeding and diabetic

rats.[58-60] Collectively, these data underscore the significance of metabolic plasticity of the mammalian heart and its innate ability to respond to physiologic and pathophysiologic fuel substrate switches.

1.2. Metabolic remodeling

1.2.1. An odyssey of metabolic remodeling

Metabolic remodeling is suggested to be part of an adaptive response under energy-limiting conditions and is regulated in response to developmental, hormonal or physiological stimuli. During the past few decades, researchers have investigated several aspects of metabolic remodeling including fetal to adult transition, physiological remodeling, development of cardiac hypertrophy, progression of cardiac hypertrophy to heart failure and diabetic remodeling.[34, 35, 40-43]

a) Fetal to adult transition

During the fetal to adult transition, the major energy source of the heart switches from glucose to FAs. Here, fetal hearts demonstrated increased myocardial glucose oxidation and lower FAO compared to newborn hearts. In contrast, newborn hearts exhibited increased myocardial FAO and lower glucose oxidation compared to fetal hearts.[61] This metabolic switch is paralleled by induction of FAO genes and a concomitant reduction in the expression of genes involved in glucose metabolism. For example, Nau et al.[62] observed a postnatal decline in expression patterns of glycolytic genes such as α-enolase, lactate

dehydrogenase A and liver-type PFK whereas mitochondrial oxidative genes such as cytochrome c, COX Va and CPT1 were increased.

5' adenosine monophosphate protein kinase (AMPK) has previously been shown to increase FAO by phosphorylation and inhibition of ACC.[63-65] In support, Makinde et al.[66] demonstrated higher FAO rates in 7-day old rabbit hearts compared to 1-day old hearts were associated with increased AMPK and reduced ACC activities. Although these studies did not investigate the role of CPT1 in cardiac development, it appears that expression levels of muscle- type CPT1 (mCPT1) are low at birth and are upregulated during the postnatal development.[67]

These data highlight the importance of understanding the metabolic transition from fetal to adult in the heart since some pathological conditions such cardiac hypertrophy are associated with a reversion to the fetal program, proposed to be part of an adaptive mechanism.

b) Physiological remodeling and mitochondrial biogenesis

The mitochondrion is regarded as the 'power-house' of a cell since it generates about 90% of the energy required for chemical and metabolic processes that take place in a living organism.[68] Therefore, failure to respond to energy demands either due to mutations in mitochondrial DNA or functional abnormalities can result in a variety of diseases such as childhood cardiomyopathy and sudden death, diabetes mellitus, ischemic heart disease, cardiac hypertrophy and heart failure.[69-73,74]

Under energetic stress conditions such as endurance exercise, severe hypoxia, fasting or postnatal development, heart and skeletal muscle respond by increasing bioenergetic capacity.[75-78] This adaptive response is characterized by increased expression of nuclear and mitochondrial enzyme-encoding genes and an increase in number and size of the mitochondria, referred to as mitochondrial biogenesis. Since mitochondrial bioenergetic capacity is such a critical factor in the cell's survival and response to stress conditions, the regulation of nuclear and mitochondrial enzyme-encoding genes must be under stringent control. Scarpulla and co-workers highlighted the role of nuclear respiratory factors (NRF1 and NRF2) as important transcriptional modulators that induce nuclear genes encoding respiratory chain proteins and regulatory factors modulate mitochondrial-encoded genes.[79-81] In support, previous studies have demonstrated that NRF1 induces mitochondrial transcription and replication via activation of mitochondrial transcription factor A (TFAM).[82] Also, TFAM overexpressing mice were resistant to left ventricular (LV) remodeling and improved LV function post myocardial infarction.[83]

On the other hand, NRF1 augments FAO and oxidative phosphorylation enzyme-encoding genes through its interaction with a FA-responsive nuclear transcription factor, peroxisome proliferator-activated receptor (PPAR) α and its transcriptional coactivator, peroxisome proliferator-activated gamma coactivator (PGC) 1α.[42, 43, 68, 84-86] Importantly, impaired mitochondrial function observed with conditions such as obesity and diabetes has been attributed to attenuated expression of

PGC1 and NRF1.[87, 88] However, it has not been fully established whether NRF1 has direct involvement in the regulatory control mechanism of FA importation into the mitochondrion. Other investigators revealed that increased NRF1 expression was associated with elevated CPT1 gene expression.[89, 90] Pharmacological activation of AMPK was also associated with increased NRF1 promoter binding.[91] Furthermore, energy-sparing conditions such as endurance exercise have been shown to increase mitochondrial respiratory capacity and stimulate FAO in an NRF1/PGC1 dependent manner.[86] Interestingly, ACCβ, an indirect inhibitor of FAO, is suppressed following endurance exercise. Although these studies suggest a direct link between NRF1 and FAO genes, whether NRF1 is involved in transcriptional mechanisms driving regulation of ACCβ remains unclear.

c) Cardiac hypertrophy and heart failure

Metabolic remodeling has also been demonstrated as a feature of cardiac hypertrophy. Previous investigators have reported increased dependence on glucose as a substrate with a concomitant decrease in FAO in hypertrophied hearts.[92-94] This substrate switch is paralleled by a reduction in FAO enzyme-encoding genes and the re-emergence of the fetal gene program.[35, 36, 95] For example, reduction in the expression of GLUT4 and re-expression of GLUT1 (fetal isoform) has been previously reported.[96, 97] Moreover, earlier studies indicated that glycolytic enzymes such as hexokinase and lactate dehydrogenase paralleled increased preferential glucose utilization with cardiac hypertrophy,[35]

while increased expression and activity of AMPK was associated with higher basal glucose uptake with left ventricular hypertrophy.[98]

In contrast, the reduction in FAO observed with cardiac hypertrophy is paralleled by suppression of a number of FAO responsive genes such as FAT/CD36, MCD, PDK4, mCPT1, MCAD, LCAS.[43, 98-102] This coordinate downregulation of FAO responsive genes has largely been attributed to reduced PPARα expression.[99, 103] Furthermore, reduction of PPARα during cardiac hypertrophy has been directly linked to preserved contractile function since its reactivation diminished cardiac contractile function.[39]

The failing heart has also been shown to shift towards preferential utilization of glucose.[34, 104, 105] For example, Razeghi et al.[106] proposed that the failing heart switches to the fetal gene program by reduction of adult gene isoforms such as GLUT4 and muscle CPT1 rather than the induction of fetal gene expression. Here, the expression of fetal gene isoforms such as GLUT1 and l-CPT1 was unchanged in failing human hearts compared to fetal hearts. On the other hand, other investigators observed a downregulation of several FA utilization genes in the failing heart.[99, 107] However, Chandler et al. [108] found no differences in myocardial substrate metabolism in a dog model of moderate coronary microembolization-induced heart failure. The authors suggested that the switch to a fetal gene program may depend on the severity of heart failure i.e. there is no decrease in FAO in their model since it represents compensated heart failure. The observed switch to carbohydrate oxidation may therefore be a late-stage phenomenon. If this is correct, reports of overstimulation of the adrenergic

system with heart failure and the elevation of FFA levels may represent an earlier point in terms of the progression to heart failure. It has recently been hypothesized that under these conditions UCPs uncouple mitochondrial respiration leading to oxygen wastage and reduced cardiac efficiency.[109] Hence, the mechanism of action of pharmacological inhibitors of FAO, a potential treatment for heart failure, [110] is thought to be due to an indirect increase in glucose oxidation and decreased FA-induced oxygen wastage.[109, 111]

d) Diabetes

Several studies have reported that diabetic hearts demonstrate severely diminished glucose uptake and a marked reliance on FAO to meet energetic requirements.[52, 112, 113] Moreover, intracellular accumulation of NEFA levels further impair glucose tolerance and decrease muscle insulin sensitivity in high fat feeding and diabetic rats.[58-60] As a consequence of diabetic remodeling, the expression of genes involved in glucose metabolism is suppressed whereas FA-responsive genes are upregulated.[51] For example, cardiac GLUT4 expression is reduced while both hexokinase and PDC enzyme activity were diminished in diabetic rat hearts.[114, 115] Intracellular FA accumulation during diabetes is thought to activate PPARα and PGC1α, which in turn induce the expression FAO genes such as FAT, mCPT1, MCAD, PDK4, and MCD.[43, 51, 101] This could explain reduced glucose metabolism and markedly increased FAO rates exhibited by perfused diabetic hearts.[116, 117] Increased PDK4 expression is thought to be responsible for reduced PDC activity observed in diabetic rat hearts. In contrast,

some have argued that reduced PDC activity is due to accumulation of FAO products rather than the phosphorylated state of PDC complex.[118]

Lastly, chronically diabetic subjects were characterized by a reduction of genes involved in oxidative metabolism for e.g. PGC1, CPT1, citrate synthase (CS) and cytochrome C oxidase (COX) .[87, 119] In support, other investigators have further suggested that reduced PGC1 and NRF1 levels may be contribute to the accumulation of intracellular lipids and decreased FAO.[88] Again, differences in substrate metabolism may be due to the stage of the disease. It has also been suggested that reduced FAO observed in certain type 2 diabetic patients may be attributed to increased gene expression of ACCβ and subsequent accumulation of malonyl-CoA levels.[120, 121]

1.3. Transcriptional regulation of metabolic remodeling

As described in the previous section, metabolic remodeling may occur in several physiologic and pathophysiologic contexts. Several investigators suggested that metabolic remodeling is driven by changes in the expression of metabolic genes. However, the molecular mechanisms underlying regulation and control of several putative metabolic genes are currently being investigated. For example, at the transcriptional level recent studies have suggested that nutrient-sensing transcriptional modulators that regulate glucose and FA utilization genes may play a pivotal role in driving metabolic remodeling processes.[20, 41, 122-125]

1.3.1. Fatty acid mediated transcriptional regulation

Recent studies have identified PPARs, the ligand-activated nuclear receptor family of transcription factors, as key modulators that induce the expression of FAO genes.[20, 41, 124, 125] Thus far, three PPAR isoforms have been identified, namely: PPARα, β/δ and γ. PPARα is predominantly expressed in highly oxidative tissues like liver, heart and skeletal muscle, while PPARβ/δ is ubiquitously expressed and has been implicated in the regulation of FAO.[125, 126] Conversely, PPARγ is highly expressed in lipogenic tissues such liver, white and brown adipose tissue. PPARγ is thought to play a role in thermogenesis, lipid storage and glucose metabolism.

PPARs consist of a DNA-binding domain containing two Zn-finger motifs and a C-terminal domain for ligand binding. Upon binding its ligand, PPAR heterodimerizes with its obligate partner, retinoid X receptor (RXR), subsequently binding to peroxisome-proliferator response elements (PPRE) located within the regulatory region of target genes. PPARs can bind both endogenous and exogenous ligands such as long chain FAs and fibrates, respectively. RXR is also activated in a ligand-dependent manner i.e. 9-cis retinoic acid serving as its ligand.

a) Ligand-activated PPARα regulates fatty acid oxidation

The use of synthetic ligands such as fibrates and Wy-14,643, and PPARα null mice have contributed tremendously in identifying a key role for PPARα in cardiac FA utilization.[127-129] Moreover, endogenous ligands such as LCFAs

mediate metabolic changes via activation of PPARα.[124, 130] Here, PPARα has been shown to induce gene expression of cardiac FA transport proteins such as FATP, FAT/CD36 and FABP[131] and FAO via the induction of CPT1 and MCAD gene expression.[42] PDK4 was also identified as a PPARα target gene since its gene expression and activity was increased in response to PPARα stimulation.[85] High fat feeding, fasting and diabetes have been shown to increase FFA levels, PPARα and subsequent activation of FA responsive genes such as CPT1, MCAD and PDK4.[118, 129] On the contrary, downregulation of FAO genes during hypoxic exposure has been attributed to deactivation of PPARα, a major transcriptional modulator of FA responsive genes in the rat heart.[41, 108] Moreover, PPARα null mice exhibit low FAO rates paralleled by a marked reduction in several FA responsive PPARα-regulated genes.[128, 129] Together these data show that PPARα is a pivotal regulator of several cardiac FA utilization genes.

b) Ligand-activated PPARβ/δ regulates fatty acid oxidation

Thus far, the transcriptional role of PPARβ/δ is not as well understood. PPARβ/δ is ubiquitously expressed, with higher levels of expression in the brain, adipose tissue and skin.[126, 127, 132] Recently, PPARβ/δ has been demonstrated to regulate the expression of genes involved in FAO in skeletal muscle cells.[133, 134] Moreover, both PPARα and PPARβ/δ activate FAO in cultured neonatal and adult myocytes.[108] PPARβ/δ expression is regulated by nutritional status.[135] Like PPARα, PPARβ/δ is activated by LCFAs and has also been implicated in the

regulation of genes involved in sarcolemmal FA uptake (FATP and FAT)[136] and mitochondrial FA uptake (CPT1).[137] These data suggest that there may be some redundancy in terms of PPAR activation of cardiac FA utilization. However, the precise interplay between PPARα and PPARβ/δ in terms of regulation of FAO genes still needs to be determined.

1.4. Glucose as a transcriptional regulator

Glucose has classically been considered as a metabolic substrate that provides energy to the cell especially under energy sparing conditions such as exercise,[138] and ischemia.[139, 140] However, recent studies show that glucose may also provide signals to glucose responsive factors to drive transcriptional activation of genes involved in glucose metabolism and lipogenesis.[122, 123] For example, the glycolytic liver type pyruvate kinase (LPK) gene was upregulated with high glucose exposure in hepatocytes[122] or high carbohydrate refeeding.[141] Furthermore, an increase in glucose levels following high carbohydrate refeeding was associated with induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression.[142] In addition, expression of lipogenic genes such as fatty acid synthase (FAS) and ACCα were reduced by an acute fast but upregulated following high carbohydrate refeeding.[122, 141] Glucose has also been shown to induce the gene expression of S14, a lipogenic protein, in the liver.[143]

These glucose-mediated gene transcriptional effects are suggested to occur via specific glucose-sensing transcriptional modulators able to bind consensus

regulatory regions within promoters of target genes. Glucose-responsive transcription factors include carbohydrate response element binding protein (ChREBP),[144, 145] sterol regulatory element binding protein 1 (SREBP1),[146, 147] stimulatory protein 1 (Sp1)[148] and upstream stimulatory factors (USF1 and USF2 isoforms).[122, 149] Moreover, ChoRE, a glucose-inducible promoter region, was demonstrated as a key transcriptional regulatory region of ACCα, FAS and S14 in response to glucose.[150, 151] For example, ChREBP was identified as the key glucose-responsive modulator that binds to ChoRE region of LPK gene promoter.[144, 145] Previous investigators have demonstrated that USF1/2 isoforms and ChREBP bind E-box sequence motifs (CANNTG) as homo and/or heterodimers on promoter regions with subsequent induction of target genes.[150, 152-157] Furthermore, Wang et al.[158] demonstrated markedly increased USF1 protein expression upon high carbohydrate refeeding following an acute fast. In mice lacking USF1, glucose mediated stimulation of LPK and S14 genes is partially reduced and compensated for by USF2.[122, 149] Furthermore, USF2 null mice displayed an impaired glucose responsiveness of glucose-induced genes. These data suggest that USFs play a crucial role in glucose-mediated transcriptional responses.[159]

Some researchers have suggested that glucose metabolites and intermediates such as G-6-P and X-5-P, and not glucose *per se*, are responsible for glucose mediated transcriptional activation of genes.[155, 160, 161] In agreement, G-6-P has been demonstrated to activate transcription of target genes.[162] In support of this concept, 3-O-methyl glucose, a glucose analog that is taken up by the cells but

cannot be phosphorylated, failed to increase the expression of FAS and ACCα.[11] In addition, 2-deoxyglucose, a glucose analog that is taken up but can be phosphorylated, stimulates FAS gene expression in cultured white adipose tissue.[155, 160] Furthermore, X-5-P, an intermediate in pentose phosphate pathway, increased transcription of genes involved in lipogenesis, hexosamine monophosphate shunt and glycolysis.[9, 163, 164] X-5-P stimulates PP2A[161] thereby promoting translocation of ChREBP into the nucleus where it binds to ChoRE on the promoter of the LPK gene.[10, 11, 13] Also, PP2A dephosphorylates and activates ACCα in most tissues.[165, 166] In agreement with this concept, ACCα was transcriptionally activated by glucose[148] in pancreatic beta cells via activation of Sp1.[148] Together these data implicate glucose and/or its metabolites as important signaling molecules in the induction of glucose responsive genes.

1.5. AMPK as a transcriptional regulator

Under conditions of increased (AMP/ATP) for e.g. exercise or hypoxia, AMPK is activated and ACC subsequently inactivated (by phosphorylation) in the liver and FAO hepatoma cells.[167, 168] It has recently been shown that AMPK is activated in response to energetic stress to increase bioenergetic capacity. For example, increased AMPK activity has been shown to elevate glucose uptake by GLUT4 translocation and stimulate FAO by inhibition of ACCβ [169-171]. Recently, AMPK has been demonstrated as a direct link between intracellular stress signals and the regulation of gene transcription. For example, addition of AICAR (AMPK activator) downregulates the expression of GLUT2, aldolase B and LPK genes in

pancreatic β cells.[172-174] AICAR administration in hepatocytes also resulted in decreased expression of FAS, PEPCK and G-6-P genes.[174, 175] Increased AMPK activity in the liver also resulted in upregulation of CPT1 [176]

These AMPK effects have been suggested to be mediated via direct activation of nuclear transcription factors that either activate or repress transcription of target genes.[177] For example, it has been shown that AMPK represses PPARγ-mediated transcriptional activity.[177] On the other hand, AMPK activates PPARα-mediated transcriptional regulation.[178] In addition, β-GPA (indirect AMPK activator) activation of AMPK was associated with increased binding activity of NRF1 to the promoter of a target gene.[91] Recent evidence has also shown that AMPK phosphorylates and inhibits transcriptional activity of ChREBP.[13] AICAR administration to hepatocytes repressed the induction of LPK gene expression by attenuating the transcriptional activity of hepatocyte nuclear factor 4 (HNF4).[173, 177] Moreover, AMPKα$^{-/-}$ mice were characterized by a downregulation of several genes involved in both glucose transport and FA utilization.[179] These data therefore indicate that AMPK could be a direct link between intracellular stress signals and the regulation of gene transcription.

1.6. The role of acetyl-CoA carboxylase in substrate metabolism

Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, a potent inhibitor of CPT1. At least two ACC isoforms (ACCα and ACCβ) exist in mammals. The ACCα gene encodes a 265-kDa isoenzymic form that is predominantly expressed in lipogenic tissues such as the liver and

adipose tissue.[23-25] In contrast, ACCβ encodes a 280-kDa isoenzymic form that is mainly expressed in non-lipogenic tissues such as the heart and the skeletal muscle.[26, 27] The catalytic portion of ACCβ is similar to that of ACCα except for an additional 150 amino acids at the N-terminus. The N-terminus is believed to be responsible for interacting with the outer membrane of mitochondria where it controls mitochondrial FA transfer by producing malonyl-CoA near its CPT1 binding site.[180, 181] Since both ACC isoforms produce malonyl-CoA, it is believed that malonyl-CoA produced by ACCα is essential for FA biosynthesis while malonyl-CoA produced by ACCβ regulates mitochondrial FA oxidation pathways.

ACCα gene regulation has been extensively characterized (reviewed by Taegtmeyer and others)[22, 95, 182] and multiple levels of control have been determined for its regulation in the FA biosynthetic pathway.[181] At the transcriptional level, ACCα is regulated by two promoters, PI and PII. PI is regulated under lipogenic conditions such as high carbohydrate feeding,[183, 184] while PII is regulated by insulin, cAMP and tumor necrosis factor.[185, 186] Interestingly, PII contains glucose responsive elements and Sp1 sites which are thought to mediated glucose-induced transcriptional activation of ACCα.[187, 188]

Since very little or *de novo* fatty acid synthesis occurs in non-lipogenic tissues and as malonyl-CoA has been demonstrated as a potent inhibitor of CPT1, this strongly suggests a distinct role for ACCβ in the regulation of mitochondrial FAO in the heart and skeletal muscle.[189-191] Therefore, ACCβ must be subject to stringent regulatory control mechanisms at both transcriptional and translational levels.

1.6.1. Metabolic regulation of the cardiac isoform of acetyl-CoA carboxylase (ACCβ)

Previous investigators have demonstrated that *in vitro* and *in vivo* ACCβ activity is inhibited by reversible phosphorylation. Here, AMPK and PKA have been identified as the two main enzymes involved in phosphorylation and inhibition of ACCβ activity (Figure 6). AMPK phosphorylates ACCα at Ser79, Ser1200 and Ser1215 phosphorylation sites while PKA phosphorylates ACCα at Ser77 and Ser1200.[192] Interestingly, these phosphorylation sites are preserved on ACCβ.[27, 165, 166, 192] In addition, phosphorylation sites for protein kinase C and casein kinase II have been identified on the N-terminal region of ACCβ.[180] It is also important to note that phosphorylation of both ACC isoforms can be reversed by protein phosphatases 2A and 2C.[165, 166, 193] The activation of protein phosphatases is thought to be mediated by glucose and/or insulin.[10, 11, 13, 194]

As shown in Figure 6, the formation of malonyl-CoA by ACC can be reversed through decarboxylation by malonyl-CoA decarboxylase (MCD). Increased MCD gene expression and activity result in reduced malonyl-CoA levels thereby promoting FAO. Some investigators have reported that FAO-promoting conditions such as fasting and high-fat feeding increase cardiac MCD gene expression.[276] MCD is also a PPARα target gene, for example, administration of PPARα agonists resulted in the induction of MCD expression (at gene and protein level) and activity.[276] Moreover, downregulation of cardiac MCD expression (gene and protein) and activity was observed in PPARα$^{-/-}$ mice.[277]

Post-transcriptionally, MCD is phosphorylated and activated by AMPK which is in turn activated in response to ischemia[278] and exercise.[252]

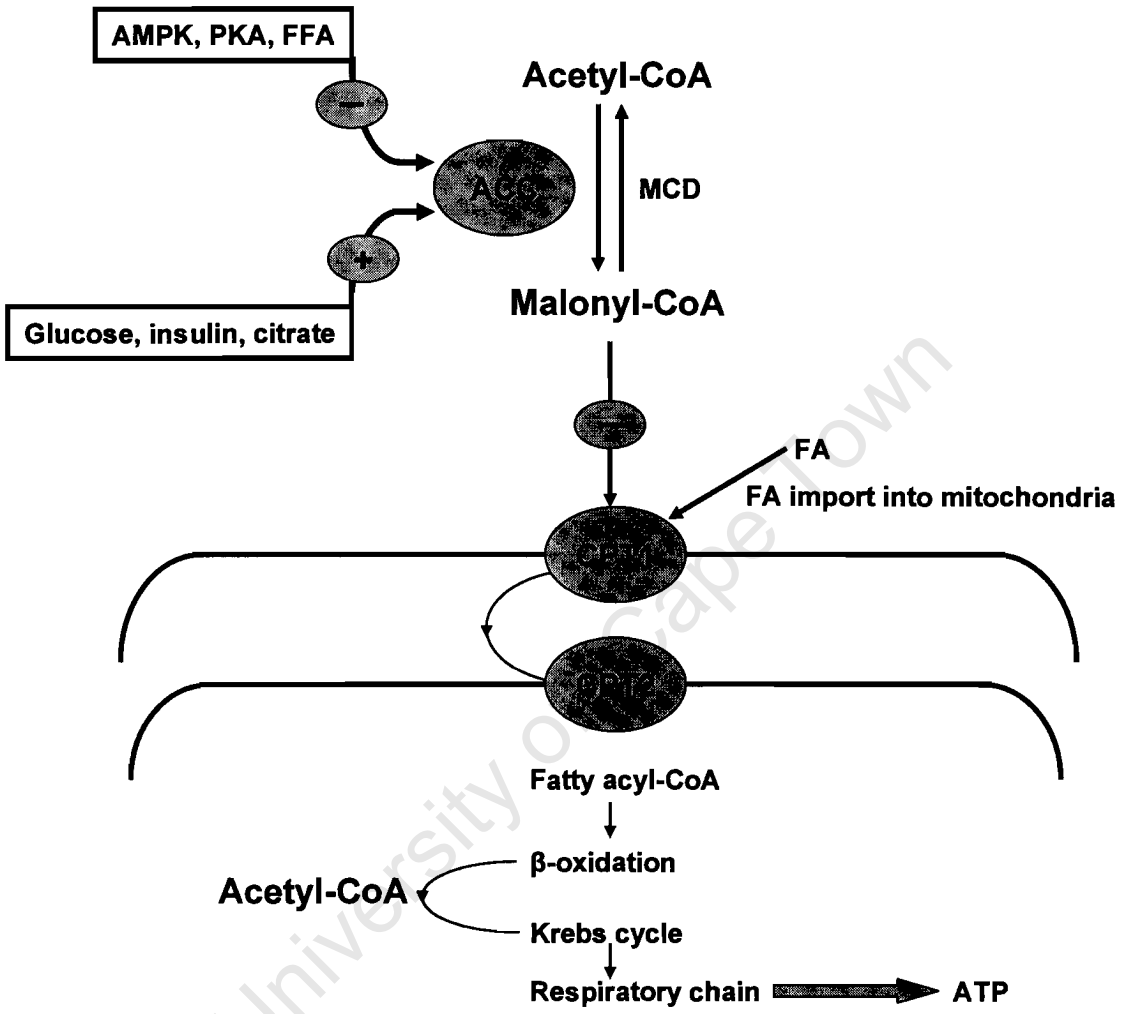


Figure 6. A schematic representation showing metabolic regulatory mechanism of acetyl-CoA carboxylase. Abbreviation: free fatty acids (FFA), 5' adenosine monophosphate protein kinase (AMPK), protein kinase A (PKA), acetyl-CoA carboxylase (ACC), malonyl-CoA decarboxylase (MCD), carnitine palmitoyl transferase 1 / 2 (CPT 1 / 2).

Under energy-sparing conditions for e.g. ischemia and exercise, activation of AMPK and PKA result in phosphorylation and inhibition of ACCβ and subsequent lowering of malonyl-CoA levels.[193, 195-199] In support, Abu-Elheiga et al.[200] observed reduced malonyl-CoA levels following a 48 hour fast in the liver, skeletal muscle and the heart, leading to the conclusion that both ACC isoforms must be regulated by nutritional flux.[200, 201] Furthermore, administration of AICAR to perfused rat hindlimbs lead to a reduction in ACCβ activity and intracellular malonyl-CoA levels, thereby increasing FAO.[202] Also, insulin, epinephrine, glucagon, leptin, citrate, acetyl-CoA levels, LCFA and malonyl-CoA have also been implicated as important post-translational regulators of ACC activity.[26, 57, 165, 181, 203-205]

Since the regulation of ACC activity by allosteric activation (citrate) and covalent modification of enzymes i.e. by phosphorylation/dephosphorylation lasts for seconds to minutes, maintenance of ACC activity for hours to days must be regulated by changes in gene expression.[165, 181, 194, 203, 204, 206] In support, ACCβ gene and protein expression were upregulated following exposure to glucose and ciprofibrate, a PPARα agonist.[180] Moreover, ACCβ gene expression in the rat liver was downregulated following a 48 hour fast and markedly increased following 24-48 hour refeeding with a fat-free high carbohydrate diet.[180, 194] Interestingly, H9C2 myoblasts do not express ACCβ while differentiated H9C2 myotubes highly express ACCβ, underscoring the importance of controlling FAO in the developing and an adult hearts.[180, 207] Moreover, ACCβ null mice are

characterized by low malonyl-CoA levels and a subsequent increase in FAO rates in skeletal and cardiac tissues.[200] When ACCβ null mice were challenged with a diabetes/ obesity-inducing diet (high-fat/high-carbohydrate), they were protected against diabetes and resistant to weight gain compared to wild-type mice.[194] This was evidenced by a number of factors including decreased fat content, efficient glucose clearance and high insulin sensitivity (reduced glucose and insulin levels) and attenuated levels.[194, 200] Collectively, these data underscore the importance of understanding transcriptional regulatory machinery of ACCβ and its role in the regulation of FAO. In this regard, characterization of the recently cloned human ACCβ promoter[180, 208] requires further attention in order to advance our understanding of the regulation of ACCβ gene expression.

1.6.2. Transcriptional regulation of human ACCβ promoter

Most muscle-specific genes have multiple E-boxes (CANNTG) in their promoters that bind muscle specific factors and regulate their gene transcription.[209] Since ACCβ is highly expressed in the skeletal muscle and the heart, Lee and coworkers[208] analyzed the human ACCβ promoter for novel CANNTG binding sites. Here, at least four E-box consensus sequences were identified.[208] These studies revealed that MyoD, a member of the muscle regulatory factor family, induces ACCβ expression by binding to E-box consensus motifs located on the ACCβ promoter.[208] Furthermore, Sp1, a glucose responsive transcriptional factor, inhibits MyoD mediated transcriptional of ACCβ.[208] Others have demonstrated that MyoD mediated transcriptional activation of ACCβ promoter was attenuated

by retinoic acid receptors, RXRα and RARα.[207] Recently, SREBP1, a glucose-responsive transcription factor thought to regulate expression of insulin and/or diet-induced genes in the liver, has been implicated in transcriptional activation of ACCβ.[194]

1.7. The role of ACCβ in metabolic remodeling

Understanding the fundamental role of fuel substrate switches in the progression of cardiovascular diseases such as diabetic cardiomyopathy, cardiac hypertrophy and heart failure is important to the development of novel therapeutic interventions. As previously noted, the diabetes-induced shift in cardiac metabolism (increased FAO rates) triggers contractile dysfunction.[116, 117] Some investigators have therefore argued that the use of pharmacological inhibitors of FAO, as a potential treatment for heart failure may be due to an indirect increase in glucose oxidation and decreased fatty acid-induced mitochondrial oxygen wastage.[109, 111]

These and other data highlight the importance of understanding the regulatory mechanisms underlying such fuel substrate switches in the heart. In light of this, ACCβ has been identified as a key regulator of FAO since its product malonyl-CoA potently deactivates CPT1. Moreover, ACCβ null mice are resistant to diabetes and obesity-inducing diets, likely due to reduced malonyl-CoA production and therefore continuous elevation of FAO rates.[194, 200] In addition to ACCβ-mediated control of FAO, preferential utilization of one particular metabolic

fuel substrate by the heart will inhibit utilization of the other (Randle cycle). For example, glucose is thought to limit FAO by inhibiting FA importation into the mitochondrion. Together these data implicate ACCβ as an important target for therapeutic interventions during pathophysiologic metabolic remodeling. Therefore, this thesis focused on further understanding transcriptional mechanisms regulating ACCβ gene expression in the heart.

Specifically, we hypothesized that (Figure 7):

1. ***Increased glucose availability induces cardiac ACCβ expression via USF1, a glucose-responsive transcription factor***
2. ***NRF1, a transcription factor that promotes FAO, inhibits ACCβ expression in the heart.***

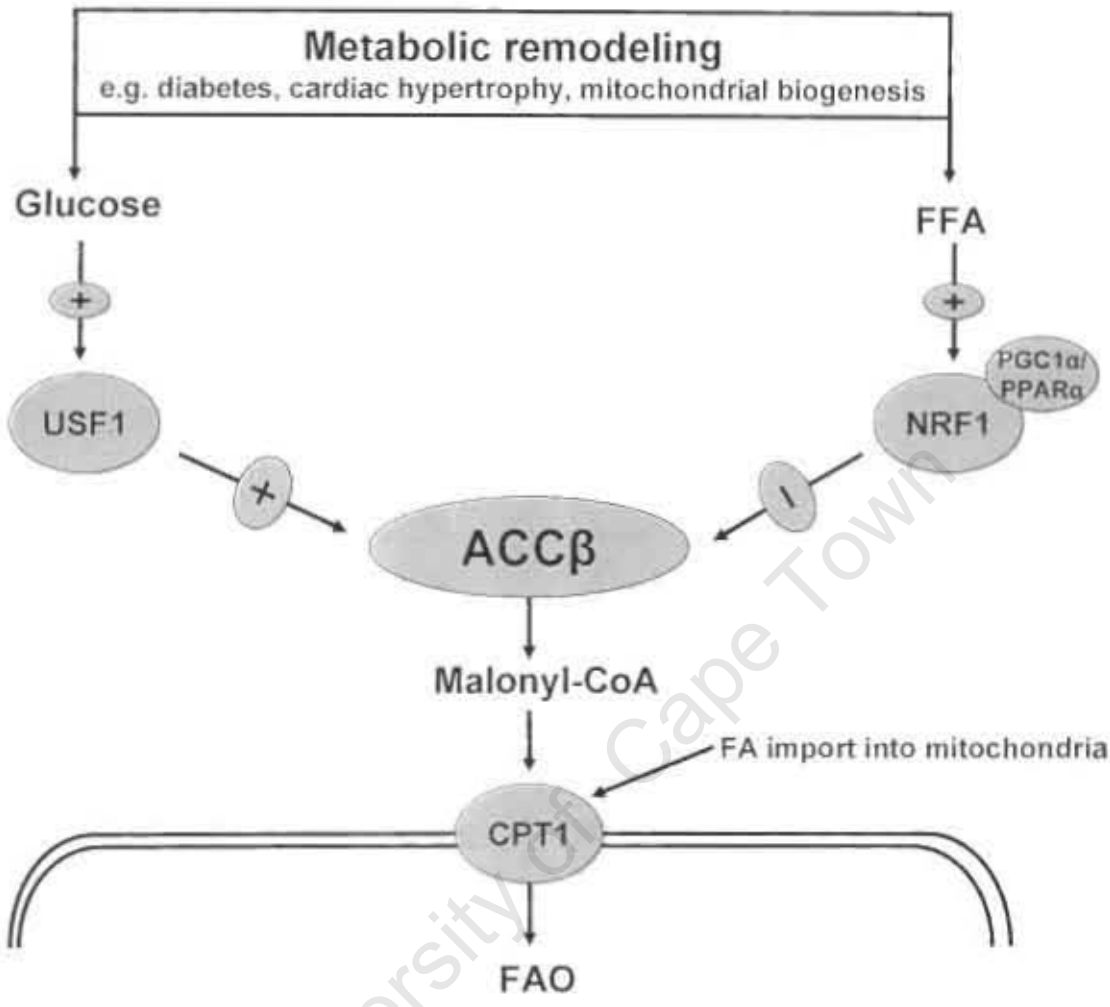


Figure 7. Schematic representation of hypotheses

The objectives of the study were to:

1) Determine in vivo cardiac ACCβ expression in response to high carbohydrate refeeding
2) Elucidate the transcriptional mechanisms regulating the human ACCβ gene promoter in response to increased glucose flux

Chapter 2

The Human Gene Promoter of the Cardiac Isoform of Acetyl-CoA Carboxylase (ACCβ) is Transactivated by Upstream Stimulatory Factor 1 (USF1) in a Glucose-dependent Manner

2.1. Introduction

The cardiac-enriched isoform of acetyl-CoA carboxylase (ACCβ) is a key enzyme regulating mitochondrial FAO in the heart. However, unlike ACCα (enriched in lipogenic tissues), molecular mechanisms directing ACCβ gene transcription are unclear. Previous studies reported increased ACCβ gene expression in the liver of mice fed a high carbohydrate diet.[180, 194] These data therefore associate elevated plasma glucose levels with increased ACCβ gene expression. In agreement, others have shown that glucose and/or metabolites of glucose metabolic pathways for e.g. the pentose phosphate and hexosamine biosynthetic pathways play a role in mediating transcription of lipogenic genes.[122, 123] For example, intermediate metabolites of the pentose phosphate pathway i.e. glucose-6-phosphate (G-6-P) and xylulose-5-phosphate (X-5-P) have been implicated in the transcriptional activation of several metabolic genes.[155, 160, 161] Here, it has been proposed that increased X-5-P levels may activate a downstream target, protein phosphatase 2A (PP2A) which in turn dephosphorylates and activates specific transcription factors for e.g. upstream stimulatory factor 1 (USF1). Subsequently, activated glucose-sensing transcriptional modulators bind specific *cis*-elements located within the promoter regions of target genes. For example, it has been demonstrated that USF1 regulates gene transcription by binding to E-box sequence motifs (CANNTG) located within the promoter regions of target genes.[222]

Recently, four E-box consensus sequences were identified on the human ACCβ gene promoter.[208] In light of this, we hypothesized that USF1 induces ACCβ gene transcription in the heart in a glucose-dependent manner. Moreover, we proposed that USF1-mediated induction of ACCβ gene transcription occurs via the pentose phosphate pathway. In order to test the hypothesis, we initially determined *in vivo* cardiac ACCβ gene and protein expression in mice fed a high carbohydrate diet after a period of fasting. In agreement with our hypothesis, we found increased cardiac ACCβ expression associated with elevated plasma glucose levels. To further investigate this, we next performed a series of transfection-based studies using the human ACCβ gene promoter and a USF1 expression construct. Here, we transiently transfected various cell lines i.e. neonatal cardiomyocytes and cardiac-derived H9c2 myoblasts, and two cardiac-null cell types (CV-1 fibroblasts and HepG2 hepatocytes) with the full-length ACCβ gene promoter construct and a USF1 expression vector under low (5.5 mM) and high glucose (25 mM) culturing conditions.

In this study, we identify USF1 as a novel transactivator of the human ACCβ gene promoter in three of the four cell lines tested (cardiomyocytes, CV-1 fibroblasts and HepG2 hepatocytes). Transient transfection studies with several ACCβ gene promoter deletion and mutant constructs show that USF1-induced human ACCβ promoter activity is mediated through region -92 to -39 and/or E-box 4 (-14 to -9) elements located in the first 569 bp upstream of the transcription start site. Moreover, we found glucose-dependent USF1 transactivation of the

human ACCβ promoter in CV-1 fibroblasts and HepG2 hepatocytes. Our *in vitro* data also suggest the involvement of a xylulose-5-phosphate/PP2A mediated signaling pathway that directs the transcriptional regulation of the ACCβ gene in CV-1 fibroblasts.

2.2. Materials and Methods

2.2.1. In-vivo studies

a) Animals

Balb/C female mice aged between 8-10 weeks were housed at room temperature on a 12-hour dark/12-hour light cycle. The metabolic cages lacked bedding and contained a metal grid at the bottom of the cage to avoid re-feeding on fecal matter, especially during fasting. Mice were stabilized for ten days with a commercially available control diet (19% protein, 10% fat, 4.3% fiber and 60.6% carbohydrates, catalog # 7024 (5755), Purina Mills, Richmond, IN). After stabilization, one group continued to receive food *ad libitum* (control group) while the other mouse group was fasted for 48 hours and re-fed a carbohydrate-enriched diet (19% protein, 0% fat, 4.3% fiber and 70.6% carbohydrates, catalog # 7576 (5803), Purina Mills, Richmond, IN) (RHC group) for an additional 48 hours. All animals had free access to clean water. In order to avoid any bias due to diurnal variations, preparations such as blood and tissue collection were routinely performed between 16h00 and 18h00. Mice were sacrificed after each experimental time point using carbon dioxide. Intact hearts were dissected out and stored at -80°C for subsequent molecular and biochemical analyses. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

b) Determination of glucose levels

Blood was collected by cardiac puncture from all experimental groups. Samples were centrifuged at 3,500 rpm for 10 min and the supernatant collected. Plasma (50 µl) glucose levels were measured using a spectrophotometric glucose oxidase method described previously by Trinder et al. (1969). [214]

c) RNA isolation and Northern blot analysis

RNA was extracted and purified as described previously.[99] Northern blot analysis was performed by loading 20 µg of total RNA in each lane, separated on a denaturing gel and transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech, UK). After fixation, the membrane was probed with radiolabeled probes derived from cDNA fragments encoding for ACCβ. A 1,122-bp fragment of ACCβ was generated by RT-PCR using primers based on the published rat ACCβ cDNA sequence (5'-TTCGATGTCCTGCCCACTTT-3' (forward) and 5'-TGGTGATGAAGAGGCGAAT-3' (reverse).[215] 28 S rRNA transfer to nitrocellulose membrane was used to assess RNA integrity and to control for variance in gel loading. The relative abundance of mRNA was determined by autoradiography and quantified by laser densitometric analysis using the UVITec autoradiography analyzer with uviband software v.97 (Cambridge, UK).

d) Protein extraction and Western blot analysis

Frozen heart tissue (60-80 mg) was briefly sonicated in 400 μl lysis buffer (10 mM Tris, 5 mM EGTA, 0.1 mM DTT, pH 7.2) supplemented with protease inhibitors (2 mM PMSF, 0.125 μg/ml leupeptin, 0.5% SDS). The homogenate was centrifuged at 5,000 rpm for 5 min, the supernatant collected and protein concentrations determined using the Lowry method.[216] Sixty μg of protein extract was used for ACC protein blotting analysis. Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (5%) and protein levels of the ACC 280-kDa isoform detected by probing blots with horse radish peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD), which binds biotin-containing carboxylases. Equal protein loading was confirmed using Ponceau Red staining as described previously.[217] Protein bands were detected using a chemiluminescence system (Amersham Pharmacia Biotechnology, UK) and quantified by laser densitometric analysis using the UVITec autoradiography analyzer with uviband software v.97 (Cambridge, UK).

2.2.2. *In vitro* studies

a) Cardiomyocytes

Cardiomyocytes were isolated from 1-3 day old Wistar rats by collagenase/ pancreatin digestion and purified using percoll density gradient centrifugation. Briefly, rats were intraperitoneally anaesthetized with 0.2 ml sodium pentobarbitone. The rat hearts were exercised out and immersed in 1X ADS buffer (6.8 g NaCl, 0.4 g KCl, 1 g glucose, 0.17 g NaH_2PO_4, 0.2 g $MgSO_4$, 4.76g HEPES in 1 L H_2O, pH 7.4). The hearts were cut in half and washed twice with

fresh 1X ADS buffer to remove blood. For the collagenase digestion, the hearts were finely cut into small pieces, placed into a 25 cm^2 flask containing 10 ml collagenase solution (0.0225g collagenase D (394 U/mg), 0.045 g pancreatin in 50 ml 1X ADS buffer) and incubated in a shaking water bath at 37°C for 15 minutes. The supernatant was transferred into a 15 ml tube and centrifuged at 300 x g at room temperature for 3 minutes. The pellet was resuspended in 2 ml newborn calf serum (NBS) (Gibco, New Zealand) and incubated at 37°C (the collagenase digestion step was repeated until all of the tissue was digested). The combined suspension was centrifuged at 300 x g for 3 minutes at room temperature. The pellet was resuspended in 4 ml of 1.082 g/ml of percoll solution (Amersham, South Africa). An equal volume of 1.062 g/ml of percoll solution was then added on top of the suspension followed by centrifugation at 1,000 x g at room temperature for 3 minutes. Thereafter, cardiomyocytes were transferred into 1X ADS buffer and centrifuged at 300 x g at room temperature for 3 minutes. The pellet was suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Highveld Biological, South Africa) supplemented with 20% fetal calf serum (v/v) and 100 µg/ml of penicillin/streptomycin. Each group of 20 neonatal rat hearts yielded approximately 16 x 10^6 cells/ml. Cardiomyocytes were subsequently plated in 12-well plates pre-coated with fibronectin (35 µg/ml) at a density of ~8 x 10^4 cells per well in DMEM.

b) CV-1 fibroblasts, H9C2 myoblasts and HepG2 hepatocytes

H9C2 rat heart-derived myoblasts (ECACC no. 88092904), CV-1 monkey kidney fibroblasts (ECACC no. 87032605) and HepG2 human Caucasian hepatocytes (ECACC no. 85011430) were seeded in 75 cm^2 flasks until they reached 70-80% confluency. Thereafter, the medium was removed and cells were washed with 5 ml 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L, pH 7.4). The cells were trypsinized with 5 ml of 0.25% trypsin (Highveld Biological, South Africa) and 0.2% EDTA (Sigma, Germany) and incubated at 37°C for 3 minutes. Following incubation, 10 ml of medium was added and cells were transferred into a 50 ml tube and centrifuged at 1,000 rpm for 5 minutes. Cells were then plated in 12-well plates at a density of 3.5 x 10^4 cells per well for H9C2 myoblasts and ~7 x 10^4 cells per well for CV-1 fibroblasts and HepG2 hepatocytes, respectively. H9C2 myoblasts and CV-1 fibroblasts were maintained in DMEM (Highveld Biological, South Africa) supplemented with 10% fetal calf serum (v/v) and 100 µg/ml of penicillin/streptomycin. HepG2 hepatocytes were maintained in Earle's Base Minimum Eagle's Medium (EMEM) (Highveld Biological, South Africa) supplemented with 10% fetal calf serum (v/v) without penicillin/streptomycin. All cells were incubated at 37°C (95% humidity, 5% CO_2).

c) Transfection studies

The cell transfection studies were carried out using FuGENE 6 Transfection Reagent (Roche, Mannheim, Germany) following the manufacturer's manual. Briefly, twenty four hours following plating, neonatal cardiomyocytes, H9C2, CV-1

and HepG2 cells were transiently transfected for 48 hours with 0.38, 0.2, 0.6 and 0.5 µg, respectively, of the full-length pPIIβ-1317 human ACCβ promoter-reporter luciferase construct (ACCβ plasmid DNA) and 10 ng of pRL CMV-Renilla was added to correct for transfection efficiency. FuGENE 6 transfection reagent was diluted in serum-free medium and added to the ACCβ plasmid DNA. A FuGENE 6:DNA complex was allowed to form by gently mixing and incubation for 15 minutes at room temperature. Following incubation, the FuGENE6:DNA complex was carefully added (drop-wise) into the wells containing medium. Forty eight hours after transfection, cells were subsequently harvested by washing twice with cold 1X PBS, lysed by adding 100 µl of 1X Passive Lysis Buffer (PLB) (Promega, Madison, WI). The lysates were centrifuged in a microfuge at 12,000 rpm for 2 minutes at 4°C. The supernatant was stored at -80°C for subsequent measurement of luciferase activities.

For cotransfection studies, full-length pPIIβ-1317 human ACCβ promoter-reporter luciferase construct was transfected together with 0.19, 0.8, 0.3 and 0.25 µg of the USF1-pUC-SRα expression (generously provided by Dr. Tetsuya Kamataki, Hokkaido University, Japan) for neonatal cardiomyocytes, H9C2, CV-1 and HepG2 cells, respectively. In additional experiments, promoter deletion constructs pPIIβ-569/+65, pPIIβ-m569/+65, pPIIβ-349/+65, pPIIβ-93/+65, pPIIβ-38/+65 and pPIIβ-18/+65 were transfected as earlier described (deletion constructs shown in Figure 1). The serial deletion human ACCβ promoter-

reporter luciferase constructs have been previously described[208] and were kindly provided by Dr. K-S Kim (Yonsei University College of Medicine, Seoul, Korea).

Transfection studies also included 0.38 µg of the USF1 reporter construct (pUSF1-Luc) (Panomics, Redwood City, CA) containing multiple copies of USF1-specific enhancer elements in order to provide a measure of endogenous USF1 transcriptional activity in cardiomyocytes, H9C2 myoblasts and CV-1 fibroblasts.

To determine luciferase activity, the dual luciferase reporter-assay system was used according to the manufacturer's instructions (Promega, Madison, WI). Total DNA concentrations were equalized with pGL3-Basic (vector only). Results are expressed as relative luciferase units normalized to the activity of pGL3-Basic for ACCβ promoter-reporter luciferase construct and pControl-Luc for USF1 reporter vector (vector only = 1). These data represent the mean ± SEM of 2 or more independent experiments performed in triplicates. To further assess the mechanisms responsible for the USF1-mediated induction of ACCβ promoter activity, we performed various treatments. For the low versus high glucose experiments, cells were plated in charcoal-stripped fetal calf serum (sFCS) to avoid possible interference of growth factors with the glucose response. Twenty four hours after transfection, cells were washed twice with phosphate-buffered saline followed by an exposure to either low (5.5 mM) or high glucose (25 mM) for an additional 24 hours. 2-Deoxyglucose (25 mM) was added to cells for 24 hours under high glucose (25 mM) conditions. For the okadaic acid experiments,

cells were treated (48 hours after transfection) with 50 nM, 75 nM and 100 nM okadaic acid (Sigma-Aldrich, Germany) for 6 hours. In a separate experiment, cells were incubated with 1 mM and 1.5 mM oxythiamine (Sigma-Aldrich, Germany) for twenty four hours.

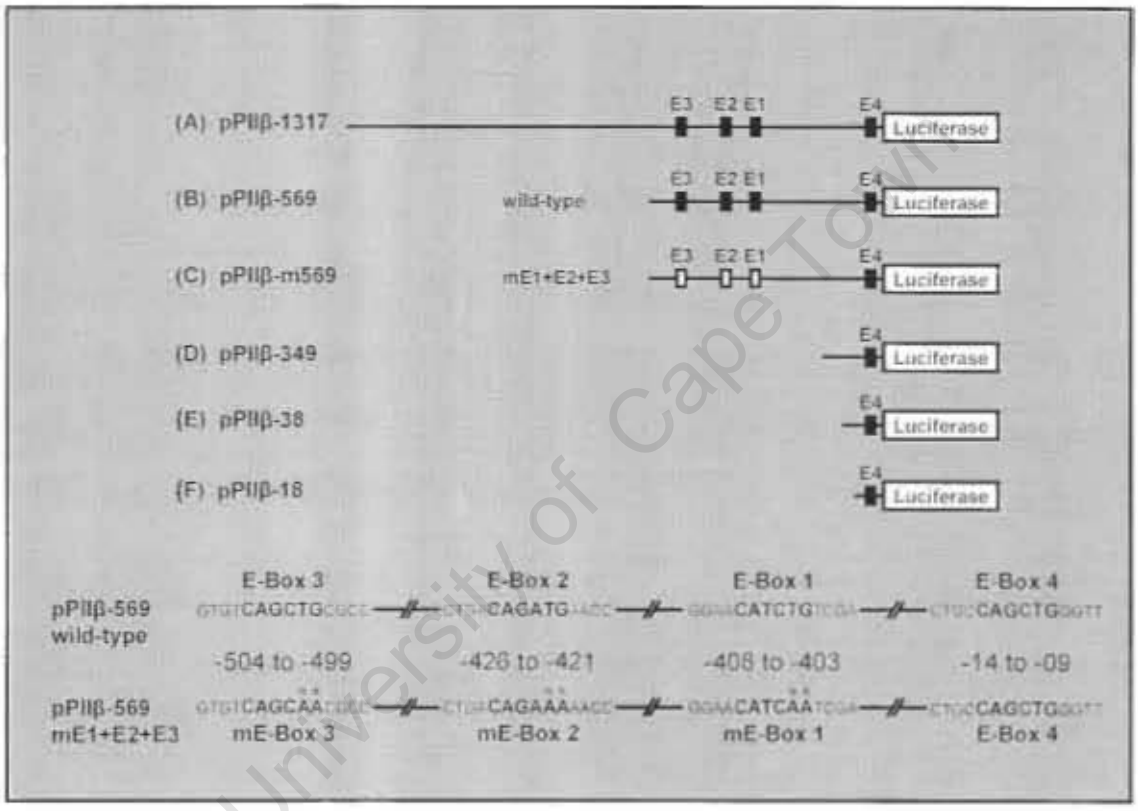


Figure 1. Schematic representation of human ACCβ promoter II (pPIIβ-1317) serial deletion reporter constructs.
(A) Full-length pPIIβ-1317/+65, with E-boxes 1-4 (CANNTG). E-box mutants were generated for E-boxes 1-3 by site-directed mutagenesis, substituting TG with AA in each CANNTG (denoted by asterisk). (B) pPIIβ-569/+65, with E-boxes 1-4 (wild-type). (C) pPIIβ-m569/+65, with mutated E-boxes 1-3 and intact E-box 4 (mE1+E2+E3). (D) pPIIβ-349/+65, with E-box 4 only. (E) pPIIβ-18/+65, with E-box 4 only.

2.2.3. Statistical analysis

These data are expressed as mean ± SEM. The mRNA and peptide levels are reported in arbitrary units (AU) determined by densitometry and normalized to controls (mean control value is set to 100). The unpaired Student's t test was used to determine differences between two groups. ANOVA one–way analysis of variance was used to determine significance between control and experimental groups where more than two sample populations were compared. All post-hoc determinations (when ANOVA showed significant differences) were made by the Bonferroni test. Values were considered significant when $p<0.05$.

2.3. Results

2.3.1. Induction of in vivo ACCβ gene and protein expression in response to elevated glucose levels

To investigate our hypothesis that ACCβ gene expression is glucose-responsive in the heart, mice were fasted and re-fed a high carbohydrate diet (RHC) for 48 hours. Circulating plasma glucose levels and ACCβ gene and protein expression were thereafter determined. Plasma glucose levels were significantly elevated from 6.6 ± 0.2 to 10.3 ± 1.6 mmol/L ($p<0.01$ vs. control, $n\geq6$) in the RHC group (Figure 2A).

In parallel, ACCβ steady-state gene ($n\geq5$, $p<0.001$ vs. control) and protein ($n=5$, $p<0.001$ vs. control) expression were significantly elevated following high carbohydrate re-feeding (Figures 2B & 2C). These data suggest that the induction of ACCβ gene expression in the mouse heart is associated with elevated systemic glucose levels.

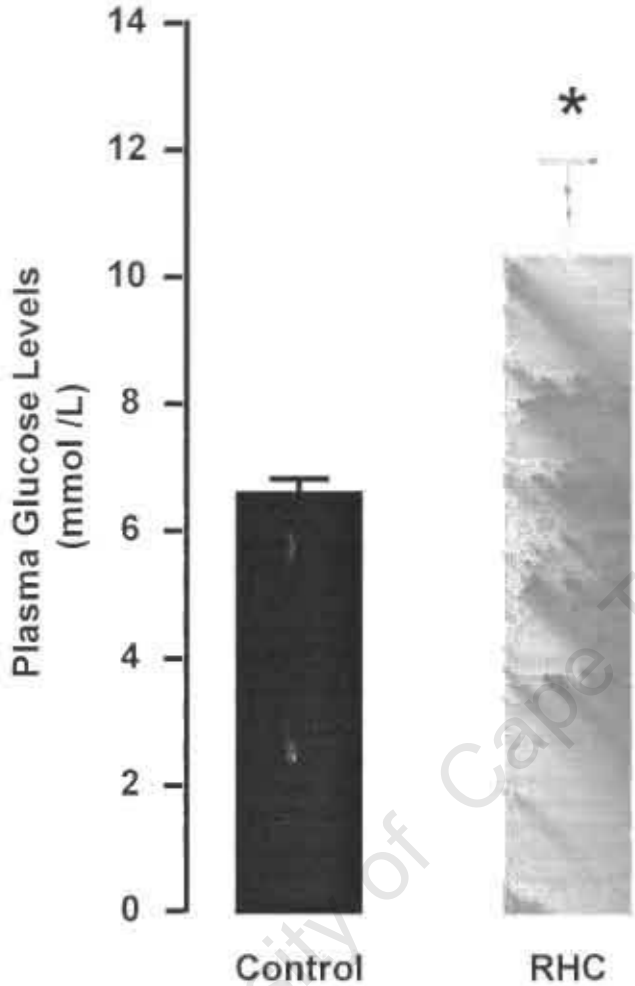


Figure 2A. Elevated glucose levels following high carbohydrate refeeding. Blood was collected by cardiac puncture and plasma glucose levels measured in re-fed high carbohydrate (RHC) diet and control mice. Data are represented as means ± SEM (n≥6). The asterisk denotes a significant difference between RHC and control mice, *p<0.01 vs. control.

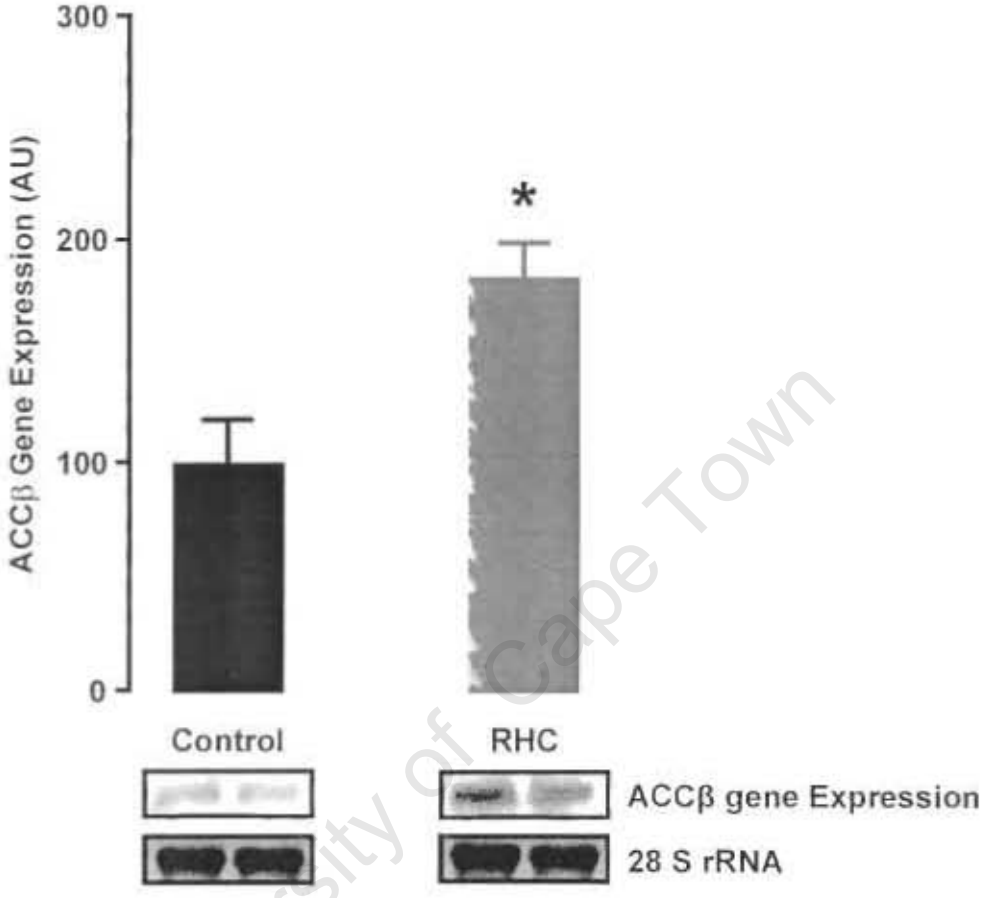


Figure 2B. Induction of *in vivo* ACCβ gene expression in response to elevated glucose levels. Cardiac ACCβ mRNA levels in response to a refed high carbohydrate (RHC) diet. Inset - representative Northern blot is shown for ACCβ. Ethidium bromide stained 28 S rRNA is included to demonstrate unchanged RNA expression. Densitometric analysis of Northern blots was performed and data normalized to 28 S rRNA (mean control value set at 100). The bars represent mean ± SEM (n≥5). The asterisk denotes a significant difference between RHC and control mice, *p<0.001. AU – arbitrary units.

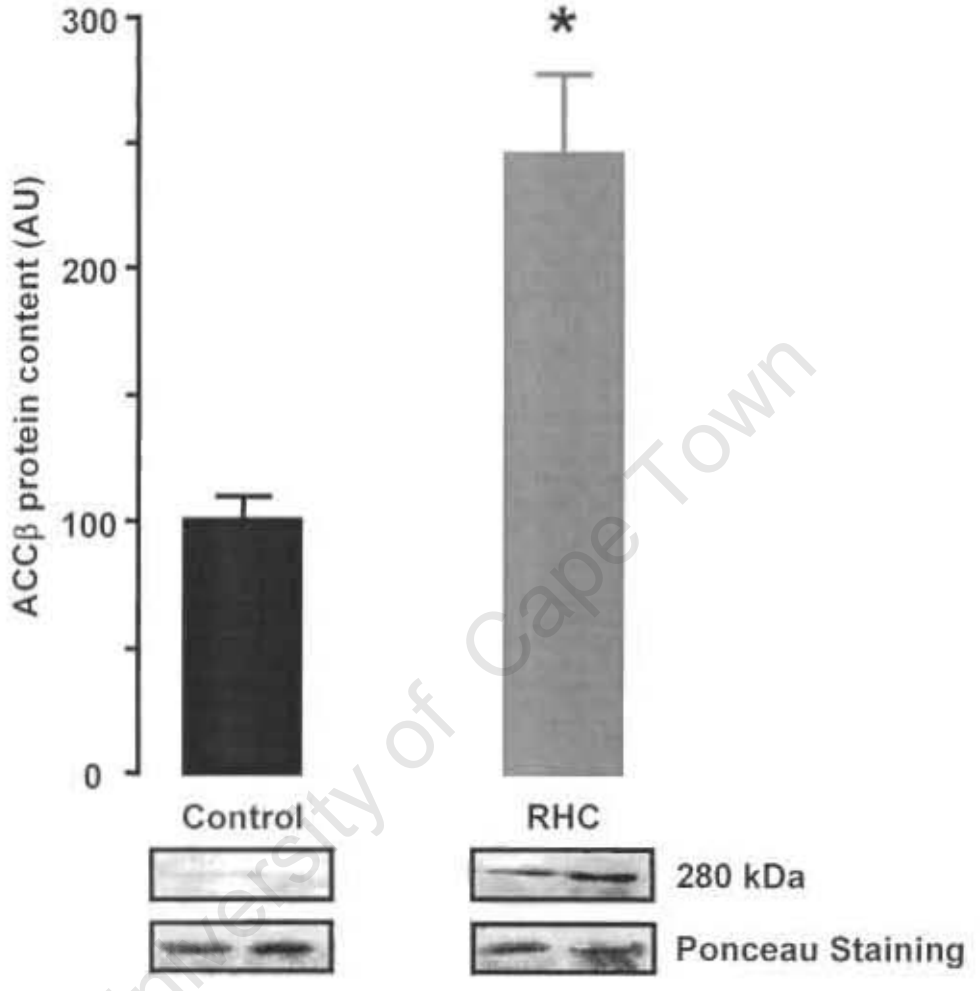


Figure 2C. Induction of *in vivo* ACCβ protein expression in response to elevated glucose levels. ACCβ protein (280 kDa) levels in response to RHC. Bar graphs represent the mean cardiac acetyl-CoA carboxylase (ACCβ) peptide levels for RHC and control mice normalized to Ponceau-stained protein bands (mean control value set at 100) (n=5). The asterisk denotes a significant difference between RHC and control mice, *p<0.001. Inset - A representative Western blot for ACCβ. Ponceau-stained bands are shown to indicate unchanged protein loading. AU – arbitrary units.

2.3.2. Glucose induces human ACCβ promoter activity

To further elucidate the role of glucose in the induction of ACCβ gene expression, we began by transiently transfecting a 1,317 bp human ACCβ promoter-luciferase construct (pPIIβ-1317/+65) into neonatal cardiomyocytes, H9C2 myoblasts, CV-1 fibroblasts and HepG2 hepatocytes. Twenty four hours after transfection, cells were exposed to either low (5.5 mM) or high glucose (25 mM) for an additional 24 hours. High glucose exposure resulted in a moderate but non-significant increase in ACCβ promoter activity in neonatal cardiomyocytes and CV-1 fibroblasts. Moreover, high glucose exposure significantly elevated ACCβ promoter activity from 1.87 ± 0.26 to 3.91 ± 0.37 ($n=9$, $p<0.001$) and 1.4 ± 0.2 to 3.5 ± 0.2 fold ($n=3$) in H9C2 myoblasts and HepG2 hepatocytes, respectively (Figure 3). In agreement with our gene and protein expression data, these analyses demonstrate that the human ACCβ promoter activity can be induced by elevated glucose levels in a cell type-specific manner.

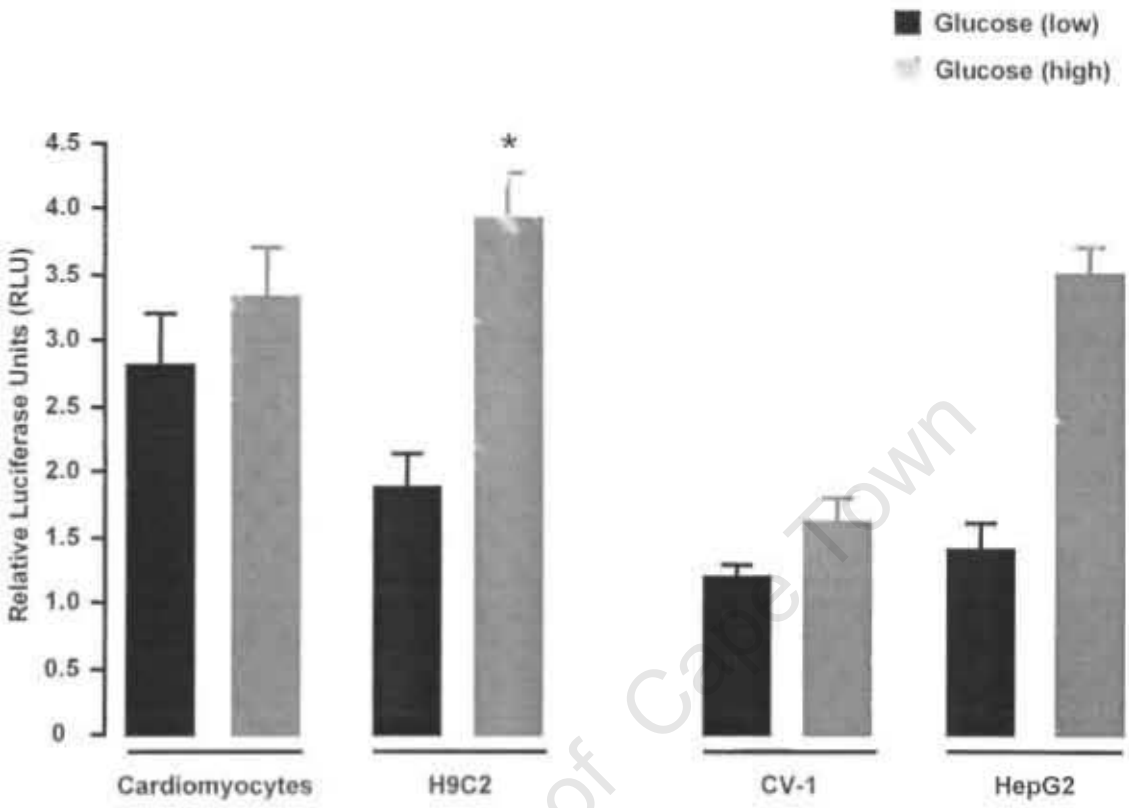


Figure 3. ACCβ promoter luciferase activity in response to low/high glucose levels. Cardiomyocytes, H9C2 myoblasts, CV-1 fibroblasts and HepG2 hepatocytes were transiently cotransfected with the full-length ACCβ promoter-reporter construct (pPIIβ-1317/+65). Cells were exposed to low (5.5 mM) or high glucose (25 mM) levels for an additional 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates, unless stated. HepG2, n=1 independent experiment. *$p<0.05$ vs. pPIIβ-1317/+65 (low glucose).

2.3.3. USF1 transactivates ACCβ promoter activity

To delineate transcriptional pathways mediating glucose-induced ACCβ promoter activity, we next performed transfection assays employing an expression vector of USF1, a candidate glucose-responsive transcription factor. Recently, four E-box consensus sequences have been identified on the 1,317 bp human ACCβ promoter[208] i.e. E-box 1, E-box 2 and E-box 3 located further upstream of the transcription start site (-569 to -348) and E-box 4 located immediately adjacent to it (-14 to -9) (Figure 1). Since high glucose exposure failed to increase ACCβ promoter activity in neonatal cardiomyocytes and CV-1 fibroblasts, we first tested whether USF1 would induce ACCβ promoter activity under low glucose. Secondly, we tested whether high glucose exposure would further increase USF1-mediated ACCβ promoter activity. In neonatal cardiomyocytes, USF1 overexpression increased ACCβ promoter activity from 2.75 ± 0.37 to 5.91 ± 0.47 -fold ($n=6$, $p<0.05$) and 3.23 ± 0.38 to 4.66 ± 0.35 -fold ($n=6$, $p<0.05$) under low and high glucose exposure, respectively, (Figure 4A). In CV-1 cells, USF1 markedly increased ACCβ promoter activity by 19.2 ± 1.33 -fold ($n=15$, $p<0.001$), under low glucose exposure. A further USF1-mediated increase of ACCβ promoter activity was observed with high glucose exposure ($p<0.01$ vs. USF1 + high glucose) (Figure 4B). However, USF1 overexpression failed to increase ACCβ promoter activity in H9C2 myoblasts whereas induction of the ACCβ promoter activity was noted in HepG2 hepatocytes ($n=3$) (Figure 4C). Collectively, these data suggest that USF1 transactivates the human ACCβ promoter activity in a cell type-specific manner and further implicate the

involvement of glucose and/or USF1 responsive regions in the regulation of the ACCβ promoter.

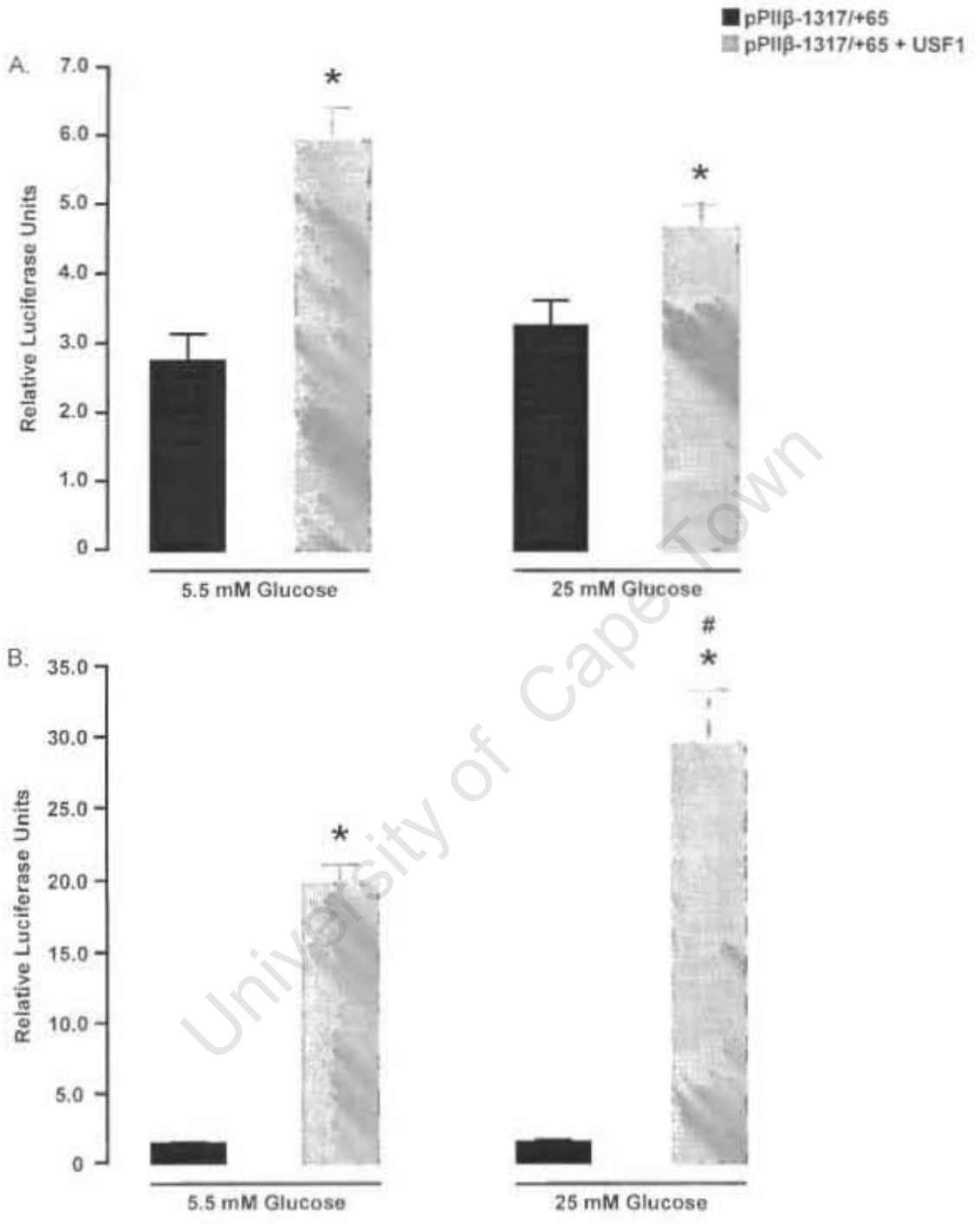


Figure 4A and 4B. USF1 transactivates human ACCβ promoter activity. A) Cardiomyocytes and B) CV-1 fibroblasts were transiently cotransfected with the full-length ACCβ promoter-reporter construct (pPIIβ-1317/+65) and USF1 expression vector. After 24 hours, cells were exposed to low (5.5 mM) or high glucose (25 mM) levels for an additional 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic=1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.001$ vs. pPIIβ-1317/+65; #$p<0.01$ vs. pPIIβ-1317/+65 + USF1 (low glucose).

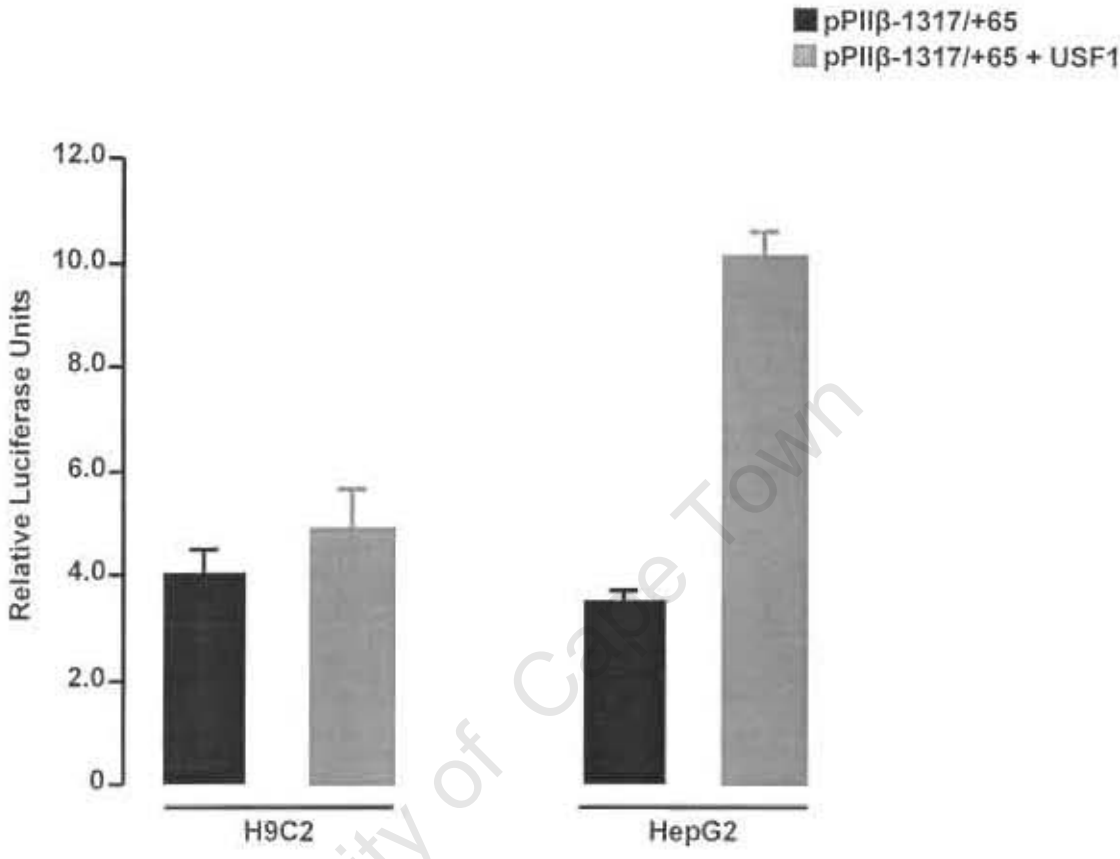


Figure 4C. USF1 transactivates human ACCβ promoter activity in a cell type-specific manner. H9C2 myoblasts and HepG2 hepatocytes were transiently cotransfected with the full-length ACCβ promoter-reporter construct (-1317/+65) and USF1 expression vector. Cells were exposed to high glucose (25 mM) levels for 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data represent mean ± SEM of 2-5 independent experiments performed in triplicates, unless stated. HepG2, n=3 or 1 independent experiment.

2.3.4. Delineation of the transcriptional machinery pathways directing human ACCβ promoter activity

a) 2-Deoxyglucose (2-DG) inhibits human ACCβ promoter activity

Previous studies have shown that xylulose-5-phosphate, an intermediate in pentose phosphate pathway (PPP), increases transcription of glucose-responsive genes via stimulation of PP2A.[10, 11, 13] To investigate the transcriptional mechanisms directing USF1-mediated ACCβ promoter activity, we reasoned that inhibition of the PPP pathway should result in a reduction of USF1-mediated transactivation of ACCβ promoter activity. To accomplish this, we used two pharmacological compounds known to inhibit the PPP pathway i.e. 2-deoxyglucose and oxythiamine. 2-Deoxyglucose (2-DG), a nonmetabolizable glucose analogue, has previously been used as a glucose deprivation mimetic[218, 219] and has been shown to inhibit G-6-PD activity, a critical step in the PPP pathway, in the liver and skeletal muscle of rats.[218] Furthermore, 2-DG administration in HeLa cells strongly inhibited the activity of Sp1, another glucose-responsive transcription factor.[220] To test our hypothesis, we treated neonatal cardiomyocytes and CV-1 fibroblasts with 25 mM 2-DG for 24 hours. In neonatal cardiomyocytes, 2-DG did not inhibit USF1-induced ACCβ promoter activity (Figure 5A). However, in CV-1 cells, USF1-induced ACCβ promoter activity was strongly suppressed in the presence of 2-DG ($p<0.01$ vs. ACCβ + USF1) (Figure 5B). Moreover, oxythiamine, a transketolase inhibitor,[221, 222] did not have any effect on USF1-induced ACCβ promoter activity in both cell lines

employed (Figures 6A and B). These data implicate the involvement of glucose and its metabolite, X-5-P in the transcriptional regulation of the ACCβ promoter in CV-1 cells.

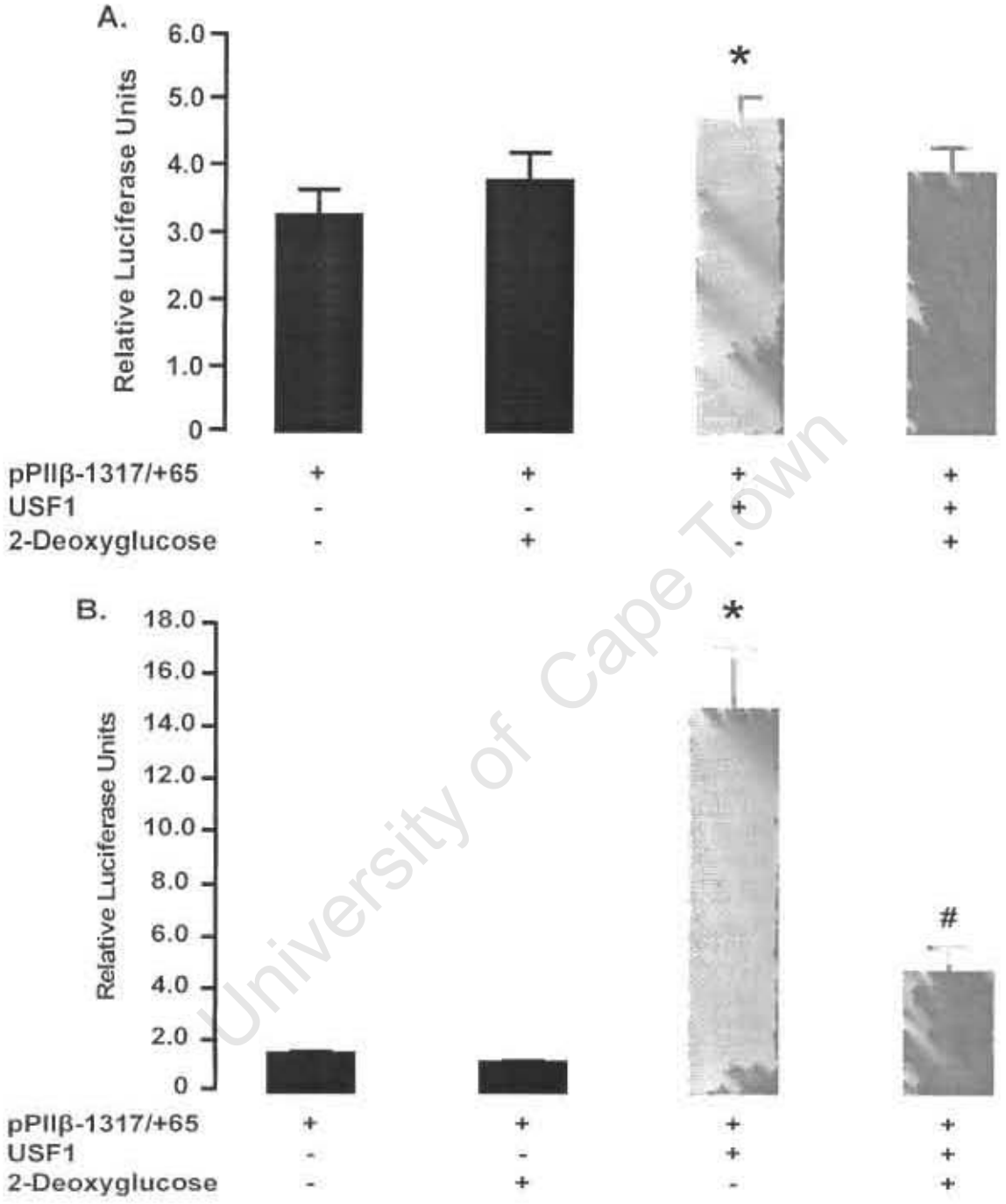


Figure 5. The effect of 2-deoxyglucose on USF1-induced activation of the human ACCβ promoter activity. (A) Cardiomyocytes and (B) CV-1 cells were transiently cotransfected with the full-length ACCβ promoter-reporter construct (pPIIβ-1317/+65) and USF1 expression vector under high glucose (25 mM) conditions. The following day, cells were exposed to 25mM 2-deoxyglucose for 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic=1). These data represent mean ± SEM of 2 independent experiments performed in triplicates. *$p<0.05$ vs. pPIIβ-1317/+65; #$p<0.001$ vs. pPIIβ-1317/+65 + USF1.

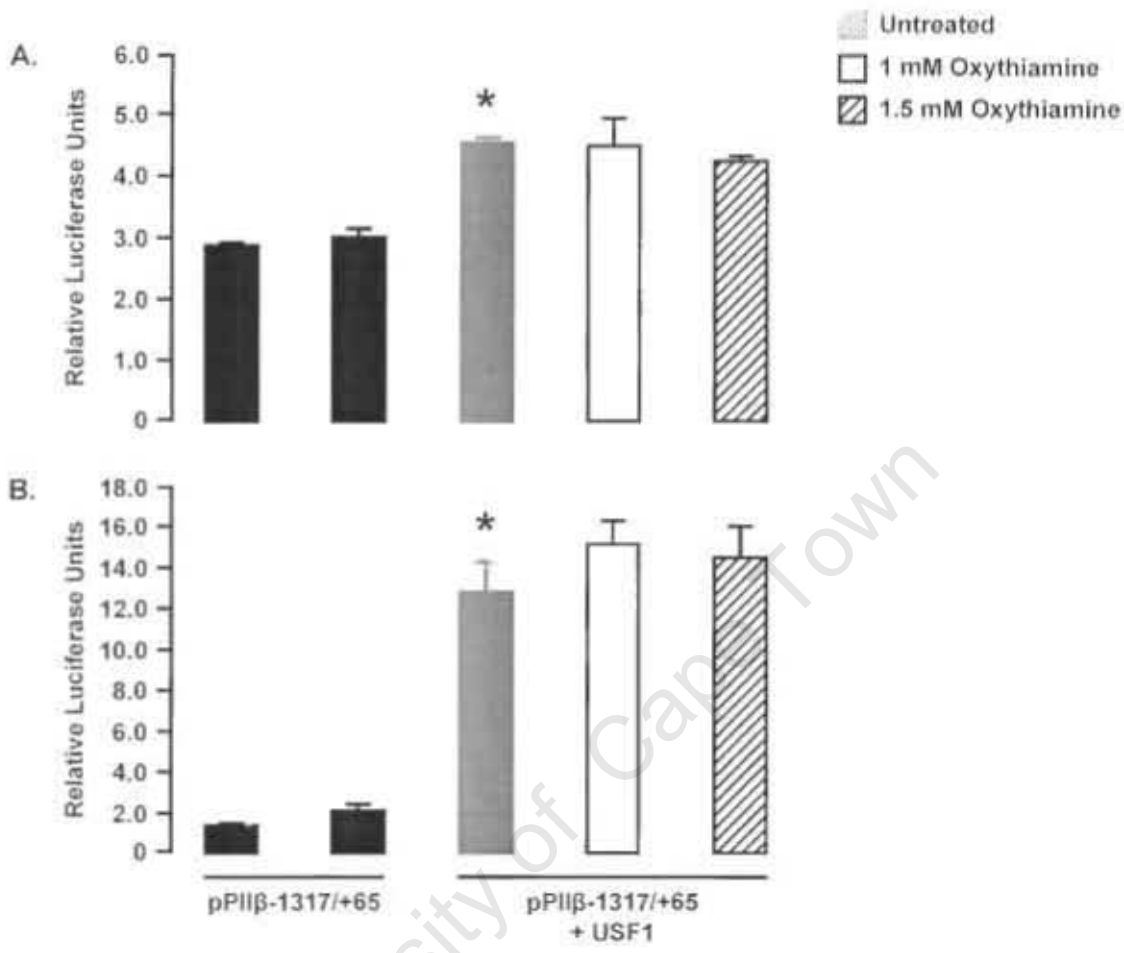


Figure 6. The effect of oxythiamine on human ACCβ promoter activity.
(A) Cardiomyocytes and (B) CV-1 fibroblasts were transiently cotransfected with full-length ACCβ promoter and USF1 expression vector. The following day, cells were treated with the indicated concentrations of oxythiamine for 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data represent mean ± SEM of 2 independent experiments performed in triplicates. *p<0.05 vs. pPIIβ-1317/+65.

b) Inhibition of PP2A reduces human ACCβ promoter activity

Protein phosphatase 2A (PP2A), a downstream target of X-5-P, has been implicated in increased gene transcription by promoting translocation of carbohydrate response element binding protein (ChREBP) into the nucleus where it binds to the promoter region of target genes.[10, 11, 13] In this study, I investigated whether PP2A phosphorylates and activates USF1 to subsequently promote ACCβ transcriptional activation. I therefore proposed that inhibition of PP2A would reduce USF1-mediated ACCβ promoter activity. Okadaic acid, a PP2A inhibitor, was used to confirm whether X-5-P mediated transcriptional inhibition of USF1-induced ACCβ promoter activity involved activation of PP2A. In this study, I found that okadaic acid suppressed USF1-induced ACCβ promoter activity in a dose-dependent manner in neonatal cardiomyocytes and CV-1 cells (Figures 7A and B). These data confirmed the involvement of PP2A in the USF1-mediated transcriptional regulation of ACCβ promoter.

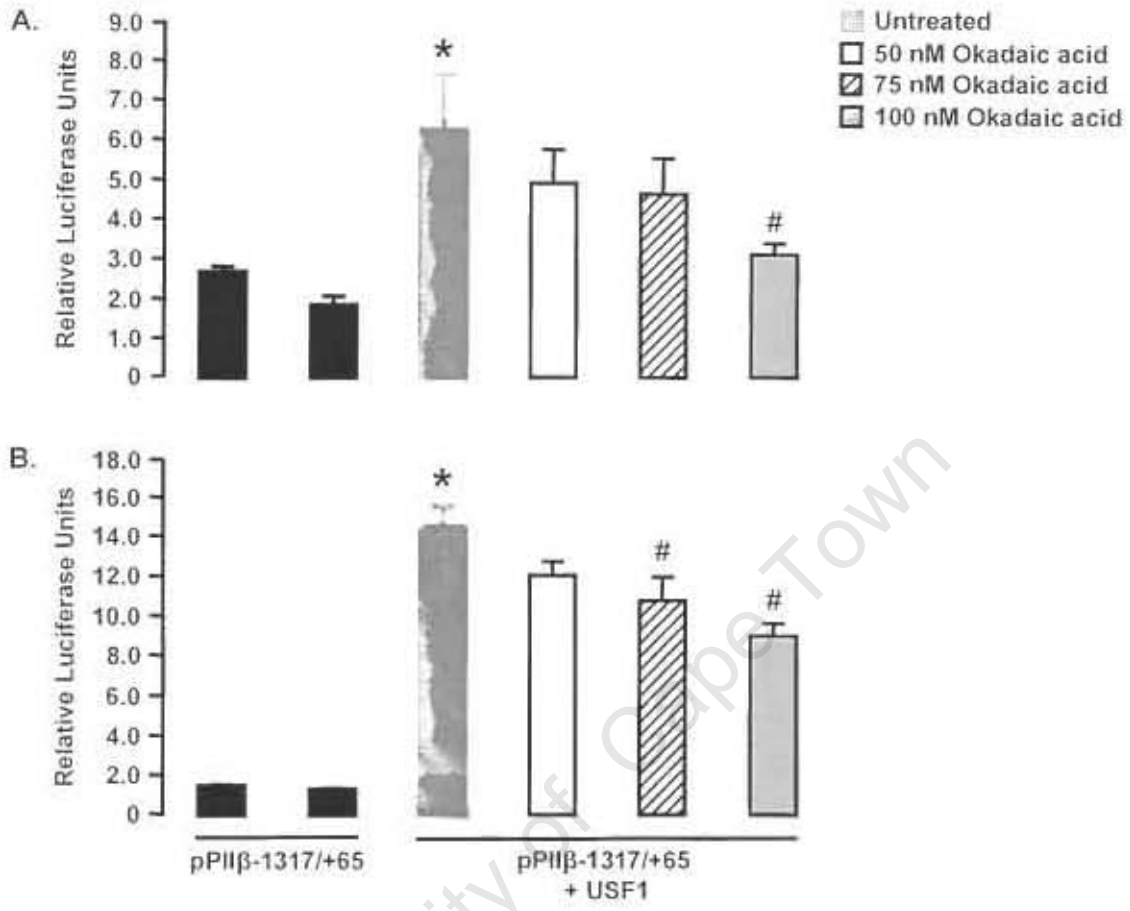
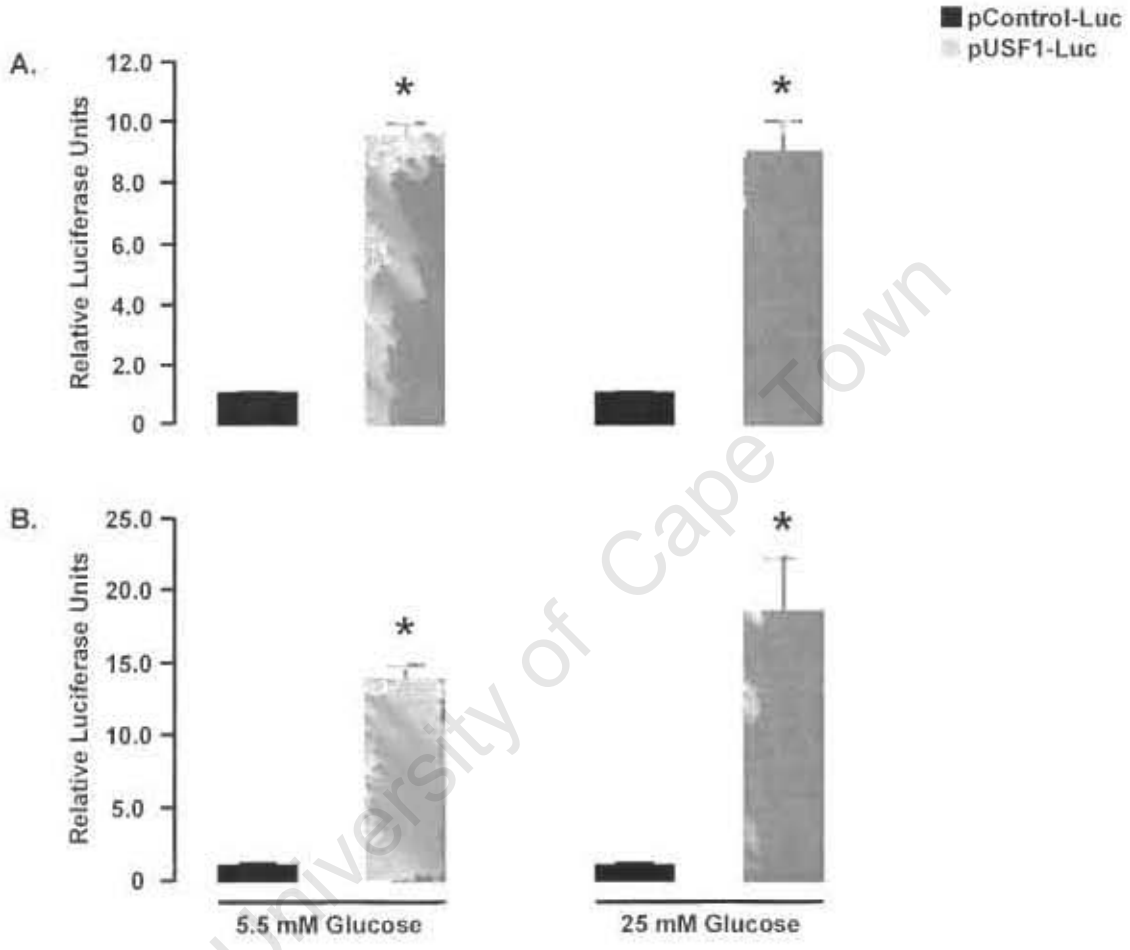


Figure 7. The effect of okadaic acid on human ACCβ promoter activity.
The full-length ACCβ promoter (pPIIβ-1317/+65) and USF1 expression vector were transiently cotransfected into cardiomyocytes (A) and CV-1 cells (B). After 48 hours, the cells were treated with okadaic acid for 6 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic=1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates. *p<0.05 vs. pPIIβ-1317/+65; #p<0.05 vs. pPIIβ-1317/+65 + USF1 (untreated).

2.3.5. Assessment of endogenous USF1 transcriptional activity in cardiomyocytes and CV-1 fibroblasts

a) Endogenous USF1 transcriptional activity is independent of glucose levels.

To determine whether endogenous activity of USF1 transcription factor is under the control of glucose, we transiently transfected neonatal cardiomyocytes and CV-1 fibroblasts with a USF1 reporter construct (containing multiple copies of USF1-specific enhancer elements) under low (5.5 mM) and high (25 mM) glucose conditions. The transcriptional activity of the USF1 reporter construct was significantly higher compared to control reporter construct in both neonatal cardiomyocytes and CV-1 fibroblasts ($p<0.001$ vs. pControl-Luc in both cell lines). However, exposure of the USF1 reporter construct to either low or high glucose conditions showed a similar effect, thereby implying that endogenous USF1 transcriptional activity is independent of glucose levels (Figures 8A and B).



Figures 8A and B. Endogenous USF1 transcriptional activity is independent of glucose levels. The USF1 reporter vector (pUSF1-Luc) was transiently transfected into cardiomyocytes (A) and CV-1 cells (B). After 24 hours, cells were exposed to low (5.5 mM) or high (25 mM) glucose for a further 24 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pControl-Luc = 1). These data represent mean ± SEM of 2 independent experiments performed in triplicates, *p<0.01 vs. pControl-Luc.

b) Endogenous USF1 transcriptional activity is inhibited by okadaic acid

We have previously demonstrated that USF1-mediated induction of ACCβ promoter activity is inhibited by okadaic acid (Figure 7). To further verify these results, we transiently transfected the USF1 reporter construct in the presence of okadaic acid, a PP2A inhibitor. Again, okadaic acid significantly attenuated USF1 transcriptional activity in a dose-dependant manner ($p<0.05$) (Figure 9). These data further support the role of glucose metabolites in the transcriptional activation of the human ACCβ gene promoter.

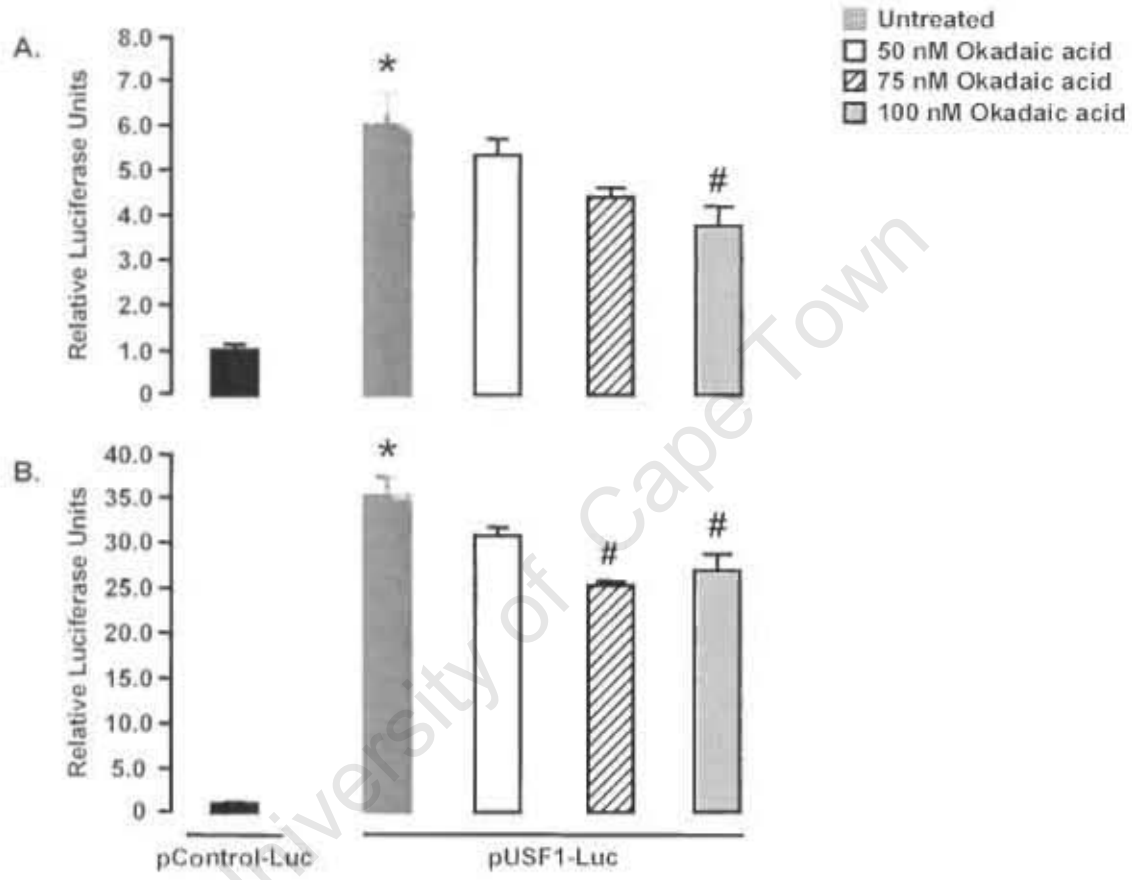


Figure 9. Okadaic acid suppresses endogenous USF1 transcriptional activity. The USF1 reporter vector (pUSF1-Luc) was transiently transfected into cardiomyocytes (A) and CV-1 cells (B). After 48 hours, cells were treated with okadaic acid for 6 hours and luciferase activities were measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pControl-Luc = 1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates, unless stated. *$p<0.001$ vs. pControl-Luc; #$p<0.05$ vs. pUSF1-Luc (untreated).

2.3.6. Assessment of USF1-dependent transactivation sites of the human ACCβ promoter

a) USF1 transactivation of the human ACCβ promoter does not occur via E-Boxes 1-3.

Previous investigators have demonstrated that USF1 and USF2 isoforms bind E-box sequence motifs (CANNTG) as homo and/or heterodimers on promoter regions, increasing expression of several target genes.[159] Moreover, the human ACCβ promoter is transcriptionally activated by the muscle specific factor, MyoD, via the E-boxes1-3 located at positions -569 to -348.[208] To further investigate whether E-boxes1-3 are involved in USF1-induced ACCβ promoter activity, I employed two human ACCβ promoter deletion constructs i.e. pPIIβ-569/+65 (with E-boxes 1-4) and pPIIβ-m569/+65 (mutations within E-box 1, E-box 2 and E-box 3) (Figure 1). Here, USF1 overexpression induced ACCβ promoter activity ($p<0.05$ vs. -569/+65). The mutations in E-boxes1-3 showed no significant changes for basal or USF1-induced ACCβ promoter activity in both cell lines (Figure 10). These data indicate that E-boxes 1-3 are not involved in the basal or USF1-induced ACCβ promoter activity, thereby raising the possibility that E-box 4 (-14 to -9) and/or an identified region on the promoter may play a significant role in the induction of ACCβ promoter activity.

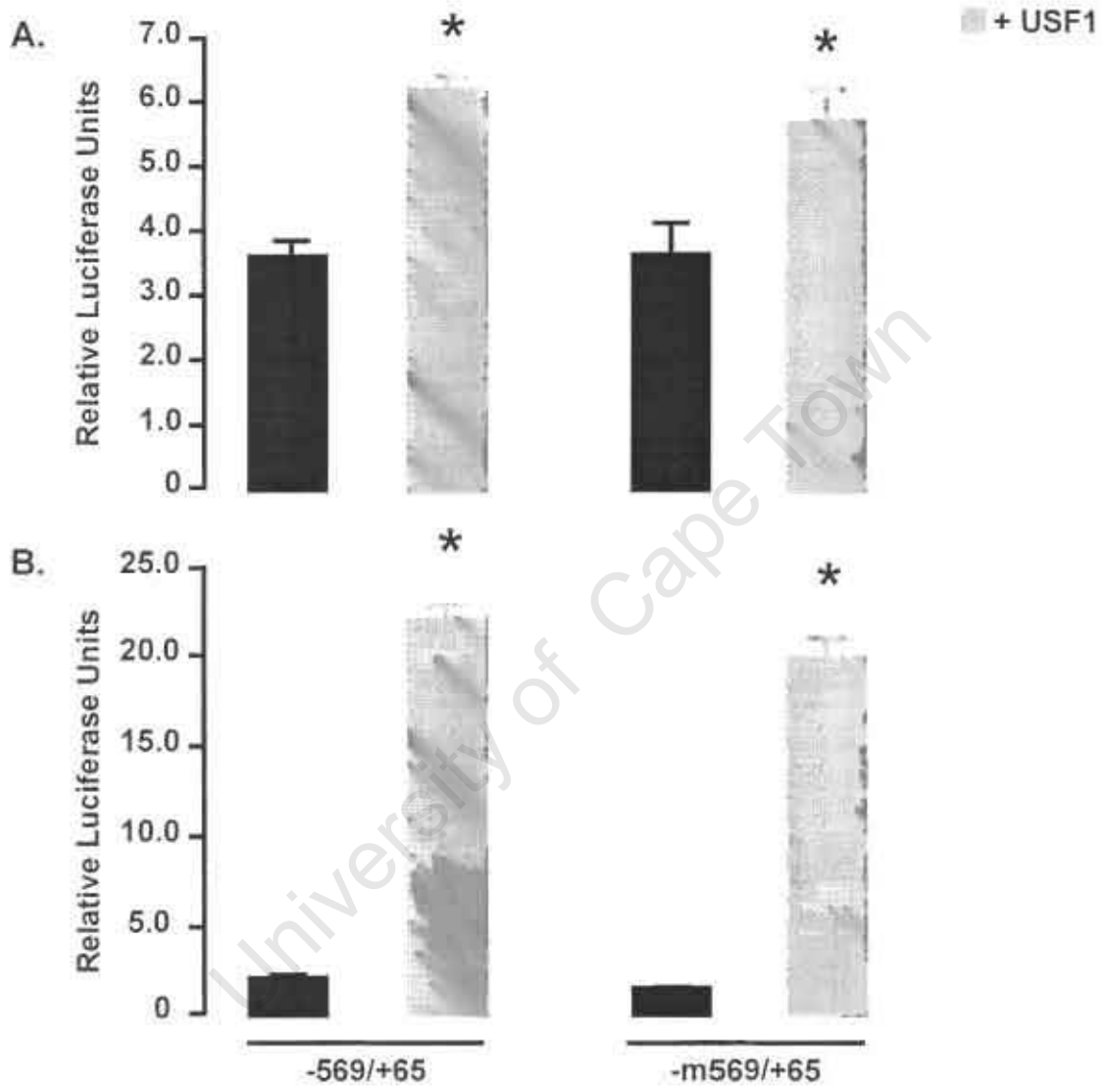


Figure 10. USF1 transactivation of the human ACCβ promoter does not occur via E–boxes1-3. Cardiomyocytes (A) and CV-1 cells (B) were transiently cotransfected with either the wild-type (pPIIβ-569/+65) or E-box mutant reporter construct (pPIIβ-m569/+65), (refer to Figure 1), together with a USF1 expression vector under high glucose (25 mM) conditions for 48 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic=1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates. *p<0.05 vs. pPIIβ-569/+65 only or pPIIβ-m569/+65 only.

b) Assessment of USF1-dependent transactivation region of the human ACCβ promoter using serial deletion constructs

To identify USF1-responsive regions within the human ACCβ promoter, serial deletion constructs (Figure 1) were transiently cotransfected with the USF1 expression vector. In neonatal cardiomyocytes, USF1 overexpression led to a 6-fold induction of basal promoter activity of deletion constructs pPIIβ-1317 and pPIIβ-569 ($p<0.05$ vs. ACCβ basal promoter activity) while basal promoter activity of deletion construct pPIIβ-93/+65 was moderately increased to 8.6-fold, suggesting the presence of a repressor site between -569 and -94 region. On the other hand, deletion of region -92 to -39 resulted in about 50% loss of both basal promoter activity and USF1 responsiveness, suggesting that endogenous activators and/or USF1 may bind to this region thereby increasing ACCβ promoter activity. A further loss of basal promoter activity (~50%) was observed with deletion construct pPIIβ-18/+65 but USF1 responsiveness was similar to that of pPIIβ-38/+65 (Figure 11A).

In CV-1 cells, USF1 responsiveness was similar for deletion constructs pPIIβ-1317, pPIIβ-569 and pPIIβ-93 ($p<0.05$ vs. ACCβ basal promoter activity). Again, deletion of region -92 to -39 resulted in a significant reduction of USF1 responsiveness. In addition, deletion constructs pPIIβ-38 and pPIIβ-18 had a similar response to USF1 overexpression (Figure 11B). These data indicate that region -92 to -39 could be a possible target for USF1 responsiveness. Moreover, USF1 responsiveness was not completely lost with deletion of region -92 to -39, thereby suggesting E-box4 could be involved.

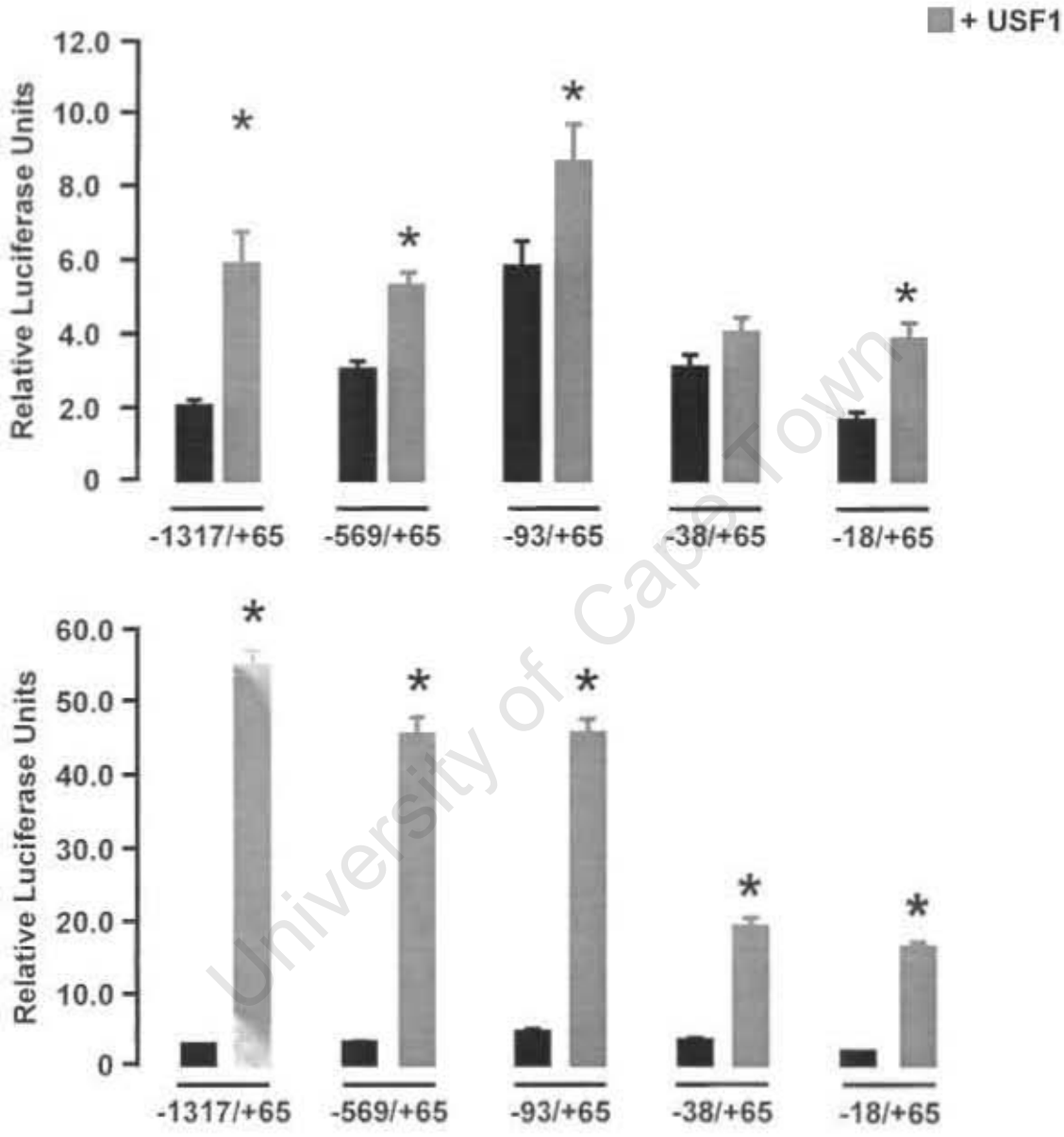


Figure 11. Assessment of USF1-dependent transactivation of the human ACCβ promoter using serial deletion constructs. Serial deletion constructs of the human ACCβ promoter were transiently cotransfected with the USF1 expression vector into cardiomyocytes (A) and CV-1 cells (B) for 48 hours. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data represent mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.05$ vs. ACCβ basal activity.

2.4. Discussion

The purpose of this study was to investigate transcriptional mechanisms directing glucose-mediated ACCβ gene expression. Here, we show an induction of *in vivo* cardiac ACCβ gene and protein expression in mice re-fed a high carbohydrate diet after an acute fast. To further investigate the association between augmented glucose levels and increased ACCβ gene expression, we transiently transfected neonatal cardiomyocytes, H9C2, CV-1 and HepG2 cells with a full-length human ACCβ promoter-luciferase construct under low or high glucose exposure. In agreement with our *in vivo* expression data, transfection studies show increased human ACCβ promoter activity in cardiac-derived H9C2 myoblasts and HepG2 hepatocytes in response to elevated glucose levels. However, no glucose-mediated increase in ACCβ promoter activity response was observed in neonatal cardiomyocytes. To delineate transcriptional mechanisms directing ACCβ gene transcription, we performed cotransfection studies employing human ACCβ promoter deletion constructs and a USF1 overexpression vector. Under high glucose conditions, USF1 overexpression further increased human ACCβ promoter activity in neonatal cardiomyocytes, CV-1 and HepG2 cells. However, USF1 overexpression did not have any effect on ACCβ promoter activity in H9C2 cells. Our data therefore show that USF1-mediated induction of the human ACCβ promoter activity occurs in a cell type-specific manner.

2.4.1 Glucose acts as a signaling molecule regulating the human ACCβ promoter

The role of metabolic fuels acting as signaling molecules regulating expression of numerous metabolic enzyme-encoding genes have recently been highlighted.[11, 51] For example, it has been demonstrated that the FA-responsive transcriptional regulator, peroxisome proliferator-activated receptor α (PPARα), activates numerous FA enzyme-encoding genes in the heart.[41] On the other hand, glucose-responsive transcriptional modulators for e.g. Sp1, SREBP1, and USFs have been implicated in the expression of several lipogenic enzyme-encoding genes.[11, 41, 159, 187] However, it is unclear whether glucose-sensing transcriptional mechanisms regulate expression of metabolic enzyme-encoding genes in the heart. Here, we demonstrate elevated *in vivo* murine cardiac ACCβ gene and protein expression associated with increased plasma glucose levels. In support, Oh et. al.[201] reported increased ACCβ gene expression following high carbohydrate refeeding in the liver. To further corroborate these findings, we performed several transfection assays. Our transfection studies show increased activity of the full-length human ACCβ promoter in H9C2 myoblasts and HepG2 hepatocytes in response to high glucose exposure. Our findings are consistent with Widmer et al.[180] who reported elevated ACCβ protein expression in H4IIE hepatoma cells following exposure to glucose. However, no significant increase of the full-length ACCβ promoter activity was observed in neonatal cardiomyocytes and CV-1 fibroblasts in response to high glucose levels.

2.4.2. USF1 mediates transactivation of the human ACCβ gene promoter

USFs are ubiquitously expressed and have been shown to regulate the transcription of several metabolic enzyme-encoding genes.[12] Wang et.al.[223] found USF1 protein levels are markedly increased in the liver of rats re-fed a high carbohydrate diet following an acute fast. Moreover, USF1 null mice displayed a reduction of the glucose-mediated induction of the liver-type pyruvate kinase and S14 genes.[159] We next performed cotransfection studies with a USF1 overexpressing vector to determine whether USF1 mediates the induction of ACCβ gene promoter activity in response to glucose flux. Here, we show that USF1 overexpression significantly elevates human ACCβ promoter activity in neonatal cardiomyocytes, CV-1 fibroblasts and HepG2 hepatocytes. We also found that high glucose exposure further increased USF1-mediated ACCβ promoter activation in CV-1 fibroblasts. In support, glucose-responsive transcription factors such as SREBP1 and Sp1 have been shown to drive transcriptional activation of human ACCβ promoter in HepG2 hepatocytes.[194] Our data therefore show that the glucose-dependent activation of USF1 can induce ACCβ gene promoter activity in a cell type-specific manner. However, based on the experiments performed, the USF1 response in neonatal cardiomyocytes appears to be glucose-independent. This does not, however, rule out a glucose-driven transcriptional program for ACCβ gene upregulation. The possibility exists that different data may have been generated using adult cardiomyocytes instead of neonatal myocytes, since these cells are expected to exhibit different metabolic profiles. Also, downstream pentose phosphate

pathway (PPP) or HBP glucose metabolites could perhaps mediate such a response. These possibilities require further investigations. Future studies are also required to test our findings in *vivo* since other investigators have indicated that USFs are not responsive to dietary changes.

USF1 overexpression did not induce full-length human ACCβ promoter activity in H9C2 myoblasts, suggesting that glucose-induced ACCβ promoter activity may occur through USF1-independent transcriptional mechanisms. However, it has previously been shown that muscle-type transcription factors for e.g. Myc and MyoD bind to E-boxes of human ACCβ promoter thereby resulting in its activation.[208] Since these muscle-type factors are abundant in cardiac-derived H9C2 myoblasts, endogenous ACCβ gene promoter activation could mask potential USF1 transcriptional effects.

2.4.3. Delineation of regulatory pathways directing ACCβ promoter activity

To further delineate the signaling pathway(s) for glucose-induced transactivation of the human ACCβ promoter, we tested the hypothesis that metabolites of the PPP regulate transcription of glucose-responsive genes via xylulose-5-phosphate/PP2A mechanism. Previous studies have shown that 2-DG directly inhibits G-6-PD, a critical step in the production of downstream intermediates such as ribulose-5-phosphate and X-5-P. However, we found that 2-DG did not inhibit USF1-mediated human ACCβ promoter activity in myocytes. This may be due to the PPP being less active in muscle and highly active in lipogenic tissue

such as adipose since it is involved in the reductive synthesis of FAs from acetyl-CoA.[6] In contrast, 2-DG administration robustly inhibited the USF1-mediated human ACCβ promoter activity in CV-1 fibroblasts. These findings strongly support the involvement of the downstream PPP intermediate X-5-P in the USF1-mediated transcriptional activation of ACCβ in CV-1 fibroblasts. These data also confirm earlier findings that the PPP regulates transcription in a cell type-specific manner. Oxythiamine, a transketolase inhibitor, did not significantly influence the USF1-mediated human ACCβ promoter activity in both neonatal cardiomyocytes and CV-1 fibroblasts. Since transketolase catalyzes the breakdown of X-5-P, its inhibition may not result in the reduction of X-5-P mediated gene transcription. Moreover, oxythiamine inhibits about 70% of transketolase activity and only 15% of G-6-PD activity.[224]

To further strengthen these findings, we tested whether PP2A, a downstream target of X-5-P is also involved in the USF1 mediated transcriptional activation of human ACCβ promoter. Here, we administered okadaic acid, a PP2A inhibitor together with USF1 cotransfections. PP2A inhibition resulted in the reduction of USF1-mediated human ACCβ promoter activity in both neonatal cardiomyocytes and CV-1 fibroblasts. In addition, okadaic acid reduced USF1 transcriptional activity in a dose-dependent manner. Collectively, these data implicate the involvement of a xylulose-5-phosphate/PP2A mediated signaling pathway that directs the transcriptional regulation of the ACCβ gene (Figure 12).

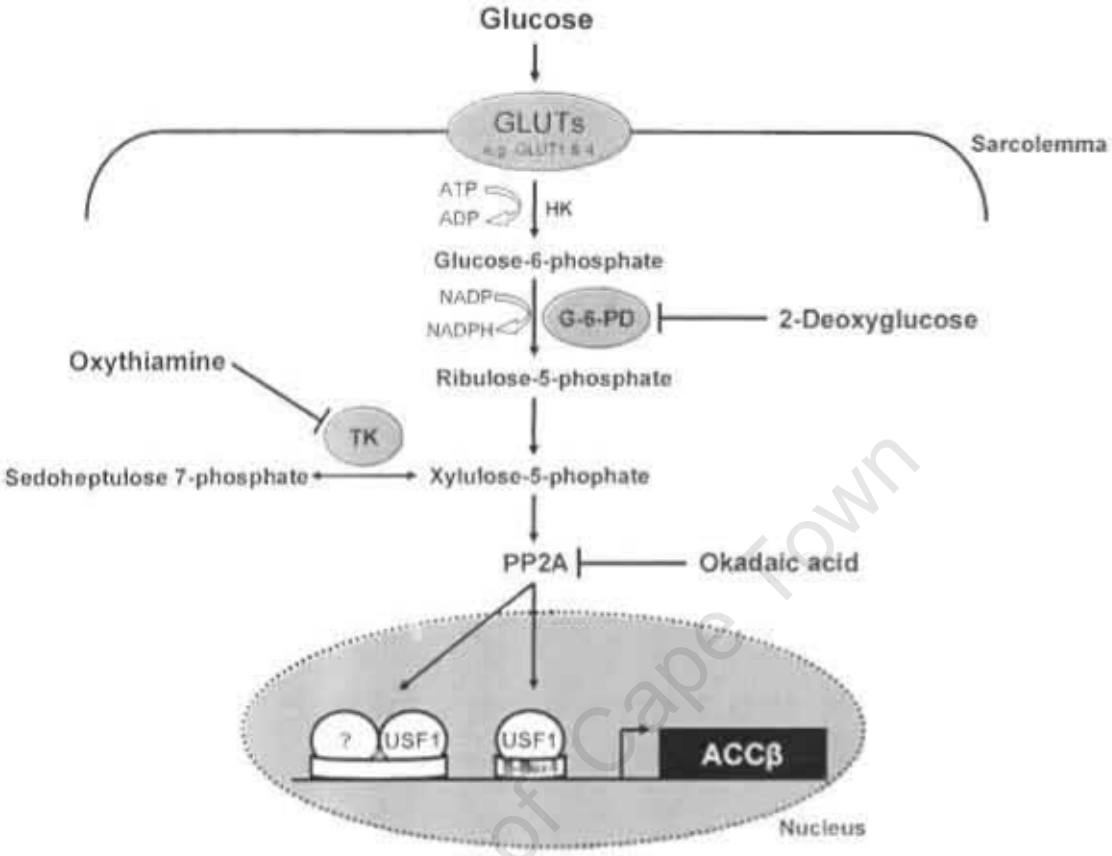


Figure 12. Proposed pathway for USF1-mediated ACCβ gene regulation

2.4.4. Assessment of USF1 responsive region(s) on the human ACCβ promoter

USFs are ubiquitously expressed and have been shown to bind consensus E-box elements located within the promoter regions of several metabolic enzyme-encoding genes.[12] Also, Moore et al.[225] demonstrated that USF-mediated regulation of the muscle-type CPT1 isoform was abolished when E-box elements were mutated. Since four E-box sequence elements were previously identified on the human ACCβ gene promoter,[208] we hypothesized that USF1-induced ACC promoter activity is mediated via the E-boxes. Cotransfection with a mutated E-box construct (E-boxes 1-3) did not diminish human ACCβ promoter activity in both neonatal cardiomyocytes and CV-1 fibroblasts, suggesting that the observed USF1-mediated induction of ACCβ promoter activity may occur via E-box 4 or another identified region on the promoter. To identify USF1-responsive regions within the human ACCβ promoter, serial deletion constructs were transiently cotransfected with the USF1 expression vector. In both neonatal cardiomyocytes and CV-1 fibroblasts, deletion of region -92 to -39 resulted in ~ 50% loss of USF1 responsiveness. Furthermore, USF1 failed to induce the promoter activity of the pPIIβ-38/+65 deletion construct. These data suggest that region -92 to -38 may be a target for USF1 responsiveness. Our data is consistent with previous studies[201] demonstrating that glucose-responsive transcription factors, SREBP1 and Sp1 stimulate the transactivation of the ACCβ promoter via an Sp1-binding site and two sterol regulatory elements located in regions -71 to -66 and -62 to -44, respectively. It has previously been proposed

that USFs function in a cell type-specific manner, through different ratios of USF1 and USF2 homo- and/ or heterodimers, or by interaction with cell type-specific transcription cofactors.[225, 226] Therefore, we cannot exclude USF1 interaction with other transcription factors for e.g. Sp1 or SREBP1 known to also bind to this promoter region. Collectively, our deletion analyses show that USF1-induced human ACCβ promoter activity is mediated in a cell type-specific manner through region -92 to -39 and/or E-box 4 (-14 to -9) elements located in the first 569 bp upstream of the transcription start site. However, additional DNA binding studies are required to determine the exact contributions of individual E-box elements located on the human ACCβ promoter in the different cell lines investigated.

These data also suggest that the glucose-fatty acid cycle may be regulated at a transcriptional level, since one would expect that glucose-induced ACCβ gene expression should result in increased malonyl-CoA levels, thereby inhibiting CPT1 and mitochondrial FAO (Figure 13). It has previously been shown that USFs inhibit the PPARγ coactivator 1 (PGC1)-dependent transactivation of the muscle-type CPT1 promoter in neonatal cardiomyocytes.[225] Also, a recent study demonstrated that elevated glucose levels resulted in a reduction of PPARα mRNA levels and some of its target genes in pancreatic islets.[227] These findings may therefore have an important bearing on the pathogenesis of cardiac disease, particularly when glucose utilization is impaired for e.g. with type 2 diabetes. Abu-Elheiga et al.[194] demonstrated that ACCβ null mice, unlike wildtype controls, did not develop diabetes when fed an obesity-inducing diet. Moreover, Debard

et al.[121] reported increased ACCβ gene expression in Type 2 diabetic patients. Therefore, glucose-mediated induction of ACCβ gene expression together with enhanced FA uptake by the diabetic heart may result in intracellular lipid accumulation and detrimental lipotoxic effects, including apoptosis and eventually contractile dysfunction.[228, 229] This study highlights the importance of investigating transcriptional mechanisms directing ACCβ gene expression in the heart, since this may eventually lead to the development of novel ACCβ inhibitors, thereby potentially diminishing the number of pathologic cardiac events occurring in diabetic patients.

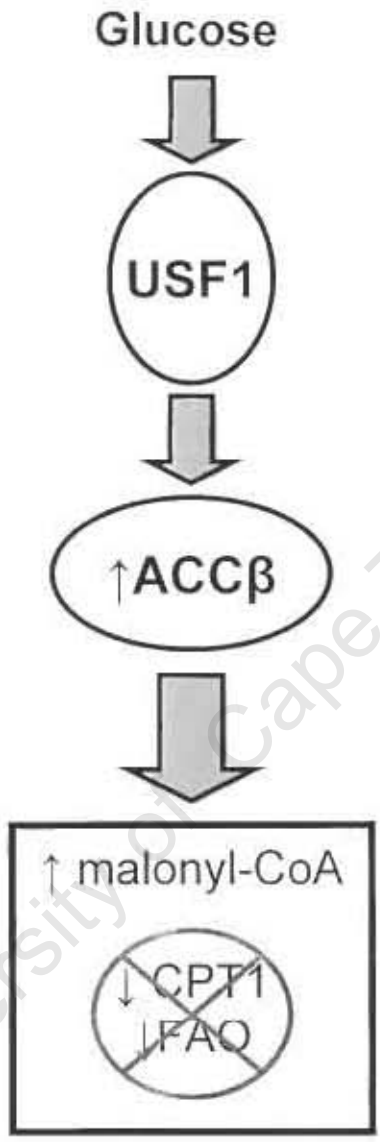


Figure 13. Proposed mechanisms through which USF1-mediated activation may attenuate fatty acid oxidation

2.4.5. Limitations and future directions

H9C2 cells are widely used as a model for cardiomyocytes to advance our knowledge of molecular and biochemical mechanisms of the heart. [101, 207, 208, 264, 265, 266] This cell line exhibits different morphological characteristics to neonatal cardiomyocytes but preserve hormonal and electrical properties found in adult cardiac cells.[264, 265] Despite the use of H9C2 cells to study various aspects of heart function, this cell line differs from neonatal cardiomyocytes in several respects. In the first instance, the H9C2 cell line is a heterogeneous mixture of cells,[265] whereas cardiomyocytes are a homogeneous collection of heart cells. Thus, like the intact heart, H9C2 cells consist of various cell types for e.g. cardiomyocytes, fibroblasts, and endothelial cells. The transfection studies on neonatal cardiomyocytes only examined the response of a single cell type, razing the possibility that other cardiac cell types may in fact mediate the ACCβ response. Further transfection studies are needed to investigate this possibility. For instance, we are planning to do flow cytometry / FACS analysis to sort different heart cells i.e. cardiac fibroblasts and endothelial cells. We will subsequently investigate whether glucose-mediated induction of ACCβ promoter activity occurs in these cells.

Secondly, H9C2 cells are embryonically derived whereas cardiomyocytes are derived from newborn rat heart tissue. Thirdly, H9C2 cells exhibit both skeletal and cardiac muscle features.[264, 265] These differences may have resulted in the distinct response to stimuli especially in substrate metabolism studies. For

example, Gilde et.al.[266] have shown that neonatal cardiomyocytes exhibit a more pronounced expression of FA metabolic genes such as UCP2 and ACS compared to H9C2 cells. Moreover, the well-described FA-responsive transcription factor PPARα is abundant in neonatal cardiomyocytes but not detectable in H9C2 cells.[101] These differences may have contributed to differential ACCβ transcriptional activity observed with low and high glucose exposure.

Lastly, I did not measure endogenous ACCβ gene expression levels since I had difficulty with the Northern blotting technique at the time (as did the rest of our laboratory). This is one of the limitations of this study since there may be a disconnection between the promoter activity and actual ACCβ gene expression levels. However, we are in the process of performing additional experiments (using real-time quantitative RT-PCR) to clarify this important issue.

Chapter 3

Nuclear Respiratory Factor 1 (NRF1) inhibits transactivation of the human gene promoter of the cardiac isoform of acetyl-CoA carboxylase (ACCβ)

3.1. Introduction

The rate limiting FA mitochondrial transfer enzyme, carnitine palmitoyl transferase 1 (CPT1) plays a key role in the regulation of cardiac mitochondrial FAO. Moreover, CPT1 gene regulation is (in part) under the control of PGC1α, a transcriptional 'master modulator'. However, PGC1α does not directly bind to the promoter regions of target genes; rather, it promotes FAO by acting as a transcriptional co-activator together with PPARα or NRF1. Both PPARα and NRF1 have been associated with increased gene expression of key modulators of FA utilization, such as AMPK and CPT1.[42, 43, 77] Upon activation by its ligands, such as oleic acid and Wy-14.643, PPARα upregulates FAO-inducing genes such CPT1 and MCAD to ultimately stimulate FAO.[231-233] Likewise, NRF1 was associated with increased gene expression of CPT1 in neonatal cardiomyocytes.[89] These studies therefore highlight the role of PPARα and NRF1 in the transcriptional regulation of FAO metabolic genes.

Conversely, ACCβ catalyzes the production of malonyl-CoA, a potent inhibitor of CPT1, thereby acting as an indirect inhibitor of FAO in non-lipogenic tissues such as the heart and skeletal muscle.[190, 237] In terms of the FA-glucose cycle, increased FAO rates should result in a concomitant decrease in glucose oxidation. However, it is not clear whether this sequence will transpire at the transcriptional level i.e. if transcriptional activators of FA-responsive genes like PPARα and NRF1, will repress gene transcription of an inhibitor of FAO for e.g. ACCβ.[57, 181, 208]

In light of this, we therefore hypothesized that PPARα and NRF1 inhibit ACCβ gene transcription thereby resulting in elevated FAO rates. To test this hypothesis, I set out to establish an *in vivo* FA utilization mouse model by activation of PPARα, a well-known FA responsive transcription factor. Here, I administered natural (olive oil) and synthetic PPARα ligands (Wy-14,643) to mice and measured ACCβ gene expression after three days of treatment. Moreover, I performed several transfection-based studies overexpressing PPARα or NRF1 in different cell lines and determining its effects on ACCβ promoter activity. In this study, we found that 1) administration of olive oil and Wy-14,643 (PPARα ligands) to mice over a period of 3 days promotes systemic FFA clearance and induce ACCβ gene expression in the heart. However, transfection of a PPARα expression vector together with an ACCβ promoter construct did not have a direct effect on ACCβ promoter activity, suggesting that ACCβ is not a PPARα target gene. 2) NRF1 inhibits both basal and USF1-induced ACCβ promoter activity in cardiac derived cell lines i.e. neonatal cardiomyocytes and H9C2 myoblasts. 3) NRF1 induces basal ACCβ promoter activity in non-cardiac derived cell lines i.e. CV-1 fibroblasts and HepG2 hepatocytes. 4) NRF1 inhibits endogenous USF1 transcriptional activity in all cell lines tested.

3.2. Materials and Methods

3.2.1. *In-vivo* studies

a) An *in-vivo* FA utilization model by olive oil and Wy-14,643 administration

PPARα has been previously demonstrated to be an important regulator of several FA utilization enzyme-encoding genes[42, 43] and is activated by endogenous (oleic acid) and synthetic ligands (Wy-14,643).[231-233] Based on our hypothesis, we therefore investigated whether PPARα activation inhibits ACCβ gene expression in the mouse heart. To activate PPARα, we administered olive oil (enriched with oleic acid), and the specific PPARα synthetic ligand, Wy-14,643 (Biomol, Hamburg, Germany) to mice. The mice were divided into three separate groups. The control group received basal diet (no treatment), the second group received basal diet and olive oil (0.25 ml)[233] and the last group received a combination of olive oil and Wy-14,643 (100 mg/kg), by oral gavage on a daily basis for a continuous period of 3 days as described before.[233] Olive oil also allowed the agonist to be readily and homogeneously mixed, thereby ensuring less variation regarding the actual dose administered to each animal. Moreover, olive oil has no toxic side effects that could be detrimental to the general well-being of the treated mice. The Wy-14,643 dose used has previously been reported.[233] At the end of the experiments, tissue was collected for RNA isolation and Northern blot analyses as previously described (chapter 2, section 2.2.1.).

b) Free fatty acids measurements

Blood was collected by cardiac puncture from all experimental groups. Samples were centrifuged at 3,500 rpm for 10 min and the supernatant collected to determine plasma levels as described previously.[217] Plasma FFA levels were spectrophotometrically determined using a commercially available kit for free fatty acids (Half-micro test kit, Roche Mannheim, Basel, Switzerland).

3.2.2. *In-vitro* studies

a) Transfection studies

Transfections were carried out as previously described (chapter 2, section 2.2.2.). For this study, the full-length pPIIβ-1317 human ACCβ promoter-reporter luciferase construct[208] was transfected with equal amounts of either pSG5rPPARα or NRF1-pSG5 expression vector. To assess whether USF1-dependent activation of the ACCβ promoter is regulated by NRF1, I co-transfected the ACCβ promoter-reporter construct ± 0.19, 0.8, 0.3 and 0.25 µg of the USF1-pUC-SRα expression vector ± NRF1 expression vector for neonatal cardiomyocytes, H9C2 myoblasts, CV-1 fibroblasts and HepG2 hepatocytes, respectively. Additional transfections with ACCβ promoter deletion constructs pPIIβ-569/+65, pPIIβ-93/+65, pPIIβ-38/+65 and pPIIβ-18/+65 together with NRF1-pSG5 expression vector were also performed (deletion constructs are shown in chapter 2, Figure 1).

To assess whether endogenous USF1 transcription factor transcriptional activity is under the control of NRF1, neonatal cardiomyocytes, H9C2 myoblasts and CV-

1 fibroblasts were transiently transfected as previously described (chapter 2, section 2.2.2) ± equal amounts of the NRF1 expression vector, respectively. The pSG5rPPARα expression vector was generously provided by Dr. W. Wahli of the University of Lausanne, Switzerland. The NRF1-pSG5 expression vector was constructed by Dr. Richard Scarpulla and made available to us by Dr. Dan Kelly (Washington University School of Medicine, MO).

3.3. Results

3.3.1. The role of PPARα in the transcriptional regulation of ACCβ

a) Activation of PPARα induces ACCβ gene expression in the mouse heart

The lipid-activated transcription factor, PPARα is a pivotal transactivator of several FA enzyme-encoding genes, including CPT1 and MCAD.[42, 43] Furthermore, PPARα is activated by both endogenous and synthetic ligands such as oleic acid [231, 232] and Wy-14,643, respectively. In light of this, olive oil, a natural source of oleic acid, and Wy-14,643 were orally administered to mice for 3 days. To confirm whether our *in vivo* mouse model promotes FA utilization, we first measured plasma FFA levels together with MCAD mRNA expression, as markers of FA utilization.

Olive oil administration alone did not have any effect on plasma FFA levels. However, the combination of olive oil and Wy-14,643 resulted in a significant reduction in FFA levels ($p<0.05$ vs. control mice) (Figure 1) and maintenance of glucose levels (data not shown). The transcript levels of MCAD were increased by both olive and the combination of olive oil and Wy-14,643 ($p<0.05$) (Figure 2A). We next investigated whether PPARα-mediated whole-body metabolic changes affected ACCβ gene expression. The administration of olive oil resulted in a moderate induction of ACCβ gene expression versus control (NS). Surprisingly, the combination of olive oil and Wy-14,643 further increased ACCβ gene expression versus the olive oil alone group in the mouse heart ($p<0.05$) (Figure 2B). These data suggest that activation of PPARα by olive oil and Wy-

14,643 promotes FAO thereby increasing systemic FFA clearance. However, it is not clear whether PPARα activation directly induced ACCβ gene expression in the heart.

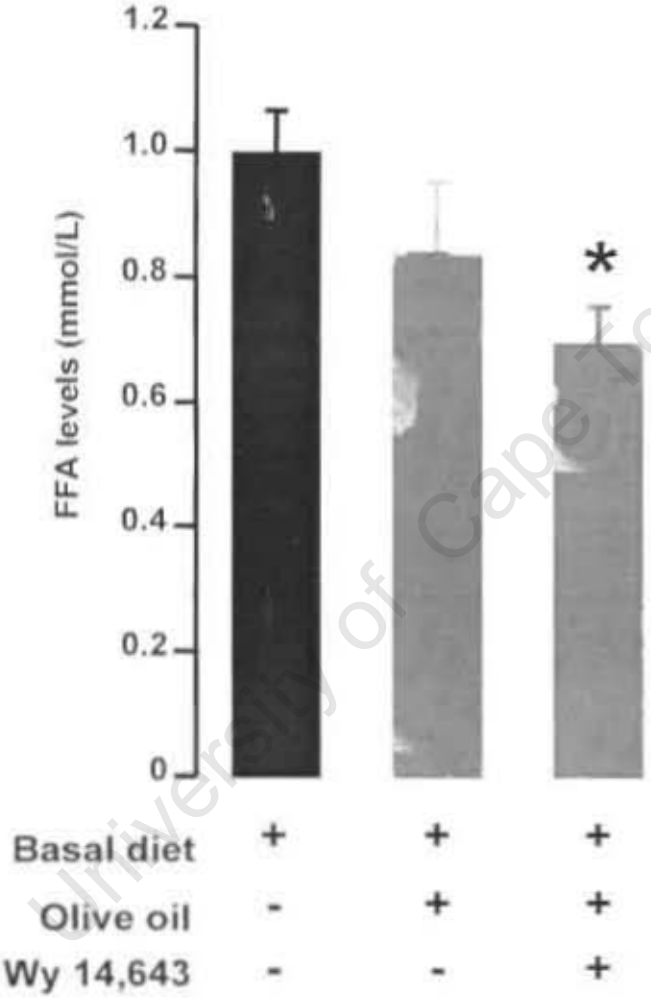


Figure 1: The effects of PPARα activation on plasma free fatty acids levels. Blood was collected by cardiac puncture and plasma FFA levels measured in control, olive oil and olive oil + Wy-14,643 treated mice. These data are represented as means ± SEM ($n \geq 6$). The asterisk denotes a significant difference between olive oil + Wy-14,643 and control (basal diet only) mice ($p < 0.05$).

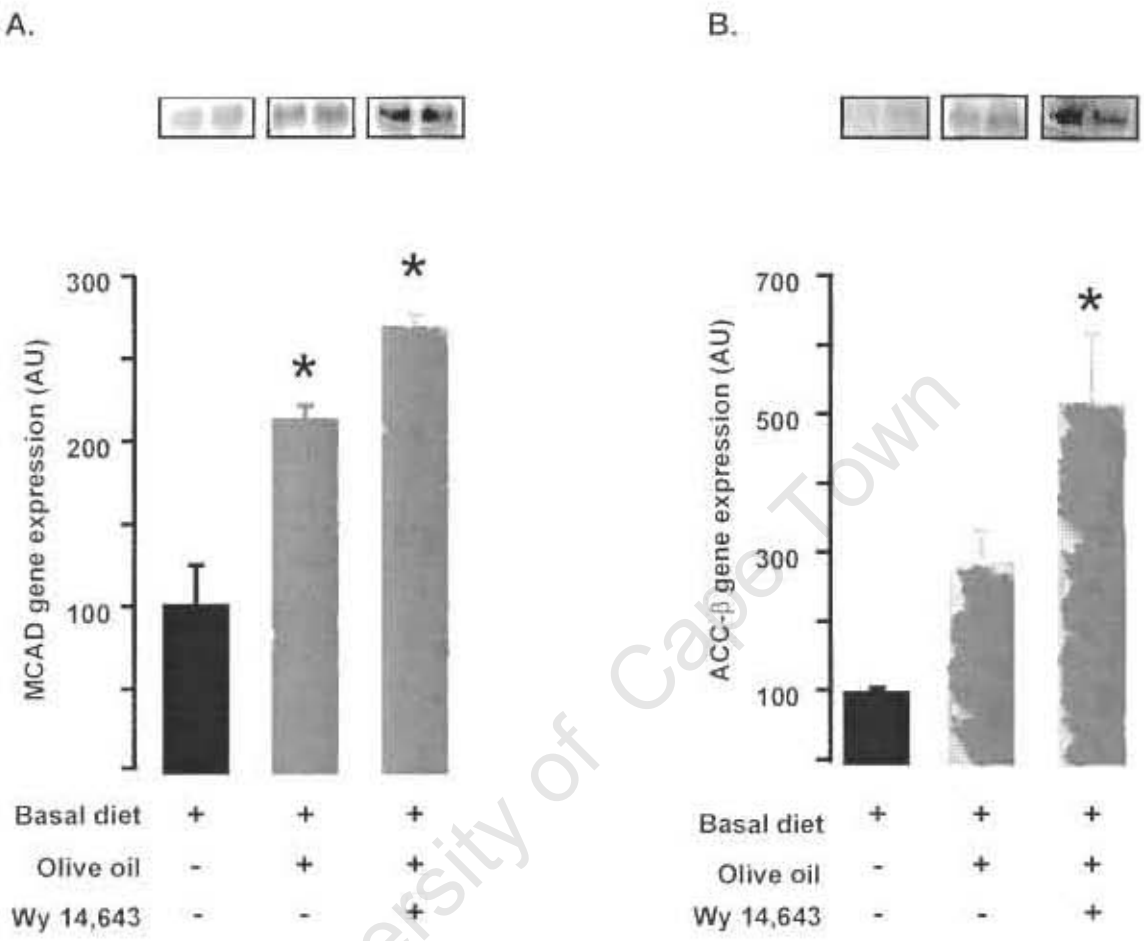


Figure 2A and B: Increased ACCβ gene expression in response to *in vivo* administration of the synthetic PPARα ligand, Wy-14,643. Olive oil (250 μl) and/or Wy-14,643, a specific PPARα agonist, was administered (100 mg/kg/day) to mice by oral gavage for a period of 3 days. Control mice were only given the basal diet. Inset - representative Northern blot is shown for medium-chain acyl-CoA dehydrogenase (MCAD) and cardiac-enriched acetyl-CoA carboxylase (ACCβ). The bars represent mean ± SEM ($n \geq 4$). The asterisk denotes a significant difference between mice administered Wy-14,643 (with olive oil) versus olive oil-treated animals ($p < 0.05$). AU – arbitrary units.

b) PPARα overexpression does not induce ACCβ promoter activity in cardiac- derived cells

To further investigate the concept that PPARα may induce ACCβ gene expression, I transiently co-transfected neonatal cardiomyocytes, H9C2 myoblasts and CV-1 cells with the full-length human ACCβ promoter-luciferase reporter construct and a PPARα expression vector. In contrast to the *in vivo* data, PPARα overexpression did not have any effect on ACCβ promoter activity in cardiac-derived cells i.e. cardiomyocytes or H9C2 myoblasts (Figure 3). However, PPARα overexpression significantly elevated ACCβ promoter activity in CV-1 fibroblasts (Figure 3). Together, these data suggest that PPARα may regulate ACCβ promoter activity in a cell type-specific manner.

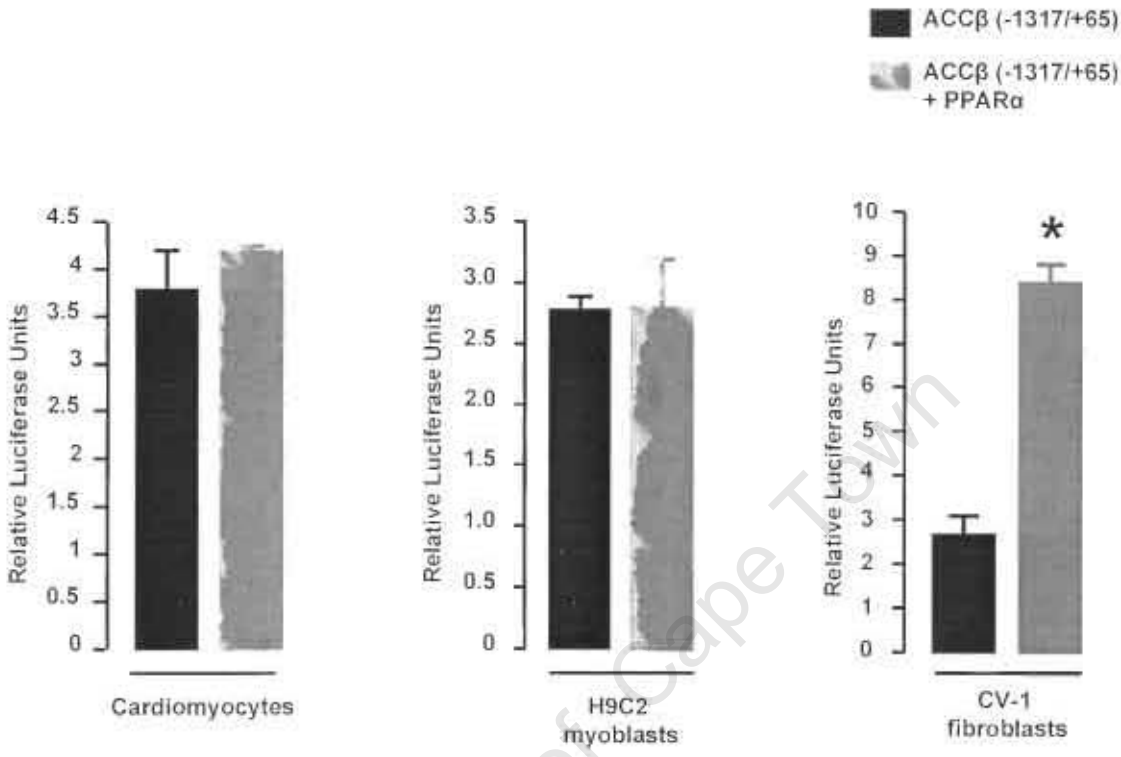


Figure 3: PPARα overexpression does not increase ACCβ promoter activity in heart-derived cells.

Cells were cotransfected with the full-length pPIIβ-1317 human ACCβ promoter-reporter construct together with the pSG5rPPARα expression vector. Cells were subsequently cultured for an additional 48 hours where after lysates were collected and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.05$ vs. pPIIβ-1317 (only).

3.3.2. The role of NRF1 in the transcriptional regulation of ACCβ

a) NRF1 overexpression inhibits basal ACCβ promoter activity in neonatal cardiomyocytes and H9C2 myoblasts

NRF1 has been previously associated with genes regulating FAO.[89, 90] However, since ACCβ inhibits FAO via malonyl-CoA production, we reasoned that NRF1 promotes FAO, in part, via inhibition of ACCβ gene expression. We began to test this hypothesis by employing transfection-based studies to analyze the effect of NRF1 overexpression on ACCβ promoter activity. As previously demonstrated, ACCβ promoter activity was induced basally for the full-length pPIIβ-1317/+65 construct in neonatal cardiomyocytes ($p<0.05$ vs. pGL3-Basic). Interestingly, cotransfection with NRF1 attenuated basal ACCβ promoter activity ($p<0.05$ vs. pPIIβ-1317/+65) (Figure 4A).

To confirm the transcriptional inhibition of ACCβ by NRF1 in the heart, we repeated these experiments using H9C2 myoblasts, a heart-derived cell line. Here, ACCβ promoter basal activity was significantly induced for the pPIIβ-1317/+65 construct ($p<0.05$ vs. pGL3-Basic). Again, NRF1 overexpression suppressed basal ACCβ promoter activity ($p<0.01$ vs. pPIIβ-1317/+65) (Figure 4B). Together these data demonstrate that NRF1 overexpression inhibits basal ACCβ promoter activity in the heart.

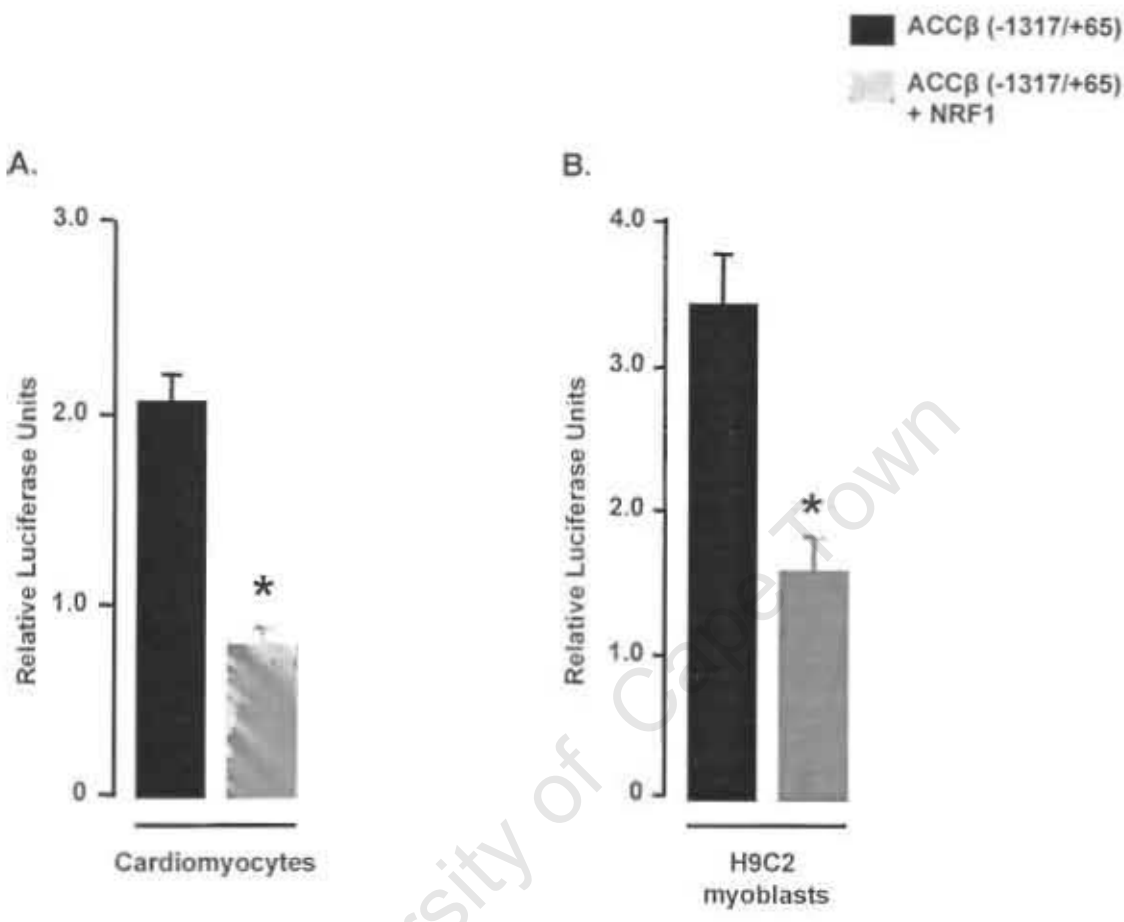


Figure 4A and B: NRF1 overexpression inhibits ACCβ promoter activity in heart-derived cells. Cells were cotransfected with the full-length pPIIβ-1317 human ACCβ promoter-reporter construct together with the NRF1-pSG5 expression vector. Cells were subsequently cultured for an additional 48 hours where after lysates were collected and luciferase activities. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.05$ vs. pPIIβ-1317.

b) NRF1 overexpression induces basal ACCβ promoter activity in CV-1 fibroblasts and HepG2 hepatocytes

In light of these findings, we next tested whether NRF1 inhibits ACCβ promoter activity in non-cardiac derived cell lines i.e. CV-1 fibroblasts and HepG2 hepatocytes. Here, CV-1 fibroblasts were transiently transfected with the pPIIβ-1317/+65 promoter construct, resulting in the induction of basal promoter activity ($p<0.05$ vs. pGL3-Basic) (Figure 5A). NRF1 overexpression further induced basal ACCβ promoter activity for the full-length pPIIβ-1317/+65 promoter construct ($p<0.001$). Of note, no basal induction of ACCβ promoter activity was observed with transfection of promoter constructs into HepG2 hepatocytes. However, NRF1 overexpression also induced ACCβ promoter activity in HepG2 hepatocytes ($p<0.01$ vs. pPIIβ-1317/+65) (Figure 5B). These data show NRF1 promotes ACCβ promoter activity in non-cardiac derived cell lines.

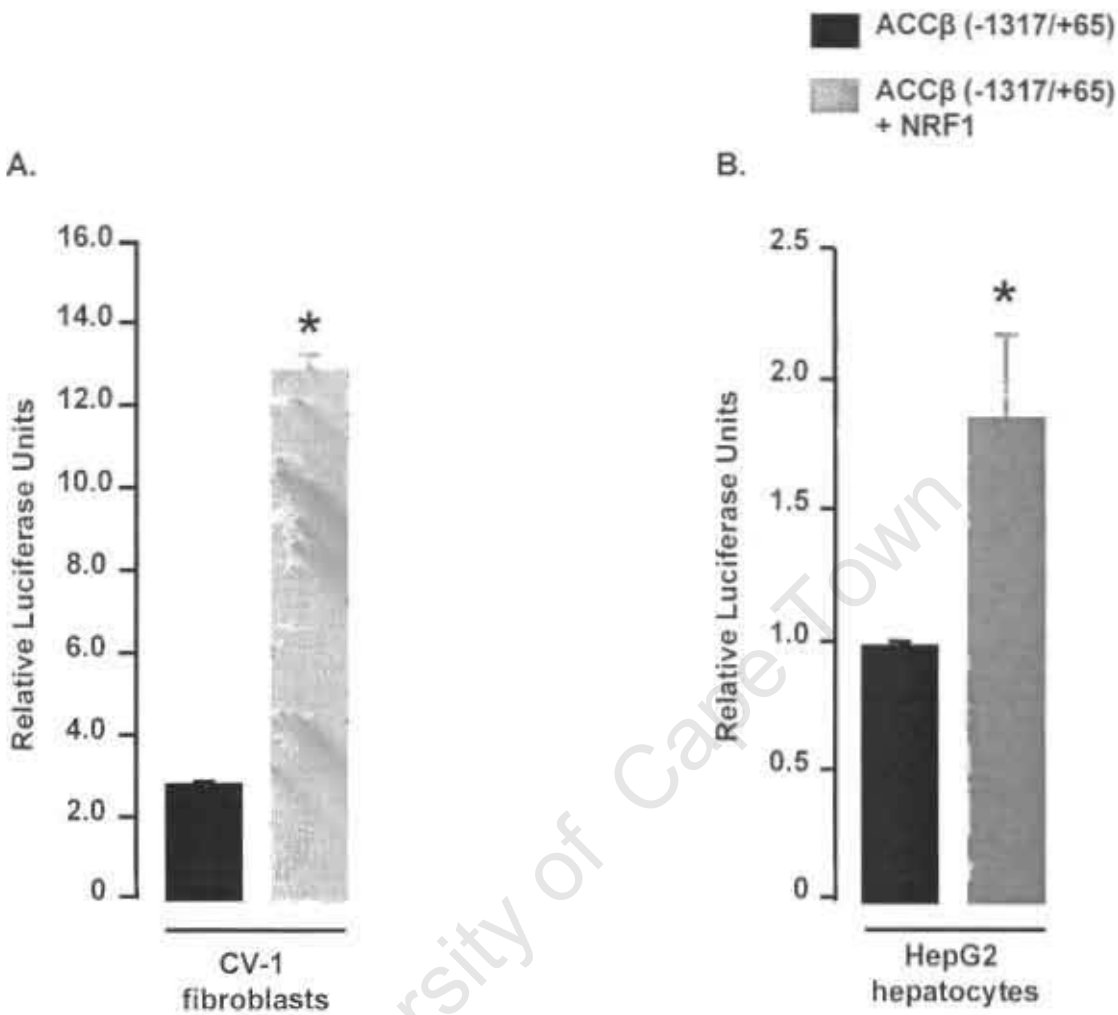


Figure 5A and B: NRF1 overexpression induces basal ACCβ promoter activity in CV-1 fibroblasts and HepG2 hepatocytes.

Cells were cotransfected with the full-length pPIIβ-1317 human ACCβ promoter-reporter construct together with the NRF1-pSG5 expression vector. Cells were subsequently cultured for an additional 48 hours where after lysates were collected and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates. *p<0.05 vs. pPIIβ-1317.

c) Assessment of putative NRF1 regulatory regions on the ACCβ promoter in neonatal cardiomyocytes and H9C2 myoblasts

As far as we are aware, there are no classic NRF1 recognition sites located on the human ACCβ gene promoter. However, NRF1 has been shown to regulate expression of genes that are devoid of NRF1 recognition sites by interacting with myocyte enhancer factor-2 (MEF-2) and/or E-box consensus *cis*-elements.[234-236] For work relating to this thesis we did not find ACCβ promoter activity to be regulated via E-boxes 1-3 and speculated that E-box 4 and/or the -92 to -39 regions may be responsible for induction of ACCβ promoter activity (refer to chapter 2, Figure 10). We reasoned that the inhibitory effect of NRF1 may be mediated via interaction with E-box 4 and/or the -92 to -39 regions of the human ACCβ gene promoter. To further pursue this concept we transiently cotransfected various ACCβ promoter deletion constructs (chapter 2, Figure 1) with an NRF1 expression vector into cardiomyocytes and H9C2 myoblasts.

In both cardiomyocytes and H9C2 myoblasts, cotransfection with NRF1 overexpression attenuated basal ACCβ promoter activity for all deletion constructs ($p<0.05$ vs. all deletion constructs) except for the pPIIβ-18/+65 construct (Figure 6A). Together these data demonstrate that NRF1 overexpression potently inhibits basal ACCβ promoter activity in the heart and that NRF1 possibly interferes with factors that bind in the -38 to -18 region of the ACCβ gene promoter.

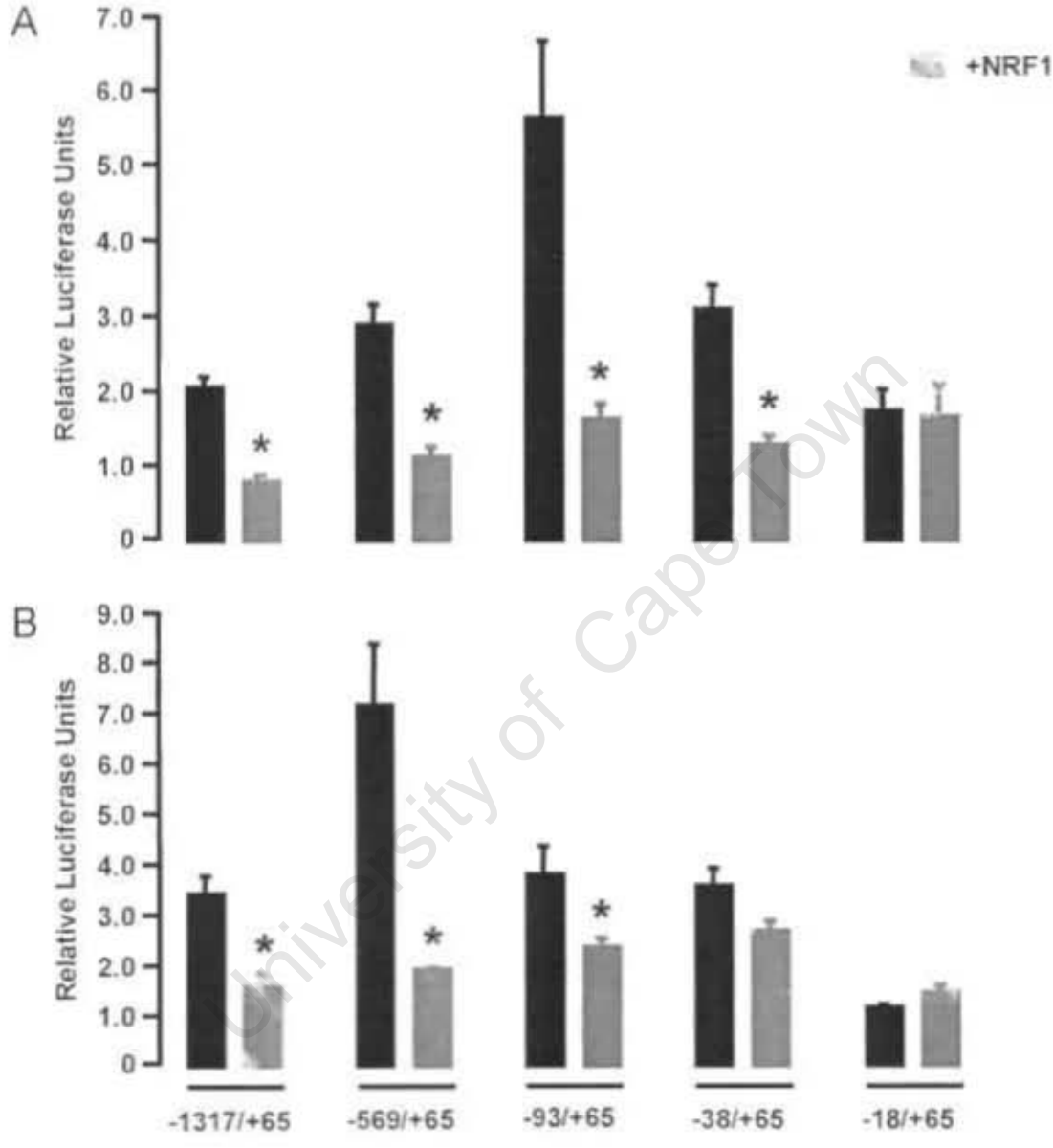


Figure 6: NRF1 overexpression inhibits basal ACCβ promoter activity in cardiac-derived cells lines.

(A) Neonatal cardiomyocytes and **(B)** H9C2 myoblasts were transiently transfected with ACCβ promoter deletion constructs (black bars) and in the other experimental group, ACCβ promoter deletion constructs were cotransfected with an NRF1 expression vector (gray bars) for 24 hours and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.05$ vs. basal ACCβ promoter activity.

d) Assessment of putative NRF1 regulatory regions on the ACCβ promoter in CV-1 fibroblasts and HepG2 hepatocytes

CV-1 fibroblasts were transiently cotransfected with various ACCβ promoter deletion constructs together with the NRF1 expression vector. NRF1 overexpression induced basal ACCβ promoter activity for all deletion constructs ($p<0.001$) (Figure 7A). However, in HepG2 hepatocytes NRF1 overexpression induced ACCβ promoter activity only for the pPIIβ-1317/+65, pPIIβ-38/+65 and pPIIβ-18/+65 constructs ($p<0.01$ vs. pPIIβ-1317/+65, pPIIβ-38/+65 and pPIIβ-18/+65) (Figure 7B). NRF1 overexpression had no effect on pPIIβ-569/+65 and pPIIβ-93/+65 promoter activity.

Collectively, these findings implicate NRF1 in the transcriptional regulation of ACCβ. Furthermore, NRF1 regulates ACCβ in a cell type-specific manner indicating differential interaction with cell type-specific transcriptional cofactors and/or metabolic substrate preference and physiological functions between the cardiac-derived tissues and the other tissues such as smooth muscle.

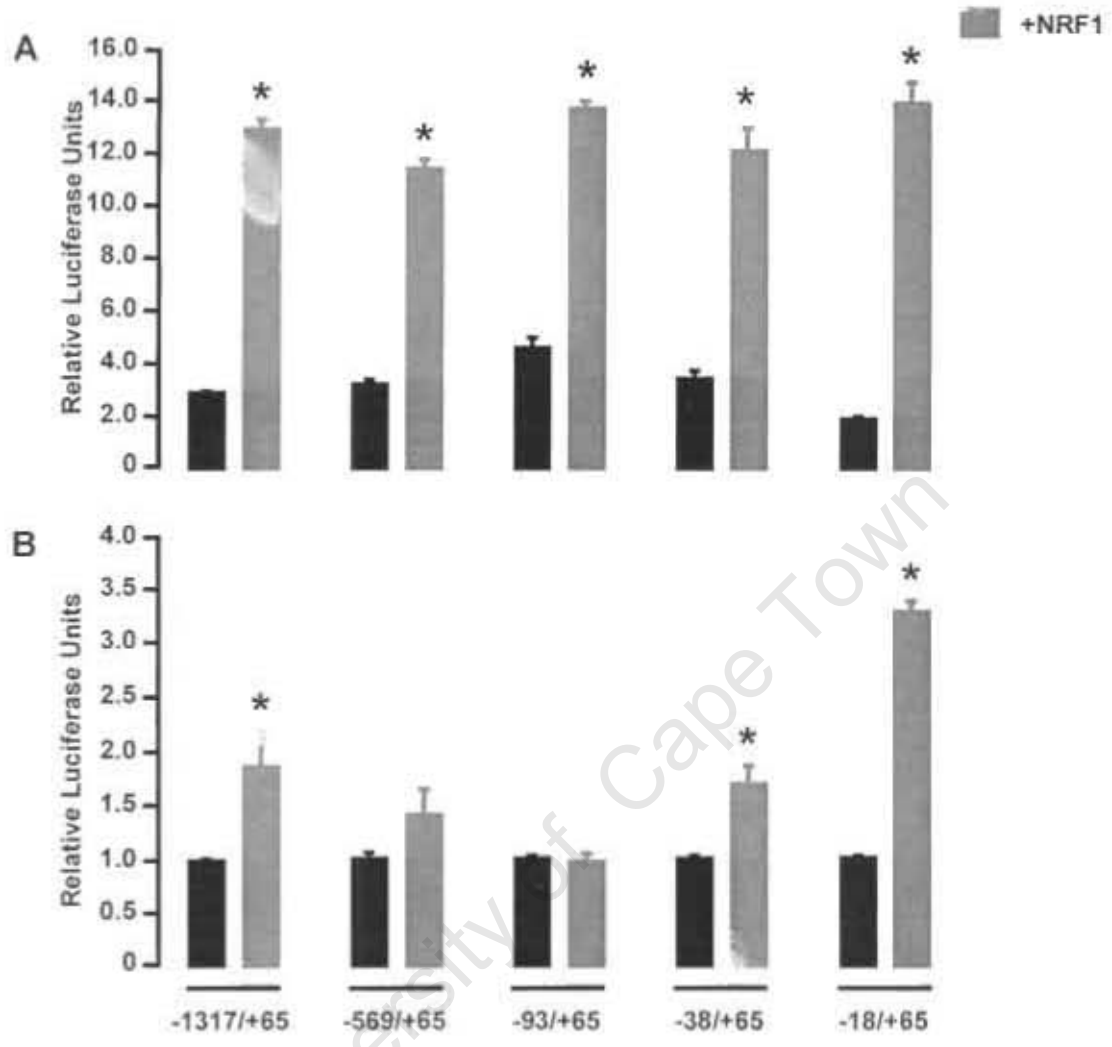


Figure 7A and B: NRF1 overexpression induces basal ACCβ promoter activity in non-cardiac derived cell lines.

(A) CV-1 fibroblasts and **(B)** HepG2 hepatocytes were transiently transfected with ACCβ promoter deletion constructs (black bars). ACCβ promoter deletion constructs were also cotransfected with an NRF1 expression vector (gray bars) for 24 hours and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates, *$p<0.05$ vs. basal ACCβ promoter activity.

3.3.3. NRF1 overexpression inhibits USF1-mediated induction of ACCβ promoter activity

We have previously demonstrated that USF1, a glucose-responsive transcription factor, induces ACCβ promoter activity.[273] To further investigate the inhibitory role of NRF1 in the transcriptional regulation of ACCβ promoter activity, we next cotransfected ACCβ promoter deletion constructs ± USF1 ± NRF1 expression vectors into neonatal cardiomyocytes, CV-1 fibroblasts and HepG2 hepatocytes.

a) NRF1 inhibits USF1-dependent induction of ACCβ promoter activity

As previously shown, cotransfection with USF1 further increased basal ACCβ promoter activity for all the deletion constructs in cardiomyocytes ($p<0.01$ vs. basal ACCβ promoter activity) (Figure 8A). NRF1 overexpression inhibited USF1-dependent induction of ACCβ promoter activity in all cell lines tested ($p<0.05$ vs. pPIIβ-1317/+65, pPIIβ-569/+65, pPIIβ-93/+65, pPIIβ-18/+65). However, the inhibitory effect of NRF1 overexpression in cardiomyocytes was abolished with the pPIIβ-38/+65 deletion construct, possibly due to severe loss of USF1 responsiveness as indicated before (chapter 2, Figure 8A). We have previously reported that NRF1 or USF1 overexpression induces basal ACCβ promoter activity in non-cardiac derived cell lines. We therefore reasoned that simultaneous cotransfection of NRF1 and USF1 would further induce USF1-mediated ACCβ promoter activity. Unexpectedly, NRF1 overexpression markedly attenuated USF1-induced ACCβ promoter activity for several deletion constructs ($p<0.05$ vs. pPIIβ-1317/+65, pPIIβ-569/+65, pPIIβ-93/+65, pPIIβ-38/+65) except

for the pPIIβ-18/+65 construct in CV-1 fibroblasts and the pPIIβ-38/+65 construct in HepG2 hepatocytes (Figures 8B and C). These data therefore strongly suggest an interaction between NRF1 and USF1 in regulating ACCβ promoter activity in these cell lines.

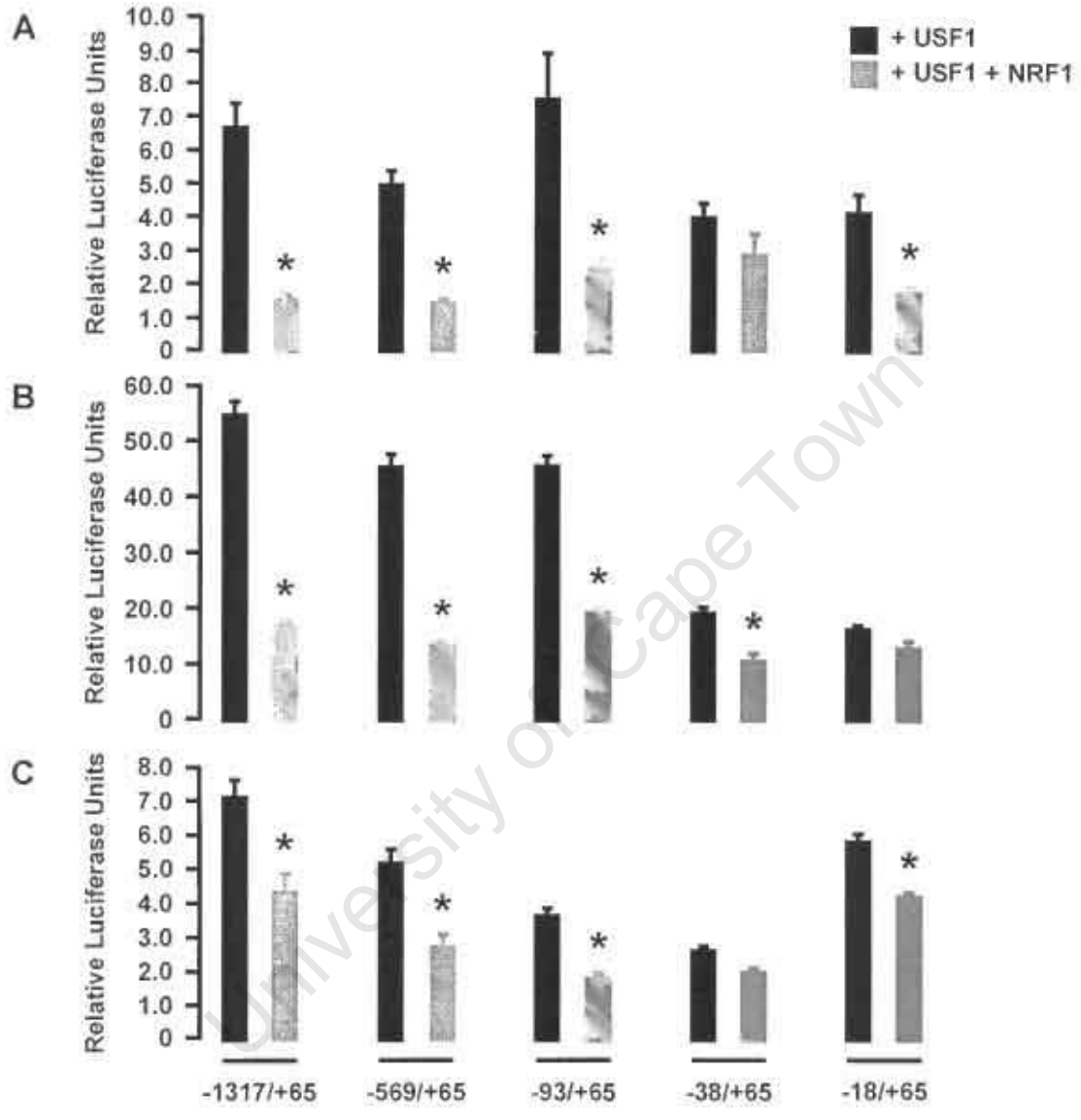


Figure 8: NRF1 overexpression inhibits USF1-dependent induction of ACCβ promoter activity.

(A) Neonatal cardiomyocytes (B) CV-1 fibroblasts and (C) HepG2 hepatocytes were transiently cotransfected with ACCβ promoter deletion constructs and a USF1 expression vector (black bars). ACCβ promoter deletion constructs were also simultaneously cotransfected with USF1 and NRF1 expression vectors (gray bars) for 24 hours and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pGL3-Basic = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates, *p<0.05 vs. ACCβ + USF1.

3.3.4 NRF1 overexpression inhibits endogenous USF1 transcriptional activity

We have previously demonstrated that the endogenous transcriptional activity of the USF1 reporter construct was significantly higher compared to control reporter construct in neonatal cardiomyocytes and CV-1 fibroblasts (chapter 2, Figure 8). To assess whether endogenous USF1 transcriptional activity is under the control of NRF1, we cotransfected a USF1 reporter vector ± an NRF1 expression vector into neonatal cardiomyocytes, H9C2 myoblasts and CV-1 fibroblasts. In agreement with our USF1 overexpression studies, endogenous USF1 transcriptional activity was significantly higher than the control vector in all cell lines ($p<0.001$ vs. USF1-Luc). Moreover, cotransfection with NRF1 significantly diminished endogenous USF1 transcriptional activity in all cell lines tested ($p<0.001$ vs. pUSF1-Luc + NRF1) (Figures 9A, 9B and 9C).

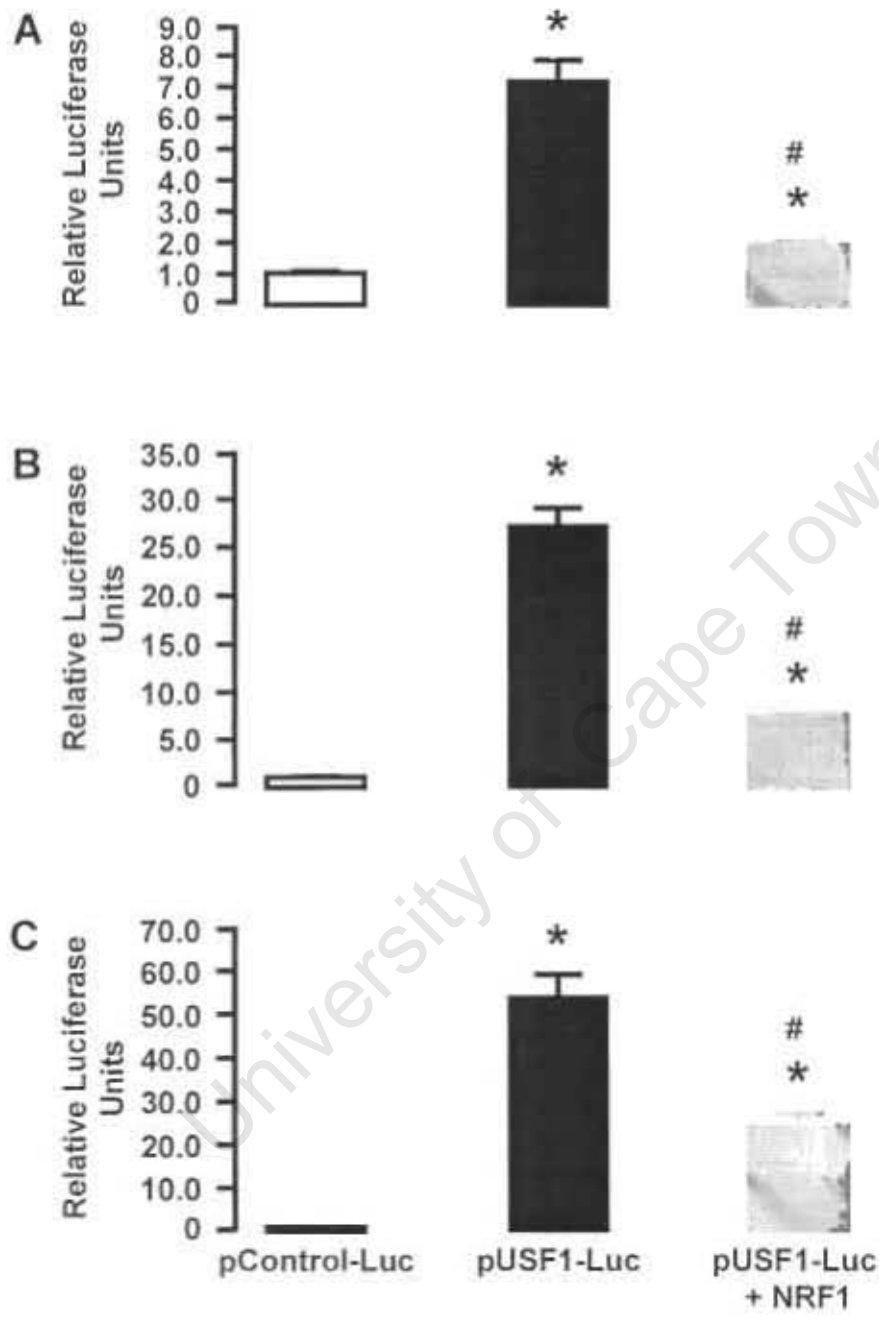


Figure 9: NRF1 overexpression inhibits endogenous USF1 transcriptional activity.

(A) Neonatal cardiomyocytes; (B) H9C2 myoblasts and (C) CV-1 fibroblasts were transiently cotransfected with a pUSF1-Luc reporter vector ± an NRF1 expression vector for 24 hours and luciferase activities measured. Results are expressed as relative luciferase units normalized to the activity of vector only (pControl-Luc = 1). These data are represented as mean ± SEM of 2 or more independent experiments performed in triplicates. *$p<0.05$ vs. pControl-Luc; #$p<0.05$ vs. pUSF1-Luc.

3.4. Discussion

3.4.1. ROLE OF PPARα IN TRANSCRIPTIONAL REGULATION OF ACCβ

a) *In vivo* fatty acid utilization mouse model

Previous investigators have shown that physiological and pathophysiological conditions that promote FAO inhibit ACCβ protein expression thereby relieving CPT1 inhibition.[238, 239] Since malonyl-CoA (ACCβ product) inhibits CPT1 under conditions favoring glucose metabolism,[267] I investigated the molecular regulation of ACCβ under FAO-inducing conditions.

To achieve this, I set out to establish an *in vivo* FA utilization mouse model by activation of PPARα, a well-known FA responsive transcription factor. PPARα is activated by several endogenous and synthetic ligands (e.g. long-chain fatty acids and fibrates).[240] In this study, I administered olive oil, a natural source of oleic acid[241] ± Wy-14,643, a specific PPARα activator[242] to mice for three consecutive days. I observed a moderate reduction in systemic FFA levels with olive treatment, reaching statistical significance with a combination of olive oil and Wy-14643 treatment. Since Wy-14,643 is a hypolipidemic drug, these results are not unexpected. Similar lipid-lowering effects were observed in obese mice[243] and lipoatrophy-induced diabetes.[244] However, systemic glucose levels were unchanged by either olive oil alone or in combination with Wy-14643 (data not shown). I also found that administration of olive oil alone or in combination Wy-14,643 increased MCAD gene expression, an indication of increased FA β-oxidation in the heart. These data are in agreement with Van der Lee et al.[101]

who also found that that administration of FAs (oleic and palmitic acids) or Wy-14,643 induced expression of several FA utilization genes *in vitro*. These data therefore support the establishment of an *in vivo* FA utilization mouse model. However, a limitation of this part of the study is that we did not measure *ex vivo* cardiac fuel substrate oxidation rates for the various treatment groups. At the time, the method was not established in our laboratory (due to lack of apparatus and costs). However, the laboratory is currently in the process of acquiring the necessary equipment to establish these methods for the mouse heart.

b) PPARα-mediated metabolic changes regulate ACCβ gene expression

To further investigate the regulation of ACCβ gene expression, I next measured transcript levels of ACCβ in the mouse heart. Here, PPARα activation by olive oil resulted in a non-significant induction of ACCβ gene expression. Moreover, a combination of olive oil together with Wy-14,643 resulted in a significant increase of ACCβ gene expression. These findings were rather unexpected since PPARα-driven FAO conditions such as exercise, fasting and high fat feeding have been shown to downregulate ACCβ protein expression.[238, 239, 268] Also, others have demonstrated that accumulation of intracellular long-chain FAs inhibit ACCβ gene expression in the pancreatic beta cells.[269]

In this study, Wy-14,643 and olive oil administration lead to significant reduction in systemic FFA levels in mice. Here, I speculate that, the observed upregulation of ACCβ gene expression following treatment with olive oil and Wy-14,643 may

be a consequence of reduced circulating FFA levels. I propose that with abundant supply of FFAs, accumulation of intracellular long-chain FAs may inhibit ACCβ gene transcription, thereby promoting FAO through a) CPT1 activation and b) ligand-induced PPARα activation.[270] However, if FAO is continuously stimulated through PPARα activation, this may lead to reduced circulating FFA levels thereby relieving the long-chain FA inhibition on ACCβ. Such a negative regulatory effect could be beneficial, especially under FFA-limiting conditions e.g. prolonged fasting or chronic activation of PPARα. Although a direct effect of PPARα cannot be excluded, FFA clearance may therefore be responsible for the induction of ACCβ gene expression observed with olive oil and Wy-14643 administration. In agreement, others have reasoned that PPARα-independent transcriptional regulation of non-PPARα target genes may be due to cellular metabolic changes induced by administration of PPARα agonists such as Wy-14,643.[20, 84] For example, decreased cardiac GLUT4 gene expression has been observed following Wy-14,643 treatment.[20] Also, Panagia et.al.[271] consistently demonstrated decreased cardiac GLUT4 protein levels in three different models of elevated circulating FFAs i.e. diabetes, high fat feeding and fasting. Furthermore, FFA clearance induced by Wy-14,643 has also been associated with improved insulin stimulated glucose uptake.[243, 245, 246]

Increased ACCβ expression is also associated with elevated glucose levels[273] and should therefore result in increased malonyl-CoA levels and inhibition of FAO. This interplay between glucose and FA availability may be implicated in the

mechanism of cellular adaptation to various stimuli as proposed by Young et. al.[51] With the dynamic nature of fuel substrate supply to the heart, it is possible that fuel substrates (acting as signaling intermediates) relay the message of decreased FFA supply to the cell's transcriptional machinery which in turn increases malonyl-CoA levels by upregulating ACCβ. In this manner, it is possible that the cell may "anticipate" the reduced FFA supply and prepares in advance by increasing ACCβ gene transcription and thereby decreasing FAO rates. Here, glucose-sensing transcriptional modulators may play a role to elevate ACCβ gene transcription. In summary, these data suggest that PPARα-mediated metabolic changes such as reduced systemic FFA and sustained glucose levels may be responsible for upregulation of ACCβ gene expression observed. However, further studies are required to fully delineate the precise molecular mechanisms that underpin these interesting findings.

c) PPARα does not directly activate ACCβ promoter activity in cardiac-derived cells

To gain further insight into the regulation of ACCβ gene transcription by PPARα, I cotransfected a PPARα expression vector together with the full-length ACCβ promoter construct into cardiomyocytes and H9C2 myoblasts. PPARα overexpression did not significantly alter ACCβ promoter activity in neonatal cardiomyocytes or H9C2 myoblasts. These data confirm my earlier proposals for the mouse *in vivo* data i.e. that the upregulation observed with Wy-14,643 and olive oil administration is not due to direct activation of PPARα.

Conversely, PPARα overexpression significantly induced ACCβ promoter activity in the 'cardiac null-background' CV-1 fibroblasts. It is well documented that PPARα heterodimerizes with its obligate partner, RXRα, binding to peroxisome proliferator response elements (PPRE) on promoters of target genes.[247, 248] For example, it was previously shown that overexpression of RXRα further activated muscle regulating factor 4 (MRF-4)-induced ACCβ promoter activity in NIH3T3 fibroblasts.[207] Here, RXRα alone did not have any effect on basal ACCβ promoter activity[207] whereas I have demonstrated that PPARα alone induce ACCβ promoter activity in CV-1 cells. These discrepancies necessitate further investigations in order to elucidate the role of PPARα/RXRα interaction in the regulation of ACCβ gene expression.

In summary, the first part of this chapter shows that ACCβ gene regulation is controlled at multiple levels in the mouse heart, underscoring the complexity of molecular regulatory mechanisms. We propose that systemic PPARα activation does not directly influence ACCβ gene expression in the heart. However, we suggest that its secondary effects on FFA clearance and improved insulin-stimulated glucose uptake may be responsible for the induction of ACCβ transcript levels. Intriguingly, as yet unidentified transcriptional mechanisms directing molecular regulation of ACCβ in non-cardiac tissues merits further investigation.

d) Limitations and future directions

Olive oil is a natural source of oleic acid. Oleic acid is an endogenous ligand of PPARα, therefore olive oil administration to mice was chosen as model of endogenous PPARα activation *in vivo*. On the other hand, the synthetic PPARα ligand, Wy-14,643 has been extensively used as a specific activator of PPARα. In our laboratory, another study investigating the effect of PPARα activation by Wy-14,643 administration alone found that it did not significantly alter rat cardiac ACCβ gene transcription compared to controls (data not shown). However, in this study, we were limited due to the prohibitive costs of the Wy-14,643 compound. In light of this, we thought that the best way to activate PPARα and cardiac FAO would be to employ Wy-14,643 together with a natural ligand. However, future experiments will be conducted in order to clearly show whether Wy-14,643 alone affects mouse ACCβ gene expression. The following experiments will be considered: administration of oleic acid, oleic acid with Wy-14,643 and Wy-14,643 alone to a) mice and b) cardiomyocytes, H9C2 myoblasts, CV-1 fibroblasts and HepG2 cells. Subsequently, I will measure: 1) ACCβ gene transcript levels using real-time quantitative RT-PCR, 2) ACCβ promoter activity using reporter assays and 3) DNA:protein binding activity using the electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assays.

The role of FA-responsive transcriptional factors, PPARα and RXRα in the regulation of ACCβ gene transcription deserves further investigation. In this

regard, the following experiments will be considered i.e. cotransfection of RXRα expression vector alone and cotransfection of RXRα/PPARα expression vectors together with the ACCβ promoter in both cardiac and non-cardiac cell lines. Neither PPARα nor RXRα expression vector alone had an effect on basal ACCβ promoter activity, therefore I will investigate whether PPARα and RXRα can affect USF1-induced ACCβ promoter activity.

3.4.2. THE ROLE OF NRF1 IN TRANSCRIPTIONAL REGULATION OF ACCβ

An important aspect of the transcriptional control of mitochondrial biogenesis is mediated by the master regulator PGC1α that induces target genes together with coactivators such as NRF1 and PPARα.[85, 86, 249, 250] NRF1 induces several nuclear-encoded mitochondrial respiratory enzymes,[80] while PPARα regulates the expression of numerous FA utilization genes for e.g. CPT1 and MCAD.[129] Furthermore, exercise-mediated stimulation of FAO is believed to be transcriptionally driven by NRF1 and PPARα.[77, 86] Whether these transcription factors promote FAO, in part, by repressing the inhibitory mechanism that controls long-chain FA (LCFA) importation into the mitochondria is largely unknown. Here, ACCβ regulates mitochondrial transfer of LCFAs via production of malonyl-CoA, a potent inhibitor of CPT1, thereby leading to a reduction in FAO. Of note, exercise-mediated stimulation of FAO is associated with elevated levels of NRF1 and PPARα accompanied by the increased expression of FA enzyme-encoding genes such as AMPK and CPT1.[77] In this study, we explored the role of NRF1 in regulating ACCβ gene regulation in the heart. To our knowledge, this is the first study to implicate NRF1 as a novel inhibitor of ACCβ promoter activity.

We found that NRF1 overexpression markedly attenuated ACCβ promoter activity in neonatal cardiomyocytes and cardiac-derived H9C2 myoblasts. Elevated NRF1 gene expression was previously shown to be associated with

upregulation of CPT1 gene expression in neonatal cardimyocytes.[89, 90] Moreover, previous studies have shown that the expression of NRF1[86] and CPT1 [251] is upregulated in response to exercise. It has also been established that exercise induces FAO by activation of AMPK which in turn phosphorylates and inhibits ACC enzyme activity.[199, 252, 253] Hence, AMPK has been recognized as a key regulator of FAO. Whether AMPK, in addition to phosphorylation of ACCβ, also inhibits transcriptional regulation of ACCβ is still unclear. A recent study reported that administration of β-guanadinopropionic acid (β-GPA), an AMPK activator, was associated with increased NRF1 promoter binding activity.[91] These findings therefore provide a possible transcriptional mechanism through which AMPK could inhibit ACCβ gene expression in the heart. However, two studies argue against this concept. In the first instance, NRF1 overexpressing mice did not show significant changes in basal palmitate oxidation.[86] We propose that since these mice were not challenged with various stimuli that induce FAO, the role of NRF1 as a positive regulator of FAO cannot be excluded. Secondly, Nau et al. [52] reported that fetal hearts exhibited elevated NRF1 levels associated with reduced CPT1 gene expression. We argue that this discrepancy may be due to the reduced ability of the fetus to utilize FAs and not necessarily due to reduced upregulation of CPT1 gene expression by NRF1.

To investigate whether the inhibitory effect of NRF1 overexpression on ACCβ is cardiac-specific, we repeated experiments employing non-cardiac cell lines i.e. CV-1 fibroblasts and HepG2 hepatocytes. Surprisingly, we found that NRF1

overexpression induced ACCβ promoter activity in both CV-1 fibroblasts and HepG2 hepatocytes. However, the NRF1-induced ACCβ promoter activity was significantly higher compared to the USF1-mediated increase in ACCβ promoter activity. In light of these findings, we further tested whether cotransfection of NRF1 and USF1 would inhibit or further induce USF1-mediated induction of ACCβ promoter activity. We found that USF1-mediated induction of ACCβ promoter activity was markedly attenuated in both cell lines. Intriguingly, NRF1-mediated induction of ACCβ promoter activity was maintained while the USF1-mediated induction of ACCβ promoter activity was abolished. This was rather unexpected since NRF1 differentially regulated basal ACCβ promoter activity. Nonetheless, these data clearly indicate that NRF1 may interfere with USF1 binding to the ACCβ promoter. To further explore the inhibitory effect of NRF1 on USF1-mediated ACCβ promoter activity we transfected cells with a USF1 translucent reporter vector and a NRF1 expression vector. Attenuation of endogenous USF1 transcriptional activity with NRF1 cotransfection confirmed that NRF1 interferes with USF1-mediated induction of ACCβ promoter activity. Overall, these data point to a novel role for NRF1 in regulating ACCβ expression in the heart and raises the possibility that NRF1 may coordinately regulate genes involved in mitochondrial FA uptake.

As far as we could ascertain, there are no classic NRF1 binding elements [(T/C)GCGCA(C/T)GCGC(A/G)] located within the human ACCβ gene promoter. However, it has been suggested that certain transcriptional coactivators do not

bind directly to promoter sequence elements but rather interact with multiple cofactors to activate or repress gene transcription.[80, 86, 235, 236] In agreement with this concept, NRF1 increased gene expression of GLUT4 and muscle-specific COXVIaH and COXVIII despite a lack of NRF1 promoter binding elements.[86, 235, 236] Instead, the NRF1-mediated effects are dependent on MEF-2 and/or E-box consensus elements for their expression.

The human ACCβ promoter contains several E-boxes. In light of this, we employed ACCβ promoter deletion constructs to investigate whether NRF1 can interact with USF1 (E-box binding protein), to regulate ACCβ promoter activity. Since ACCβ promoter activity was similar when transfecting with the pPIIβ-569/+65 or pPIIβ-93/+65 deletion constructs, the inhibitory effect of NRF1 was not mediated via a cluster of three E-boxes (E-box1-3) located between -569 and -349. Similarly, in chapter 2, we reported that E-boxes(1-3) were not involved in USF1-mediated induction of ACCβ promoter activity. However, the USF1-mediated increase of pPIIβ-18/+65 promoter activity (containing E-box4 only) was markedly attenuated by NRF1 overexpression in cardiomyocytes. However, this induction of ACCβ promoter activity was maintained or moderately inhibited by NRF1 in CV-1 fibroblasts and HepG2 hepatocytes, respectively. These data indicate that NRF1 may be interfering with USF1 binding to E-box4 in cardiomyocytes. However, in CV-1 fibroblasts and HepG2 hepatocytes, NRF1 probably increases ACCβ promoter activity via E-box4. Notably, USF1-mediated induction of pPIIβ-18/+65 promoter activity (containing E-box4 only) was reduced

blot assays to confirm my *in vitro* work (transfection assays). However, our laboratory is currently considering the purchasing a thermal cycler in order to perform real-time quantitative RT-PCR measurements of ACCβ transcript levels.

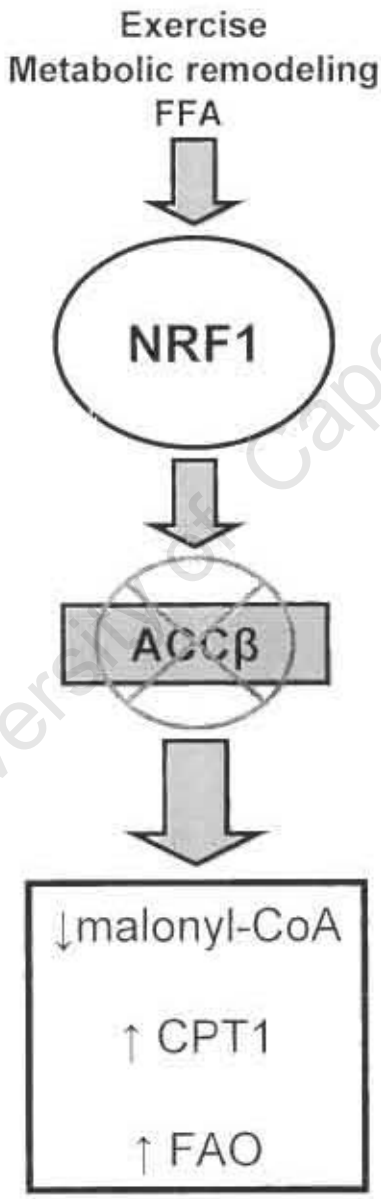


Figure 11: Proposed molecular mechanism for NRF1 in the transcriptional inhibition of ACCβ and regulation of fatty acid oxidation

Chapter 4

Conclusion

reduced fatty acid oxidation observed in type 2 diabetic patients may be attributed to increased ACCβ gene expression and the subsequent accumulation of malonyl-CoA levels.[121, 263] These data raise the interesting possibility that USF1 levels could be increased within the diabetic context resulting in the induction of ACCβ gene expression, increased malonyl-CoA levels and decreased CPT1 activity. However, further studies are required to investigate this proposal and the contribution of this pathway to the accumulation of damaging intracellular LCFAs in the heart.

In light of this, the identification of novel inhibitors regulating ACCβ expression may provide a mechanism through which detrimental intracellular lipid accumulation and insulin resistance may be controlled. In support of this concept, ablation of the ACCβ gene was accompanied by enhanced *in vivo* fatty acid oxidation rates and strong resistance of these transgenic mice to diabetes and obesity when challenged with a high-fat/high-carbohydrate diet.[194] We therefore investigated regulatory mechanisms that may repress ACCβ gene transcription. We hypothesized that transcription modulators controlling genes promoting energy producing pathways also inhibit ACCβ transcription in order to promote fatty acid oxidation. Here, for the first time as far as we are aware, we identified NRF1 as a novel repressor of the ACCβ gene promoter in the heart and in non-cardiac tissues. Furthermore, we report that NRF1 inhibits basal and USF1-induced human ACCβ promoter activity. Also, endogenous USF1 transcriptional activity was markedly attenuated by NRF1 overexpression. Our

findings provide further insight regarding NRF1-mediated repression of ACCβ gene transcription. Since we have suggested that USF1-mediated induction of ACCβ promoter activity may have deleterious effects due to accumulation of LCFAs, we propose that the newly identified NRF1-mediated repression of ACCβ transcription may in fact promote LCFA clearance. In this regard, exercise improves insulin resistance via LCFA clearance. Moreover, the beneficial effects of exercise have been extensively documented as a therapeutic intervention for diabetes, insulin resistance, coronary heart disease, hypertension and other metabolic disorders. Exercise also inhibits ACCβ gene expression thereby increasing fatty acid oxidation.[238] We therefore propose that NRF1-driven transcriptional inhibition of ACCβ may be a potential mechanism through which exercise exerts its beneficial effects.

Conversely, some investigators have reported the use of pharmacological inhibitors of fatty acid oxidation as a potential treatment for heart failure.[110] In support, mice lacking PPARα, a fatty acid-responsive transcriptional regulator, were protected against diabetes-induced cardiac hypertrophy,[229] whereas PPARα overexpressing mice displayed a diabetes-like metabolic phenotype.[20] Furthermore, reactivation of PPARα during the development of cardiac hypertrophy exacerbated cardiac function.[102] Therefore, despite the potential cardioprotective effects of ACCβ inhibition, it appears that under certain conditions its activation may indeed be beneficial. In this context, our identification of both positive and negative regulators of ACCβ gene transcription

may result in the development of novel therapeutic agents that will help alleviate the growing global burden of heart disease.

Chapter 5

References

1. Hellwig B, Joost HG. Differentiation of erythrocyte-(GLUT1), liver-(GLUT2), and adipocyte-type (GLUT4) glucose transporters by binding of the inhibitory ligands cytochalasin B, forskolin, dipyridamole, and isobutylmethylxanthine. Mol Pharmacol 1991; 40:383-9.

2. Abel ED. Glucose transport in the heart. Front Biosci 2004; 9:201-15.

3. 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.

4. Lavrentyev EN, He D, Cook GA. Expression of genes participating in regulation of fatty acid and glucose utilization and energy metabolism in developing rat hearts. Am J Physiol Heart Circ Physiol 2004; 287:H2035-42.

5. Aledo JC, Lavoie L, Volchuk A, Keller SR, Klip A, Hundal HS. Identification and characterization of two distinct intracellular GLUT4 pools in rat skeletal muscle: evidence for an endosomal and an insulin-sensitive GLUT4 compartment. Biochem J 1997; 325 (Pt 3):727-32.

6. Stryer L. Pentose phosphate pathway and gluconeogenesis. Biochemistry. 3rd Ed. Vol. 427-448. New York: Freeman Publishers, 1988.

7. McGilvery R. Storage of fats. In: McGilvery R, ed. Biochemistry. A Functional Approach. Philadelphia: Saunders, 1979:511-544.

8. Pilegaard H, Neufer PD. Transcriptional regulation of pyruvate dehydrogenase kinase 4 in skeletal muscle during and after exercise. Proc Nutr Soc 2004; 63:221-6.

9. Doiron B, Cuif MH, Chen R, Kahn A. Transcriptional glucose signaling through the glucose response element is mediated by the pentose phosphate pathway. J Biol Chem 1996; 271:5321-4.

10. Nishimura M, Uyeda K. Purification and characterization of a novel xylulose 5-phosphate-activated protein phosphatase catalyzing dephosphorylation of fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. J Biol Chem 1995; 270:26341-6.

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

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

13. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K. Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. Proc Natl Acad Sci U S A 2001; 98:13710-5.

14. Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport

system. Role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem 1991; 266:4706-12.

15. Ravussin E, Gautier JF. [Determinants and control of energy expenditure]. Ann Endocrinol (Paris) 2002; 63:96-105.

16. McClain DA. Hexosamines as mediators of nutrient sensing and regulation in diabetes. J Diabetes Complications 2002; 16:72-80.

17. Duplus E, Glorian M, Forest C. Fatty acid regulation of gene transcription. J Biol Chem 2000; 275:30749-52.

18. Stremmel W, Pohl L, Ring A, Herrmann T. A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. Lipids 2001; 36:981-9.

19. Memon RA, Fuller J, Moser AH, Smith PJ, Grunfeld C, Feingold KR. Regulation of putative fatty acid transporters and Acyl-CoA synthetase in liver and adipose tissue in ob/ob mice. Diabetes 1999; 48:121-7.

20. Finck BN, Kelly DP. Peroxisome proliferator-activated receptor alpha (PPARalpha) signaling in the gene regulatory control of energy metabolism in the normal and diseased heart. J Mol Cell Cardiol 2002; 34:1249-57.

21. Brown NF, Weis BC, Husti JE, Foster DW, McGarry JD. Mitochondrial carnitine palmitoyltransferase I isoform switching in the developing rat heart. J Biol Chem 1995; 270:8952-7.

22. Taegtmeyer H. Energy metabolism of the heart: from basic concepts to clinical applications. Curr Probl Cardiol 1994; 19:59-113.

23. Barber MC, Travers MT. Cloning and characterisation of multiple acetyl-CoA carboxylase transcripts in ovine adipose tissue. Gene 1995; 154:271-5.

24. Song CS, Kim KH. Reevaluation of properties of acetyl-CoA carboxylase from rat liver. J Biol Chem 1981; 256:7786-8.

25. Lopez-Casillas F, Bai DH, Luo XC, Kong IS, Hermodson MA, Kim KH. Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. Proc Natl Acad Sci U S A 1988; 85:5784-8.

26. 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.

27. 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.

28. Goodwin GW, Taegtmeyer H. Regulation of fatty acid oxidation of the heart by MCD and ACC during contractile stimulation. Am J Physiol 1999; 277:E772-7.

29. Saha AK, Schwarsin AJ, Roduit R, et al. Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta -D-ribofuranoside. J Biol Chem 2000; 275:24279-83.

30. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 1997; 244:1-14.

31. 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.

32. Jensen MD. Fate of fatty acids at rest and during exercise: regulatory mechanisms. Acta Physiol Scand 2003; 178:385-90.

33. Vahouny GV, Katzen R, Entenman C. Myocardial metabolism. II. Role of nutritional state on palmitate and glucose oxidation by isolated perfused hearts. Biochim Biophys Acta 1967; 137:181-3.

34. Wittels B, Spann JF, Jr. Defective lipid metabolism in the failing heart. J Clin Invest 1968; 47:1787-94.

35. Taegtmeyer H, Overturf ML. Effects of moderate hypertension on cardiac function and metabolism in the rabbit. Hypertension 1988; 11:416-26.

36. Christe ME, Rodgers RL. Altered glucose and fatty acid oxidation in hearts of the spontaneously hypertensive rat. J Mol Cell Cardiol 1994; 26:1371-5.

37. Makinde AO, Kantor PF, Lopaschuk GD. Maturation of fatty acid and carbohydrate metabolism in the newborn heart. Mol Cell Biochem 1998; 188:49-56.

38. Montessuit C, Papageorgiou I, Tardy I, Lerch R. Effect of nutritional state on substrate metabolism and contractile function in postischemic rat myocardium. Am J Physiol 1996; 271:H2060-70.

39. Young Z, Steele DS. Effects of cytosolic ATP on Ca^{2+} sparks and SR Ca^{2+} content in permeabilized cardiac myocytes. Circ Res 2001; 89:526-533.

40. Van der Lee KA, Willemsen PH, Samec S, et al. Fasting-induced changes in the expression of genes controlling substrate metabolism in the rat heart. J Lipid Res 2001; 42:1752-8.

41. Barger PM, Kelly DP. PPAR signaling in the control of cardiac energy metabolism. Trends Cardiovasc Med 2000; 10:238-45.

42. Brandt JM, Djouadi F, Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem 1998; 273:23786-92.

43. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc Natl Acad Sci U S A 1994; 91:11012-6.

44. 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.

45. Penas M, Benito M. Regulation of carnitine palmitoyltransferase activity in the liver and brown adipose tissue in the newborn rat: effect of starvation and hypothermia. Biochem Biophys Res Commun 1986; 135:589-96.

46. Taegtmeyer H, Hems R, Krebs HA. Utilization of energy-providing substrates in the isolated working rat heart. Biochem J 1980; 186:701-11.

47. Saha AK, Vavvas D, Kurowski TG, et al. Malonyl-CoA regulation in skeletal muscle: its link to cell citrate and the glucose-fatty acid cycle. Am J Physiol 1997; 272:E641-8.

48. Ruderman NB, Saha AK, Vavvas D, et al. Malonyl CoA as a metabolic switch and a regulator of insulin sensitivity. Adv Exp Med Biol 1998; 441:263-70.

49. Hillgartner FB, Charron T, Chesnut KA. Alterations in nutritional status regulate acetyl-CoA carboxylase expression in avian liver by a transcriptional mechanism. Biochem J 1996; 319 (Pt 1):263-8.

50. 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.

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

52. Taegtmeyer H, McNulty P, Young ME. Adaptation and maladaptation of the heart in diabetes: Part I: general concepts. Circulation 2002; 105:1727-33.

53. Taegtmeyer H. Switching metabolic genes to build a better heart. Circulation 2002; 106:2043-2045.

54. Huang B, Wu P, Bowker-Kinley MM, Harris RA. Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. Diabetes 2002; 51:276-83.

55. Wu P, Blair PV, Sato J, Jaskiewicz J, Popov KM, Harris RA. Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. Arch Biochem Biophys 2000; 381:1-7.

56. Holness MJ, Smith ND, Bulmer K, Hopkins T, Gibbons GF, Sugden MC. Evaluation of the role of peroxisome-proliferator-activated receptor alpha in the regulation of cardiac pyruvate dehydrogenase kinase 4 protein expression in response to starvation, high-fat feeding and hyperthyroidism. Biochem J 2002; 364:687-94.

57. 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.

58. Kim JY, Nolte LA, Hansen PA, et al. High-fat diet-induced muscle insulin resistance: relationship to visceral fat mass. Am J Physiol Regul Integr Comp Physiol 2000; 279:R2057-65.

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

60. 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.

61. Bartelds B, Knoester H, Smid GB, et al. Perinatal changes in myocardial metabolism in lambs. Circulation 2000; 102:926-31.

62. Nau PN, Van Natta T, Ralphe JC, et al. Metabolic adaptation of the fetal and postnatal ovine heart: regulatory role of hypoxia-inducible factors and nuclear respiratory factor-1. Pediatr Res 2002; 52:269-78.

63. Witters LA, Gao G, Kemp BE, Quistorff B. Hepatic 5'-AMP-activated protein kinase: zonal distribution and relationship to acetyl-CoA carboxylase activity in varying nutritional states. Arch Biochem Biophys 1994; 308:413-9.

64. Witters LA, Kemp BE. Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. J Biol Chem 1992; 267:2864-7.

65. Dyck JR, Lopaschuk GD. Malonyl CoA control of fatty acid oxidation in the ischemic heart. J Mol Cell Cardiol 2002; 34:1099-109.

66. 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.

67. Park EA, Cook GA. Differential regulation in the heart of mitochondrial carnitine palmitoyltransferase-I muscle and liver isoforms. Mol Cell Biochem 1998; 180:27-32.

68. McLeod CJ, Pagel I, Sack MN. The mitochondrial biogenesis regulatory program in cardiac adaptation to ischemia-a putative target for therapeutic intervention. Trends Cardiovasc Med 2005; 15:118-23.

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

70. Melov S, Coskun PE, Wallace DC. Mouse models of mitochondrial disease, oxidative stress, and senescence. Mutat Res 1999; 434:233-42.

71. Williams RS. Cardiac involvement in mitochondrial diseases, and vice versa. Circulation 1995; 91:1266-8.

72. Corral-Debrinski M, Stepien G, Shoffner JM, Lott MT, Kanter K, Wallace DC. Hypoxemia is associated with mitochondrial DNA damage and gene induction. Implications for cardiac disease. Jama 1991; 266:1812-6.

73. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. Mutat Res 1992; 275:169-80.

74. Arnheim N, Cortopassi G. Deleterious mitochondrial DNA mutations accumulate in aging human tissues. Mutat Res 1992; 275:157-67.

75. Salo DC, Donovan CM, Davies KJ. HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise. Free Radic Biol Med 1991; 11:239-46.

76. Marin-Garcia J, Goldenthal MJ. Mitochondrial biogenesis defects and neuromuscular disorders. Pediatr Neurol 2000; 22:122-9.

77. Baar K. Involvement of PPAR gamma co-activator-1, nuclear respiratory factors 1 and 2, and PPAR alpha in the adaptive response to endurance exercise. Proc Nutr Soc 2004; 63:269-73.

78. Garnier A, Fortin D, Zoll J, et al. Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. Faseb J 2005; 19:43-52.

79. Escriva H, Rodriguez-Pena A, Vallejo CG. Expression of mitochondrial genes and of the transcription factors involved in the biogenesis of

mitochondria Tfam, NRF-1 and NRF-2, in rat liver, testis and brain. Biochimie 1999; 81:965-71.

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

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

82. Choi YS, Lee KU, Pak YK. Regulation of mitochondrial transcription factor A expression by high glucose. Ann N Y Acad Sci 2004; 1011:69-77.

83. Ikeuchi M, Matsusaka H, Kang D, et al. Overexpression of mitochondrial transcription factor a ameliorates mitochondrial deficiencies and cardiac failure after myocardial infarction. Circulation 2005; 112:683-90.

84. Huss JM, Kopp RP, Kelly DP. Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. J Biol Chem 2002; 277:40265-74.

85. Wu P, Inskeep K, Bowker-Kinley MM, Popov KM, Harris RA. Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. Diabetes 1999; 48:1593-9.

86. Baar K, Wende AR, Jones TE, et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. Faseb J 2002; 16:1879-86.

87. Simoneau JA, Kelley DE, Warden C. Obesity and increased contractile activity influence the protein content of UCP2 in human skeletal muscle. Int J Obes Relat Metab Disord 1999; 23 Suppl 6:S68-71.

88. 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 U S A 2003; 100:8466-71.

89. 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 U S A 1997; 94:11399-404.

90. Xia Y, Buja LM, McMillin JB. Activation of the cytochrome c gene by electrical stimulation in neonatal rat cardiac myocytes. Role of NRF-1 and c-Jun. J Biol Chem 1998; 273:12593-8.

91. 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.

92. Kagaya Y, Kanno Y, Takeyama D, et al. Effects of long-term pressure overload on regional myocardial glucose and free fatty acid uptake in rats. A quantitative autoradiographic study. Circulation 1990; 81:1353-61.

93. Doenst T, Goodwin GW, Cedars AM, Wang M, Stepkowski S, Taegtmeyer H. Load-induced changes in vivo alter substrate fluxes and insulin responsiveness of rat heart in vitro. Metabolism 2001; 50:1083-90.

94. Allard MF, Schonekess B, Henning SL, English DR, Lopaschuck GD. Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. Am. J. Physiol. 1994; 267:H742-H750.

95. Bishop SP, Altschuld RA. Increased glycolytic metabolism in cardiac hypertrophy and congestive failure. Am J Physiol 1970; 218:153-9.

96. Paternostro G, Clarke K, Heath J, Seymour AM, Radda GK. Decreased GLUT-4 mRNA content and insulin-sensitive deoxyglucose uptake show insulin resistance in the hypertensive rat heart. Cardiovasc Res 1995; 30:205-11.

97. Depre C, Shipley GL, Chen W, et al. Unloaded heart in vivo replicates fetal gene expression of cardiac hypertrophy. Nat Med 1998; 4:1269-75.

98. Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ. Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. Circulation 2001; 104:1664-9.

99. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation 1996; 94:2837-42.

100. Sack MN, Disch DL, Rockman HA, Kelly DP. A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. Proc Natl Acad Sci U S A 1997; 94:6438-43.

101. Van Der Lee KA, Willemsen PH, Van Der Vusse GJ, Van Bilsen M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. Faseb J 2000; 14:495-502.

102. Young ME, Laws FA, Goodwin GW, Taegtmeyer H. Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart. J Biol Chem 2001; 276:44390-5.

103. Sack MN, Disch DL, Rockman HA, Kelly DP. A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. Proc Natl Acad Sci 1997; 94:6438-6443.

104. Olson RE, Platnek DA. Conservation of energy in cardiac muscle. Ann N Y Acad Sci 1959; 72:466-79.

105. Recchia FA, McConnell PI, Bernstein RD, Vogel TR, Xu X, Hintze TH. Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. Circ Res 1998; 83:969-79.

106. Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH. Metabolic gene expression in fetal and failing human heart. Circulation 2001; 104:2923-2931.

107. Osorio JC, Stanley WC, Linke A, et al. Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure. Circulation 2002; 106:606-12.

108. 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.

109. Essop F, Opie LH. Metabolic therapy for heart failure. Eur Heart J 2004; 25:1765-8.

110. Lionetti V, Linke A, Chandler MP, et al. Carnitine palmitoyl transferase-I inhibition prevents ventricular remodeling and delays decompensation in pacing-induced heart failure. Cardiovasc Res 2005; 66:454-61.

111. Sabbah HH, Stanley WC. Partial fatty acid oxidation inhibitors: a potentially new class of drugs for heart failure. Eur J Heart Fail 2002; 4:3-6.

112. Ungar I, Gilbert M, Siegel A, Blain JM, Bing RJ. Studies on myocardial metabolism. IV. Myocardial metabolism in diabetes. Am J Med 1955; 18:385-96.

113. Stanley WC, Lopaschuk GD, McCormack JG. Regulation of energy substrate metabolism in the diabetic heart. Cardiovasc Res 1997; 34:25-33.

114. Chatham JC, Forder JR. Metabolic compartmentation of lactate in the glucose-perfused rat heart. Am J Physiol 1996; 270:H224-9.

115. Chatham JC, Forder JR. Relationship between cardiac function and substrate oxidation in hearts of diabetic rats. Am J Physiol 1997; 273:H52-8.

116. 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.

117. 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.

118. Hopkins TA, Sugden MC, Holness MJ, Kozak R, Dyck JR, Lopaschuk GD. Control of cardiac pyruvate dehydrogenase activity in peroxisome proliferator-activated receptor-alpha transgenic mice. Am J Physiol Heart Circ Physiol 2003; 285:H270-6.

119. 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.

120. Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD. Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. Diabetes 2001; 50:123-30.

121. 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.

122. 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.

123. Dentin R, Girard J, Postic C. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. Biochimie 2005; 87:81-6.

124. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 1990; 347:645-50.

125. Jump DB, Thelen A, Mater M. Dietary polyunsaturated fatty acids and hepatic gene expression. Lipids 1999; 34 Suppl:S209-12.

126. Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue

distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 1996; 137:354-66.

127. Kota BP, Huang TH, Roufogalis BD. An overview on biological mechanisms of PPARs. Pharmacol Res 2005; 51:85-94.

128. Djouadi F, Weinheimer CJ, Saffitz JE, et al. A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator-activated receptor alpha- deficient mice. J Clin Invest 1998; 102:1083-91.

129. 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 U S A 1999; 96:7473-8.

130. Escher P, Wahli W. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. Mutat Res 2000; 448:121-38.

131. Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N. Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. J Biol Chem 1998; 273:16710-4.

132. Finck BN, Lehman JJ, Barger PM, Kelly DP. Regulatory networks controlling mitochondrial energy production in the developing, hypertrophied, and diabetic heart. Cold Spring Harb Symp Quant Biol 2002; 67:371-82.

133. Dressel U, Allen TL, Pippal JB, Rohde PR, Lau P, Muscat GE. The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. Mol Endocrinol 2003; 17:2477-93.

134. Muoio DM, Way JM, Tanner CJ, et al. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. Diabetes 2002; 51:901-9.

135. Holst D, Luquet S, Nogueira V, Kristiansen K, Leverve X, Grimaldi PA. Nutritional regulation and role of peroxisome proliferator-activated receptor delta in fatty acid catabolism in skeletal muscle. Biochim Biophys Acta 2003; 1633:43-50.

136. Rose EA, Gelijns AC, Moskowitz AJ, et al. Long-term use of a left ventricular assist device for end-stage heart failure. N Engl J Med 2001; 345:1435-43.

137. Luquet S, Gaudel C, Holst D, et al. Roles of PPAR delta in lipid absorption and metabolism: a new target for the treatment of type 2 diabetes. Biochim Biophys Acta 2005; 1740:313-7.

138. Fueger PT, Heikkinen S, Bracy DP, et al. Hexokinase II partial knockout impairs exercise-stimulated glucose uptake in oxidative muscles of mice. Am J Physiol Endocrinol Metab 2003; 285:E958-63.

139. King LM, Opie LH. Does preconditioning act by glycogen depletion in the isolated rat heart? J Mol Cell Cardiol 1996; 28:2305-21.

140. King LM, Opie LH. Glucose and glycogen utilisation in myocardial ischemia--changes in metabolism and consequences for the myocyte. Mol Cell Biochem 1998; 180:3-26.

141. Sandberg MB, Bloksgaard M, Duran-Sandoval D, Duval C, Staels B, Mandrup S. The gene encoding acyl-CoA-binding protein is subject to metabolic regulation by both sterol regulatory element-binding protein and peroxisome proliferator-activated receptor alpha in hepatocytes. J Biol Chem 2005; 280:5258-66.

142. Colosia AD, Marker AJ, Lange AJ, et al. Induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA by refeeding and insulin. J Biol Chem 1988; 263:18669-77.

143. Vaulont S, Kahn A. Transcriptional control of metabolic regulation genes by carbohydrates. Faseb J 1994; 8:28-35.

144. Yamashita H, Takenoshita M, Sakurai M, et al. A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. Proc Natl Acad Sci U S A 2001; 98:9116-21.

145. Iizuka K, Bruick RK, Liang G, Horton JD, Uyeda K. Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. Proc Natl Acad Sci U S A 2004; 101:7281-6.

146. Im SS, Kang SY, Kim SY, et al. Glucose-stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. Diabetes 2005; 54:1684-91.

147. Tang T, Li Y. [Sterol regulatory element-binding proteins and lipid metabolism]. Sheng Li Ke Xue Jin Zhan 2005; 36:29-34.

148. Zhang S, Kim KH. Protein kinase CK2 down-regulates glucose-activated expression of the acetyl-CoA carboxylase gene. Arch Biochem Biophys 1997; 338:227-32.

149. 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.

150. Shih HM, Towle HC. Definition of the carbohydrate response element of the rat S14 gene. Evidence for a common factor required for carbohydrate regulation of hepatic genes. J Biol Chem 1992; 267:13222-8.

151. Koo SH, Dutcher AK, Towle HC. Glucose and insulin function through two distinct transcription factors to stimulate expression of lipogenic enzyme genes in liver. J Biol Chem 2001; 276:9437-45.

152. Sawadogo M. Multiple forms of the human gene-specific transcription factor USF. II. DNA binding properties and transcriptional activity of the purified HeLa USF. J Biol Chem 1988; 263:11994-2001.

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

154. Bergot MO, Diaz-Guerra MJ, Puzenat N, Raymondjean M, Kahn A. Cis-regulation of the L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP. Nucleic Acids Res 1992; 20:1871-7.

155. Marie S, Diaz-Guerra MJ, Miquerol L, Kahn A, Iynedjian PB. The pyruvate kinase gene as a model for studies of glucose-dependent regulation of gene expression in the endocrine pancreatic beta-cell type. J Biol Chem 1993; 268:23881-90.

156. Diaz Guerra MJ, Bergot MO, Martinez A, Cuif MH, Kahn A, Raymondjean M. Functional characterization of the L-type pyruvate kinase gene glucose response complex. Mol Cell Biol 1993; 13:7725-33.

157. Shih HM, Liu Z, Towle HC. Two CACGTG motifs with proper spacing dictate the carbohydrate regulation of hepatic gene transcription. J Biol Chem 1995; 270:21991-7.

158. 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.

159. 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.

160. Foufelle F, Gouhot B, Pegorier JP, Perdereau D, Girard J, Ferre P. Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. A role for glucose 6-phosphate. J Biol Chem 1992; 267:20543-6.

161. Liu YQ, Uyeda K. A mechanism for fatty acid inhibition of glucose utilization in liver. Role of xylulose 5-P. J Biol Chem 1996; 271:8824-30.

162. Brun T, Roche E, Kim KH, Prentki M. Glucose regulates acetyl-CoA carboxylase gene expression in a pancreatic beta-cell line (INS-1). J Biol Chem 1993; 268:18905-11.

163. Massillon D, Chen W, Barzilai N, et al. Carbon flux via the pentose phosphate pathway regulates the hepatic expression of the glucose-6-phosphatase and phosphoenolpyruvate carboxykinase genes in conscious rats. J Biol Chem 1998; 273:228-34.

164. Mourrieras F, Foufelle F, Foretz M, Morin J, Bouche S, Ferre P. Induction of fatty acid synthase and S14 gene expression by glucose, xylitol and dihydroxyacetone in cultured rat hepatocytes is closely correlated with glucose 6-phosphate concentrations. Biochem J 1997; 326 (Pt 2):345-9.

165. Hardie DG. Protein phosphorylation and dephosphorylation. Curr Opin Cell Biol 1989; 1:220-6.

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

167. Moore F, Weekes J, Hardie DG. Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. Eur J Biochem 1991; 199:691-7.

168. Witters LA, Nordlund AC, Marshall L. Regulation of intracellular acetyl-CoA carboxylase by ATP depletors mimics the action of the 5'-AMP-activated protein kinase. Biochem Biophys Res Commun 1991; 181:1486-92.

169. Li J, Hu X, Selvakumar P, et al. Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle. Am J Physiol Endocrinol Metab 2004; 287:E834-41.

170. Ojuka EO. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. Proc Nutr Soc 2004; 63:275-8.

171. Yamaguchi S, Katahira H, Ozawa S, et al. Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3L1 adipocytes. Am J Physiol Endocrinol Metab 2005.

172. Salt IP, Johnson G, Ashcroft SJ, Hardie DG. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. Biochem J 1998; 335 (Pt 3):533-9.

173. da Silva Xavier G, Leclerc I, Salt IP, et al. Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression. Proc Natl Acad Sci U S A 2000; 97:4023-8.

174. Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C. 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.

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

176. Dobrzyn P, Dobrzyn A, Miyazaki M, et al. Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. Proc Natl Acad Sci U S A 2004; 101:6409-14.

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

178. Bronner M, Hertz R, Bar-Tana J. Kinase-independent transcriptional co-activation of peroxisome proliferator-activated receptor alpha by AMP-activated protein kinase. Biochem J 2004; 384:295-305.

179. Jorgensen SB, Wojtaszewski JF, Viollet B, et al. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. Faseb J 2005; 19:1146-8.

180. Widmer J, Fassihi KS, Schlichter SC, et al. Identification of a second human acetyl-CoA carboxylase gene. Biochem J 1996; 316 (Pt 3):915-22.

181. Kim K. Regulation of mammalian acetyl-coenzyme A carboxylase. Annu Rev Nutr 1997; 17:77-99.

182. Louis NA, Witters LA. Glucose regulation of acetyl-CoA carboxylase in hepatoma and islet cells. J Biol Chem 1992; 267:2287-93.

183. Lopez-Casillas F, Luo XC, Kong IS, Kim KH. Characterization of different forms of rat mammary gland acetyl-coenzyme A carboxylase mRNA: analysis of heterogeneity in the 5' end. Gene 1989; 83:311-9.

184. 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.

185. 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.

186. Park K, Kim KH. The site of cAMP action in the insulin induction of gene expression of acetyl-CoA carboxylase is AP-2. J Biol Chem 1993; 268:17811-9.

187. 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.

188. Luo XC, Kim KH. An enhancer element in the house-keeping promoter for acetyl-CoA carboxylase gene. Nucleic Acids Res 1990; 18:3249-54.

189. McGarry JD, Woeltje KF, Kuwajima M, Foster DW. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. Diabetes Metab Rev 1989; 5:271-84.

190. Lopaschuk GD, Gamble J. The 1993 Merck Frosst Award. Acetyl-CoA carboxylase: an important regulator of fatty acid oxidation in the heart. Can J Physiol Pharmacol 1994; 72:1101-9.

191. 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.

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

193. Dyck JR, Kudo N, Barr AJ, Davies SP, Hardie DG, Lopaschuk GD. 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.

194. 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 U S A 2003; 100:10207-12.

195. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. 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.

196. Boone AN, Rodrigues B, Brownsey RW. Multiple-site phosphorylation of the 280 kDa isoform of acetyl-CoA carboxylase in rat cardiac myocytes: evidence that cAMP-dependent protein kinase mediates effects of beta-adrenergic stimulation. Biochem J 1999; 341 (Pt 2):347-54.

197. Ruderman NB, Saha AK, Vavvas D, Witters LA. Malonyl-CoA, fuel sensing, and insulin resistance. Am J Physiol 1999; 276:E1-E18.

198. 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.

199. Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE, McConell GK. Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. Am J Physiol Endocrinol Metab 2002; 282:E688-94.

200. 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.

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

202. 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.

203. 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.

204. Thampy KG, Wakil SJ. Regulation of acetyl-coenzyme A carboxylase. II. Effect of fasting and refeeding on the activity, phosphate content, and aggregation state of the enzyme. J Biol Chem 1988; 263:6454-8.

205. Steinberg GR, Rush JW, Dyck DJ. AMPK expression and phosphorylation are increased in rodent muscle after chronic leptin treatment. Am J Physiol Endocrinol Metab 2003; 284:E648-54.

206. Allred JB, Reilly KE. Short-term regulation of acetyl CoA carboxylase in tissues of higher animals. Prog Lipid Res 1996; 35:371-85.

207. 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.

208. Lee JJ, Moon YA, Ha JH, Yoon DJ, Ahn YH, Kim KS. Cloning of human acetyl-CoA carboxylase beta promoter and its regulation by muscle regulatory factors. J Biol Chem 2001; 276:2576-85.

209. Amacher SL, Buskin JN, Hauschka SD. Multiple regulatory elements contribute differentially to muscle creatine kinase enhancer activity in skeletal and cardiac muscle. Mol Cell Biol 1993; 13:2753-64.

210. Opie LH, Owen P, Thomas M, Young V. Potassium loss, metabolic changes and ventricular arrhythmias following acute experimental coronary occlusion. J Physiol 1969; 202:44P-45P.

211. Holden JE, Stone CK, Clark CM, et al. Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia. J Appl Physiol 1995; 79:222-8.

212. Shipp J, Opie L, Challoner D. Fatty acid and glucose metabolism in the perfused heart. Nature 1961; 199:1018.

213. Bianchi A, Evans JL, Iverson AJ, Nordlund AC, Watts TD, Witters LA. Identification of an isozymic form of acetyl-CoA carboxylase. J Biol Chem 1990; 265:1502-9.

214. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol 1969; 22:158-61.

215. Abe K, Shinohara Y, Terada H. Isolation and characterization of cDNA encoding rat heart type acetyl-CoA carboxylase. Biochim Biophys Acta 1998; 1398:347-52.

216. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977; 83:346-56.

217. Ngumbela KC, Sack MN, Essop MF. Counter-regulatory effects of incremental hypoxia on the transcription of a cardiac fatty acid oxidation enzyme-encoding gene. Mol Cell Biochem 2003; 250:151-8.

218. Torlinska T, Ozegowski S, Paluszak J, Hryniewiecki T. In vivo effect of 2-deoxy-D-glucose on glucose-6-phosphate dehydrogenase activity in the cytosol of liver, heart and skeletal muscle of rats. Acta Physiol Pol 1990; 41:138-45.

219. Awan MM, Makaula S, Forresti S, Sack MN, Opie LH. Mechanisms whereby glucose deprivation triggers metabolic preconditioning in the isolated rat heart. Mol Cell Biochem 2000; 211:111-21.

220. Kang HT, Ju JW, Cho JW, Hwang ES. Down-regulation of Sp1 activity through modulation of O-glycosylation by treatment with a low glucose mimetic, 2-deoxyglucose. J Biol Chem 2003; 278:51223-31.

221. Boros LG, Brandes JL, Yusuf FI, Cascante M, Williams RD, Schirmer WJ. Inhibition of the oxidative and nonoxidative pentose phosphate pathways by somatostatin: a possible mechanism of antitumor action. Med Hypotheses 1998; 50:501-6.

222. Deb C, Mukherjee R, Kaul P, Mallick N. Pentose phosphate pathway and testicular steroidogenesis in rats following oxythiamine treatment. Endokrinologie 1977; 69:143-50.

223. Wang Z, Karras JG, Howard RG, Rothstein TL. Induction of bcl-x by CD40 engagement rescues sIg-induced apoptosis in murine B cells. J Immunol 1995; 155:3722-5.

224. Trebukhina RV, Mikhal'tsevich GN, Lashak LK, Petushok VG, Velichko MG. [Biochemical changes in the rat immunocompetent organs during oxythiamine involution of the thymus]. Vopr Med Khim 1985; 31:124-7.

225. 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.

226. Han SY, Kim JC, Suh JM, Chung IK. Cell type-dependent regulation of human DNA topoisomerase III alpha gene expression by upstream stimulatory factor 2. FEBS Lett 2001; 505:57-62.

227. Roduit R, Morin J, Masse F, et al. Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta -cell. J Biol Chem 2000; 275:35799-806.

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

229. 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 U S A 2003; 100:1226-31.

230. 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.

231. Hanley K, Jiang Y, He SS, et al. Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha. J Invest Dermatol 1998; 110:368-75.

232. Intrasuksri U, Rangwala SM, O'Brien M, Noonan DJ, Feller DR. Mechanisms of peroxisome proliferation by perfluorooctanoic acid and endogenous fatty acids. Gen Pharmacol 1998; 31:187-97.

233. Lawrence JW, Wollenberg GK, Frank JD, DeLuca JG. Dexamethasone selectively inhibits WY14,643-induced cell proliferation and not peroxisome proliferation in mice. Toxicol Appl Pharmacol 2001; 170:113-23.

234. Baar K, Song Z, Semenkovich CF, et al. Skeletal muscle overexpression of nuclear respiratory factor 1 increases glucose transport capacity. Faseb J 2003; 17:1666-73.

235. Lenka N, Basu A, Mullick J, Avadhani NG. The role of an E box binding basic helix loop helix protein in the cardiac muscle-specific expression of

the rat cytochrome oxidase subunit VIII gene. J Biol Chem 1996; 271:30281-9.

236. Wan B, Moreadith RW. Structural characterization and regulatory element analysis of the heart isoform of cytochrome c oxidase Vla. J Biol Chem 1995; 270:26433-40.

237. 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.

238. Schrauwen P, van Aggel-Leijssen DP, Hul G, et al. The effect of a 3-month low-intensity endurance training program on fat oxidation and acetyl-CoA carboxylase-2 expression. Diabetes 2002; 51:2220-6.

239. Gautier JF. [Physical activity and type 2 diabetes]. Rev Med Liege 2005; 60:395-401.

240. Bishop-Bailey D. Peroxisome proliferator-activated receptors in the cardiovascular system. Br J Pharmacol 2000; 129:823-34.

241. Harrison G, Somerset S. Macadamia or olive oil enriched diets induce changes in heart structure and function similar to regular exercise in rats. Asia Pac J Clin Nutr 2004; 13:S48.

242. Kume N, Murase T, Moriwaki H, et al. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. Circ Res 1998; 1998:322-327.

243. Ide T, Tsunoda M, Mochizuki T, Murakami K. Enhancement of insulin signaling through inhibition of tissue lipid accumulation by activation of peroxisome proliferator-activated receptor (PPAR) alpha in obese mice. Med Sci Monit 2004; 10:BR388-95.

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

245. Bouzakri K, Roques M, Debard C, et al. WY-14643 and 9- cis-retinoic acid induce IRS-2/PI 3-kinase signalling pathway and increase glucose transport in human skeletal muscle cells: differential effect in myotubes from healthy subjects and Type 2 diabetic patients. Diabetologia 2004; 47:1314-23.

246. Chou CJ, Haluzik M, Gregory C, et al. WY14,643, a peroxisome proliferator-activated receptor alpha (PPARalpha) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 mice. J Biol Chem 2002; 277:24484-9.

247. 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.

248. Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM. Convergence of 9-cis retinoic acid and peroxisome proliferator signalling

pathways through heterodimer formation of their receptors. Nature 1992; 358:771-4.

249. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 1998; 92:829-39.

250. Vega RB, Huss JM, Kelly DP. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 2000; 20:1868-76.

251. Jong-Yeon K, Hickner RC, Dohm GL, Houmard JA. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. Metabolism 2002; 51:460-4.

252. Park H, Kaushik VK, Constant S, et al. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. J Biol Chem 2002; 277:32571-7.

253. Winder WW, Holmes BF. Insulin stimulation of glucose uptake fails to decrease palmitate oxidation in muscle if AMPK is activated. J Appl Physiol 2000; 89:2430-2437.

254. Kumari D, Gabrielian A, Wheeler D, Usdin K. The roles of Sp1, Sp3, USF1/USF2 and NRF-1 in the regulation and three-dimensional structure

of the Fragile X mental retardation gene promoter. Biochem J 2005; 386:297-303.

255. Smith KT, Coffee B, Reines D. Occupancy and synergistic activation of the FMR1 promoter by Nrf-1 and Sp1 in vivo. Hum Mol Genet 2004; 13:1611-21.

256. Ge Y, Jensen TL, Matherly LH, Taub JW. Physical and functional interactions between USF and Sp1 proteins regulate human deoxycytidine kinase promoter activity. J Biol Chem 2003; 278:49901-10.

257. Sharma S, Taegtmeyer H, Adrogue J, et al. Dynamic changes of gene expression in hypoxia-induced right ventricular hypertrophy. Am J Physiol Heart Circ Physiol 2004; 286:H1185-92.

258. Unger RH. Hyperleptinemia: protecting the heart from lipid overload. Hypertension 2005; 45:1031-4.

259. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest 2000; 106:165-9.

260. Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 1996; 271:31372-8.

261. Zeng G, Nystrom FH, Ravichandran LV, et al. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of

nitric oxide in human vascular endothelial cells. Circulation 2000; 101:1539-45.

262. Randle PJ, Sugden PH, Kerbey AL, Radcliffe PM, Hutson NJ. Regulation of pyruvate oxidation and the conservation of glucose. Biochem Soc Symp 1978:47-67.

263. McGarry JD. Travels with carnitine palmitoyltransferase I: from liver to germ cell with stops in between. Biochem Soc Trans 2001; 29:241-5.

264. Hescheler J, Meyer R, Plant S, et. al. Morphological, Biochemmical, and Electrophysiological Characterisation of a Clonal Cell (H9c2) Line from rat heart. Circ Res 1998; 69:1476-1486.

265. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. Expt Cell Res 1976; 98:367-381.

266. Gilde AJ, van der Lee KAJM, Willemsen PHM, et. al. Peroxisome proliferator-activated receptor (PPAR) α and PPARβ/δ, but not PPARγ, modulate the expression of genes involved in cardiac lipid metabolism. Circ Res 2003; 92:518-524.

267. Alam N, Saggerson ED. Malonyl-CoA and the regulation of fatty acid oxidation in soleus muscle. Biochem J 1998; 334 (Pt 1): 233-241.

268. Bonilla S, Redonnet A, Noel-Suberville C, et.al. High-fat diets affect the expression of nuclear retinoic acid receptor in rat liver. Br J Nutr 2000; 83(6):665-671.

269. Brun T, Assimacopoulos-Jeannet F, Corkey BE and Prentki M. Long-chain fatty acids inhibit acetyl-CoA carboxylase gene expression in the pancreatic beta-cell line INS-1. Diabetes1997; 46(3):393-400.

270. Brandt JM, Djouadi F, Kelly DP. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase 1 gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem 1998; 273(37):23786-92.

271. Panagia M, Gibbons GF, et. al. PPAR-alpha activation required for decreased glucose uptake and increased susceptibility to injury during ischemia. Am J Physiol Heart Circ Physiol 2005; 288(6):H2677-83.

272. Roduit R, Morin J, Massé F, et. al. Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-α in the pancreatic β-cell. J Biol Chem 2000; 275:35799-35806.

273. 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(1):91-100.

274. Randle PJ, Newsholme EA, Garland PB. Regulation of glucose uptake. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. Biochem J 1964; 93:652-665.

275. Sidossis LS, Wolfe RR. Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. Am J Physiol Endocrinol Metab 1996; 270(33):E733-E738.

276. Young ME, Goodwin GW, Ying J, et. al. Regulation of cardiac and skeletal muscle malonyl-CoA decarboxylase by fatty acids. Am J Physiol Endocrinol Metab 2001; 280(3):E471-479.

277. Campbell FM, Kozak R, Wagner A, et. al. A role for peroxisome proliferator-activated receptor-α (PPARα) in the control of cardiac malonyl-CoA levels: reduced fatty acid oxidation rates and increased glucose oxidation rates in the hearts of mice lacking PPARalpha are associated with higher concentrations of malonyl-CoA and reduced expression of malonyl-CoA carboxylase. J Biol Chem 2002; 277:4098-103.

278. Hopkins TA, Dyck JR, Lopaschuk GD. AMP-activated protein kinase regulation of fatty acid oxidation in the ischemic heart. Biochem Soc Trans 2003; 31(Pt 1):207-12.

Chapter 6

Publications and Presentations

1. 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(1):91-100.

2. Siyanda Makaula, Tasneem Adam, L.H. Opie and M. Faadiel Essop. Upstream Stimulatory Factor 1 (USF1) transactivates human gene promoter of the cardiac isoform of Acetyl-CoA carboxylase (2005). *European Society of Cardiology*, Stockholm, Sweden - Eur H Journal 2005; 26 (abstract supplement): 100.

3. Siyanda Makaula, Tasneem Adam and M. Faadiel Essop. Nuclear Respiratory Factor 1 (NRF1) inhibits transactivation of the human gene promoter of the cardiac isoform of Acetyl-CoA carboxylase (2005), *Physiological Society of Southern Africa*, Cape Town, South Africa (Abstract).

4. Siyanda Makaula, Tasneem Adam, Nayna Manga, Martin Young and M. Faadiel Essop. Nuclear Respiratory Factor-1 inhibits Upstream Stimulatory Factor-1 dependent induction of the cardiac isoform of Acetyl CoA Carboxylase (ACC-β). *XVIII World Congress of the International Society for Heart Research.* 7-11 August 2004 (Brisbane, Australia). Journal of Molecular and Cellular Cardiology (2005) **38**, Abstract no. C83.

5. Siyanda Makaula, Tasneem Adam, Nayna Manga, Martin Young and M. Faadiel Essop. Nuclear Respiratory Factor-1 inhibits Upstream Stimulatory Factor-1 dependent induction of the cardiac isoform of Acetyl CoA Carboxylase (ACC-β). *Scientific Sessions of the 77th Annual Meeting of the American Heart Association.* 7-10 November 2004 (New Orleans, LA). Circulation (2004) **110**, no. 17, Abstract no. 39.

6. Siyanda Makaula, Tasneem Adam and M. Faadiel Essop; The human gene promoter of the cardiac isoform of Acetyl-CoA Carboxylase (ACCβ) is transactivated by Upstream Stimulatory Factor 1 (USF1) in a glucose-dependent manner. *South African Heart Journal* (2004) **1**, number 3, p 40.

7. Siyanda Makaula, Tasneem Adam, Martin E. Young and M. Faadiel Essop; Nuclear Respiratory Factor 1 dependent transactivation of the cardiac isoform of Acetyl-CoA carboxylase. *South African Heart Journal* (2004) **1**, number 3, p 76.

8. Siyanda Makaula, Gary D. Lopaschuk, Michael N. Sack, M. Faadiel Essop; PPARα Induction of Cardiac Acetyl CoA Carboxylase Expression is Nullified by Post-Translational Regulation in Response to Nutritional Flux. *Scientific Sessions of the 75th Annual Meeting of the American Heart Association.* 17-20 November 2002 (Chicago, IL). Circulation (2002) **106,** No. 19, Abstract no. 984.

9. Nayna Manga, Siyanda Makaula, Michael N. Sack, M. Faadiel Essop; USF-1 Trans-Activates the Rat Cardiac Acetyl CoA Carboxylase Gene – A Potential Regulatory Factor in Response to Nutritional Flux. *Scientific Sessions of the 75th Annual Meeting of the American Heart Association.* 17-20 November 2002 (Chicago, IL). Circulation (2002) **106,** No. 19, Abstract no. 985.

10. Nayna Manga, Siyanda Makaula, Michael N. Sack, M. Faadiel Essop; USF1 Transactivates the rat cardiac acetyl-CoA carboxylase gene – A potential regulatory factor in response to nutritional flux. *Physiological Society of Southern Africa* (2002), Stellenbosch, South Africa (Abstract).

11. Siyanda Makaula, Michael N. Sack & M. Faadiel Essop; The regulation of cardiac acetyl Co-A carboxylase in response to nutritional flux. *AstraZeneca Medical Research Day* (2002), University of Cape Town, South Africa (Abstract).

12. Siyanda Makaula, Michael N. Sack & M. Faadiel Essop; The regulation of cardiac acetyl Co-A carboxylase in response to nutritional flux. *Physiological Society of Southern Africa* (2002), Stellenbosch, South Africa (Abstract).

13. Siyanda Makaula, Michael Sack & M. Faadiel Essop; Regulation of cardiac acetyl-coA carboxylase (ACC-β) gene expression in response to different nutritional states; *1st IUBMB/SASBMB International Meeting on: Biochemical and Molecular basis of disease*, (2001), Cape Town, South Africa.