METOPROLOL AND CARDIAC CARNITINE PALMITOYLTRANSFERASE-1: UNRAVELLING A COMPLEX INTERACTION IN NORMAL AND DIABETIC HEARTS

by

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ABSTRACT

Diabetic cardiomyopathy may be initiated or compounded by the heavy reliance of the heart on fatty acids and ketones as metabolic fuels. β-blockers have been proposed to inhibit fatty acid oxidation by decreasing the activity of the enzyme carnitine palmitoyltransferase-1 (CPT-1). By inhibiting fatty acid oxidation, β-blockers could improve myocardial efficiency and ameliorate the cytoplasmic accumulation of toxic fatty acid and glucose intermediates.

In this study, we investigated whether metoprolol improves cardiac function and inhibits fatty acid oxidation in the streptozotocin (STZ) diabetic rat, a model of poorly controlled type 1 diabetes. The animals were injected with 60 mg/kg STZ and were euthanized six weeks following the induction of diabetes. We investigated the effects of chronic metoprolol treatment (75 mg/kg/day), and acute metoprolol perfusion on cardiac function, substrate utilization and three major systems of CPT-1 regulation: malonyl CoA levels, CPT-1 transcription and covalent modifications (phosphorylation, nitrosylation, glutathiolation, nitration).

Chronic metoprolol treatment improved cardiac function in the diabetic heart. Whereas, chronic metoprolol treatment increased fatty acid oxidation in control hearts but decreased it in diabetic hearts, acute metoprolol perfusion always inhibited fatty acid oxidation. Metoprolol lowered malonyl CoA levels in control hearts, and both acute metoprolol perfusion and chronic metoprolol treatment led to decreased CPT-1 maximum activity and decreased CPT-1 malonyl CoA sensitivity. CPT-1 sensitivity was increased by calcium/calmodulin-dependent protein kinase phosphorylation and decreased by protein kinase A-dependent phosphorylation in vitro. CPT-1 activity was inhibited by nitrosylation and glutathiolation, and stimulated by nitration in vitro. Chronic metoprolol treatment decreased the binding and coactivation of peroxisome-proliferator receptor-γ coactivator 1-α (PGC-1α) and peroxisome-proliferator receptor-α.
(PPAR-\(\alpha\)), and also increased the binding of the repressor protein upstream stimulatory factor-2 (USF-2).

In conclusion, metoprolol inhibited fatty acid oxidation, and acted partly by regulating malonyl CoA levels and partly by modulating the activity and malonyl CoA sensitivity of CPT-1 itself. The effects of metoprolol on CPT-1 were mediated acutely by covalent modifications and chronically by inhibition of the transcriptional complex that induces CPT-1 expression.
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<tr>
<td>+dP/ dt</td>
<td>Maximum Rate of Contraction</td>
</tr>
<tr>
<td>-dP/dt</td>
<td>Maximum Rate of Relaxation</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl CoA Carboxylase</td>
</tr>
<tr>
<td>Acyl CoA</td>
<td>Acyl CoEnzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycosylation Endproduct</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial Natriuretic Peptide</td>
</tr>
<tr>
<td>API</td>
<td>Atmospheric Pressure Ionization</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BH4</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CAMK</td>
<td>Calcium/ calmodulin dependent protein kinase</td>
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<td>CAPRICORN</td>
<td>Carvedilol Post-Infarction Survival Control in Left Ventricular Dysfunction Trial</td>
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<td>CAT</td>
<td>Carnitine Acyltransferase</td>
</tr>
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<td>CD36</td>
<td>Fatty acid translocase</td>
</tr>
<tr>
<td>CPT</td>
<td>Carnitine Palmitoyltransferase</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
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<td>DIGAMI</td>
<td>Diabetes Insulin Glucose in Acute Myocardial Infarction Trial</td>
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<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EGTA</td>
<td>Ethylene-glycol-bis(β-aminoethyl ether)tetraacetic Acid</td>
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<td>ERGO-1</td>
<td>Etomoxir for the Recovery of Glucose Oxidation Trial</td>
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<td>ERR</td>
<td>Estrogen-Related Receptor</td>
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<tr>
<td>FACS</td>
<td>Fatty Acyl CoA Synthase</td>
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<tr>
<td>FADH₂</td>
<td>Flavine Adenine Dinucleotide</td>
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<tr>
<td>FABP</td>
<td>Fatty Acid Binding Protein</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate Dehydrogenase</td>
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<tr>
<td>GLM-ANOVA</td>
<td>General linear model ANOVA</td>
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<td>GLP-1</td>
<td>Glucagon-like Peptide 1</td>
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<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
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<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
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<tr>
<td>HMG CoA</td>
<td>β-hydroxy-β-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance Liquid Chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>3-KAT</td>
<td>3-Ketoacyl Transferase</td>
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<tr>
<td>LC MS MS</td>
<td>Liquid Chromatography Tandem Mass Spectroscopy</td>
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<tr>
<td>LVDP</td>
<td>Left Ventricular Developed Pressure</td>
</tr>
<tr>
<td>LVEDP</td>
<td>Left Ventricular End-Diastolic Pressure</td>
</tr>
<tr>
<td>MCD</td>
<td>Malonyl CoA Decarboxylase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate Transporter</td>
</tr>
<tr>
<td>MEF-2A</td>
<td>Myocyte enhancer factor-2A</td>
</tr>
<tr>
<td>MERIT-HF</td>
<td>Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure Trial</td>
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<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)-propanesulfonic Acid</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to Charge Ratio</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>mtNOS</td>
<td>Mitochondrial Nitric Oxide Synthase</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
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<td>PDK</td>
<td>PDH kinase</td>
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<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
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<td>PGC-1</td>
<td>PPAR-(\gamma) coactivator protein-1</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidyl inositol-3 kinase</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for AGE</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RXR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SERCA</td>
<td>Sarcoplasmic Reticulum Calcium ATPase</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic Acid Cycle</td>
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<tr>
<td>USF</td>
<td>Upstream Stimulatory Factor</td>
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DEDICATION

Dedicated to my prescient parents whose timely nudge led me to the study of medicine and sciences, and opened up for me a new and exciting world to explore life’s secrets in all their wonder and beauty. I pray:

“Teach me, my God and King,
In all things thee to see,
And what I do in any thing,
To do it for thee.”

George Herbert (1593-1662): ‘The Elixir’

And I know:

“... experience is an arch wherethrough
Gleams that untraveled world, whose margin fades
For ever and ever when I move . . . . .

. . . . . . . . . . . . . . . . . . .
To strive, to seek, to find and not to yield.”

Alfred Lord Tennyson (1809-1892), ‘Ulysses’
INTRODUCTION

I. Diabetic Cardiomyopathy

Cardiovascular disease is the leading cause of death among diabetic patients, accounting for 80% of all deaths in this group (1). Indeed, diabetes is an independent risk factor for cardiovascular death, and mortality following myocardial infarction is increased in diabetic patients (2-5). The most common cause of this cardiovascular mortality is heart failure. The Framingham Heart study revealed that heart failure is twice as common in diabetic males and five times as common in diabetic females aged 45-74 years when compared to non-diabetic age-matched controls (6). Furthermore, according to the results of the UK prospective diabetes study, for each 1% increase in the HbA$_{1C}$, the risk of heart failure increases by 15% (7). Taken together, these data clearly establish that there is a link between diabetes and heart failure. The prognosis of heart failure in the context of diabetes is very poor. In the Diabetes Insulin Glucose in Acute Myocardial Infarction (DIGAMI) study, a prospective study of first myocardial infarctions in diabetic patients, 66% of deaths in the first year following myocardial infarction were caused by heart failure (8). Systolic dysfunction in diabetic patients carries an annual mortality of 15-20%. Three pathophysiological processes account for diabetes-associated heart failure: myocardial ischemia, hypertension, and diabetic cardiomyopathy (the "cardiotoxic triad") (9). The first two components of this triad are not unique to diabetes, but are important pathophysiological processes which diabetes can trigger, sustain and exacerbate.

Diabetic cardiomyopathy is a disease process in which diabetes produces a direct and continuous myocardial insult even in the absence of ischemic, hypertensive or valvular disease (Scheme 1). It can act synergistically with hypertension or ischemia to damage heart muscle, but can also cause heart failure in its own right. Diabetic cardiomyopathy was first described by Rubler et
al who reported four cases of heart failure in normotensive diabetic patients with no evidence of coronary artery disease, valvular pathology or congenital heart disease (10). Experimental evidence for the existence of this entity began to appear shortly thereafter, and epidemiological evidence also suggested that an additional cardiac insult was present in diabetic patients. However, the direct clinical demonstration of diabetic cardiomyopathy proved challenging until the 1990’s, when a series of studies using echocardiography and Doppler echocardiography documented evidence of left ventricular hypertrophy and diastolic dysfunction in both type 1 and type 2 diabetic patients independent of other risk factors (see (11) for review). Studies using Doppler ultrasound have revealed that the prevalence of diabetic cardiomyopathy is alarmingly high. Prevalence rates of >40% in young normotensive type 1 diabetic patients and 50-60% in well-controlled type 2 diabetic patients have been reported (12-15). In a recent case-control study, Bertoni et al reported that diabetes is independently associated with the development of idiopathic cardiomyopathy (16).

The clinical course of diabetic cardiomyopathy is long, and can be divided into three stages (17). In the early stage, the cardiomyopathy presents with mild asymptomatic diastolic dysfunction which is associated with ultrastructural changes in tissue architecture, impaired calcium handling, oxidative stress and changes in cardiac metabolism. As the disease progresses, evidence of left ventricular hypertrophy appears which is associated with more severe diastolic dysfunction and mild systolic dysfunction. Cardiomyocyte apoptosis and necrosis, myocardial fibrosis, mild autonomic neuropathy and activation of the renin-angiotensin system appear at this stage. Finally, combined systolic and diastolic dysfunction occur which are associated with cardiac microvascular disease, severe autonomic neuropathy and systemic sympathetic nervous system activation. This late stage is frequently associated with hypertension and the onset of ischemia (17). The mechanisms underlying the process are poorly understood, but an overall picture is emerging. The sustained diabetic cardiac insult appears to be produced by two major factors: hyperglycemia, a major
mediator of many diabetic complications, and a shift in energy substrate selection by the heart (18). This disease process impairs both passive and active mechanical properties of the myocardium; the compliance of the heart wall decreases (due to increased cross-linking of collagen, cardiac hypertrophy and fibrosis (18; 19)), and contractility also decreases.

Cardiac metabolism in the diabetic heart differs from that in the non-diabetic hypertrophied and failing heart. It has been shown that, in the advanced stages of disease, the hypertrophied and failing heart increases its reliance on glycolysis and glucose oxidation, although fatty acid oxidation remains the predominant fuel (20-22). The reverse is true in diabetic cardiomyopathy. Transport of glucose into the cardiomyocyte is dependent on the Glut-4 transporter whose translocation, translation and transcription are all decreased by diabetes, and the constitutive Glut-1 transporter (18). The diabetic heart is therefore less able to use glucose as an energy source, and relies more heavily on alternative substrates such as fatty acids and ketones. Plasma fatty acid levels are increased. Fatty acid delivery to the heart from the coronary lumen by the enzyme lipoprotein lipase (LPL), and its subsequent uptake by fatty acid transporters, is also increased (23).

The heavy reliance of the diabetic heart on fatty acids is harmful for two reasons: it decreases myocardial efficiency, and it induces 'lipotoxicity'. Despite the fact that fatty acid oxidation increases dramatically in the diabetic heart, the delivery of free fatty acids into the cardiomyocyte exceeds its capacity to metabolise and store them. Intermediate products of fatty acid metabolism, particularly long-chain acyl-coenzyme A's (Acyl CoA's), therefore accumulate in the cytoplasm (23). This effect could be exacerbated if ketone body utilisation is increased, as ketone bodies are competing substrates with fatty acids. Long chain acyl-CoA's are converted into ceramides, toxic substances which induce reactive oxygen species (ROS) and cardiomyocyte apoptosis (24). Interventions which selectively increase cytoplasmic fatty acid influx (overexpression of fatty
acid transport protein-1 or LPL) or which selectively increase long-chain acyl CoA synthesis (overexpression of long chain acyl CoA synthetase) produce a cardiac phenotype which is similar to diabetic cardiomyopathy (23). Taken together, these data provide convincing evidence that lipotoxicity is a significant disease-inducing myocardial injury.

Fatty acids bind and activate peroxisome proliferator-activated receptors (PPAR's) of which PPAR-α is a major isoform in the heart. PPAR-α is a transcription factor which acts as a 'lipostat', inducing genes involved in every step of fatty acid metabolism (25). PPAR-α upregulates enzymes at every step of the fatty acid oxidation pathway, but it is the transcriptional control of mitochondrial long-chain acyl CoA uptake which has the greatest impact on the overall fatty acid oxidation rate (26). When PPAR-α is overexpressed, a phenotype similar to that seen in diabetic cardiomyopathy is induced. Furthermore, when PPAR-α is overexpressed in diabetic hearts, the phenotype produced by diabetes is worsened (23). Conversely, deletion of PPAR-α is sufficient to protect diabetic hearts against the development of diabetic cardiomyopathy (23; 27). PPAR-α activation is therefore essential to the pathogenesis of diabetic cardiomyopathy. It is not clear, however, whether PPAR-α activation is a consequence or a cause of increased fatty acid oxidation. Overall, the reliance of the diabetic heart on fatty acid oxidation is harmful for three reasons: decreased myocardial efficiency, lipotoxicity and PPAR-α-activation.

Protein metabolism is also affected by diabetes, an aspect which is rarely considered. Ribonucleic acid (RNA) levels fall. Diabetes produces a marked artero-venous difference in branched chain amino acids across the myocardium, suggesting that protein catabolism is increased. This would decrease the availability of amino acids for translation. The combination of increased protein breakdown and decreased protein synthesis would be expected to produce qualitative and quantitative decreases in myocardial proteins (18).
Hyperglycemia, coupled with the marked decrease in glucose oxidation, increases the formation of reactive oxygen species (ROS) by several mechanisms (28). Prolonged exposure of proteins to hyperglycemia induces a series of chemical reactions which eventually lead to the irreversible formation of advanced-glycation end-products (AGE’s) which act on their own receptors (RAGE) to increase the synthesis of diacylglycerol (29; 30). Increased de novo synthesis of diacylglycerol, either by increased flux of glucose through the aldose reductase/ polyol pathway or by RAGE activation, leads to activation of protein kinase C (PKC) isoforms and stress signaling pathways (31; 32). Activation of these pathways increases mitochondrial ROS production, stimulates apoptosis and increases the transcription of pro-inflammatory and pro-fibrotic genes (28). In addition, flux of glucose through the hexosamine biosynthetic pathway leads to O-linked-N-acetylglucosamination and activation of transcription factors which also regulate pro-inflammatory and pro-fibrotic genes (33). Diabetic cardiomyopathy is therefore associated with widespread deleterious changes in fatty acid, protein and glucose metabolism. However, because glucose utilisation is decreased in the diabetic heart, fatty acid oxidation may be a more important regulator of oxidative stress in this setting.

In addition to the stress and PKC pathways activated by glucose, G protein-mediated signaling pathways (particularly G\textsubscript{i} and G\textsubscript{q}) activated by cell-surface receptors also contribute to the induction of pro-hypertrophic and pro-fibrotic genes (34). RhoA is a small molecular weight monomeric G-protein which activates Rho Kinase to induce cardiac hypertrophy and fibrosis. The Rho-A/ Rho-kinase pathway is known to be activated by α\textsubscript{1} adrenoceptor stimulation, as well as endothelium-derived vasoconstrictors such as endothelin-1 and thromboxane A\textsubscript{2}.

At the level of the cardiomyocyte, electromechanical coupling is impaired. Myocyte shortening and lengthening is slow because the action potential is
prolonged and calcium efflux is too slow (35). Cardiac contractile protein adenosine triphosphatase (ATPase) is crucial for the generation of cardiac force and is markedly depressed. In animal models, the so-called 'fetal gene program', which consists of re-expression of atrial natriuretic peptide (ANP), β-myosin and α-actin, and decreased expression of α-myosin and sarcoplasmic reticulum calcium ATPase-2 (SERCA-2) in the ventricle, is induced. This shift in contractile protein expression from a cardiac pattern to a skeletal muscle pattern is also observable when the pattern is assessed functionally. The V₁ isomyosin pattern seen in the normal heart gives way to a slower V₃ pattern in the diabetic heart (18). Total contractile protein levels may also be decreased because of the effects of diabetes on protein synthesis and breakdown. As diabetic cardiomyopathy progresses to symptomatic heart failure, downregulation of myocyte-enhancer factor-2 (MEF-2) and its target genes occurs, and a more severe contractile dysfunction ensues (36).

The decrease in SERCA-2 expression seen as part of the fetal gene program, combined with a concomitant decrease in SERCA-2 function and Na⁺ / Ca²⁺ exchanger expression and function, disturbs cardiomyocyte calcium handling. In the diabetic heart, the uptake, sarcolemmal binding and myofibrillar calcium-ATPase-mediated intake activity are all decreased. The calcium sensitivity of myofilaments becomes abnormally high as a result of PKC activation. β-adrenergic responsiveness decreases (28) and autonomic neuropathy decreases sympathetic nervous system input (18); as a result, the cardiomyocyte is cut off from systemic regulation of excitation-contraction coupling.

As the heart fails, the tissue renin-angiotensin system, the sympathetic nervous system and other neuro-hormonal regulatory systems are stimulated in an effort to maintain systemic perfusion. Activation of these systems increases apoptosis and necrosis (via β1-adrenoceptors) (37-41), and fibrosis (via α₁-adrenoceptors) and ROS generation (through the tissue renin-angiotensin
system) (28; 42-44). This time-course is reflected by pathological findings at autopsy and echocardiographic findings. Left ventricular mass, thickness and size are found to increase progressively as diabetic cardiomyopathy progresses from asymptomatic diastolic dysfunction to symptomatic systolic dysfunction (17).

The architecture of the myocardium is also progressively disturbed. Fibrosis is the most prominent histopathological finding in the diabetic heart and is diffuse. Perivascular and interstitial fibrosis appear during the intermediary stages of the disease process. Interstitial fibrosis decreases cardiac compliance, but perivascular fibrosis has additional functional consequences because it breaks the connection between the endothelium and the cardiomyocytes, thereby impairing relaxation (45). Progression to systolic dysfunction is associated with myocyte cell death which induces replacement fibrosis (17). Both apoptosis and necrosis are seen, but only necrotic cell death stimulates fibrosis (17). Focal microangiopathy is also present in the intermediary and advanced stages of the disease and may contribute to the observed fibrosis; it is unlikely to be the sole cause of fibrosis because, in diabetic cardiomyopathy, fibrosis is diffuse, not focal (17). The ultrastructure of the cardiomyocyte is profoundly damaged; cytoplasmic area is increased, with an increase in cytoplasmic lipid content (consistent with the presence of lipotoxicity) and increased collagen-fibre cross-sectional area (consistent with increased collagen cross-linking). Disturbance of the ultrastructure disrupts mitochondria, impairing mitochondrial function and inducing oxidative stress (18).

To summarize, diabetic cardiomyopathy is initiated by hyperglycemia and a shift in substrate selection in favour of fatty acids. Both processes increase oxidative stress and stimulate pro-apoptotic, pro-fibrotic and pro-inflammatory pathways. Necrosis also occurs, stimulating further fibrosis. Excitation-contraction coupling is impaired by disordered calcium handling, impaired ATPase activity and a shift in contractile protein expression from a cardiac to a skeletal muscle pattern. Excitation-contraction coupling is disconnected from
autonomic regulation as a result of impaired β-adrenergic responsiveness and autonomic neuropathy. These functional changes are accompanied by extensive damage to the architecture of the myocardium and the ultrastructure of the cardiomyocyte. The result is a disease process which can be initiated years before the appearance of hypertensive or ischemic disease. Initially presenting as asymptomatic diastolic dysfunction, the disease progresses to combined symptomatic systolic and diastolic dysfunction, and the myocardium is rendered more susceptible to damage from the other components of the cardiotoxic triad.

Despite recent advances in drug therapy for heart failure, this condition still carries a worse prognosis than most cancers, and is associated with significant morbidity. Current therapies are directed at symptomatic relief (diuretics, β-adrenergic agonists, phosphodiesterase inhibitors) and attenuation of left ventricular remodelling (angiotensin converting enzyme inhibitors, β-blockers, aldosterone antagonists). Agents which restore the normal balance of cardiac metabolism could improve the mechanical efficiency of the myocardium, and prevent the harmful sequelae of shifts in energy substrate selection. This mechanism has been proposed as a useful avenue to pursue in the identification of new drug targets for heart failure and may be particularly useful in diabetic cardiomyopathy (46-48).

II. Cardiac Metabolism

The heart requires a constant supply of adenosine triphosphate (ATP) for muscular contraction and the maintenance of ionic homeostasis (49). Under aerobic conditions most of this ATP (>95%) is generated by mitochondrial oxidative phosphorylation. Oxidation of energy substrates is coupled to the reduction of nicotinamide adenine dinucleotide (NADH) and flavoproteins. NADH and flavoproteins are then re-oxidised by oxygen, and the reducing equivalents they received are passed on to the electron transport chain which pumps protons out of the mitochondria; the result is the creation of an electrochemical gradient
consisting of a transmembrane pH gradient and a membrane potential. The $F_1F_0$ATPase located on the inner mitochondrial membrane allows protons to reenter the mitochondrial matrix down their concentration gradient, harnessing the electrical potential energy of the gradient to generate ATP from ADP and Pi (50). The oxygen consumed is converted to carbon dioxide, and the reducing equivalents are eventually transferred to hydrogen and oxygen, forming water. ATP is exported in exchange for ADP by the adenine nucleotide transporter, and a continuous supply of Pi is maintained by the phosphate translocator. The mitochondria are precisely fixed between two T-tubules, and both ATP and its metabolic intermediates are continuously channeled between the mitochondria, the myofibrils, the sarcoplasmic reticulum and the sarcolemma. This exquisite coupling system between oxidative phosphorylation and myofibril contraction allows ATP demand to be precisely and instantly met over a wide range of workloads (see (51) for review).

Although functional coupling between the myofibrils and oxidative phosphorylation matches ATP production to ATP consumption, further communication is required to ensure that the supply of energy substrates responds to changes in ATP demand. This role is fulfilled by AMP activated protein kinase (AMPK), which is activated by an increase in the AMP/ATP ratio (a signal of ATP depletion) and acts to deactivate ATP-consuming pathways (protein, triglyceride and glycogen synthesis) and activate ATP-producing pathways (protein, glycogen and triglyceride catabolism, glucose and fatty acid uptake and oxidation) (52).

The heart is an omnivorous organ which has the ability to use any energy substrate provided to it (lipids, carbohydrates, ketone bodies, amino acids); however, the normal heart derives most of its ATP from the metabolism of fatty acids and carbohydrates (53). Although fatty acid oxidation produces more ATP, glucose oxidation is more efficient in terms of oxygen consumption. Several
Mechanisms involved in the pathogenesis of diabetic cardiomyopathy. The cardiomyopathy arises as a result of a decrease in heart muscle compliance, produced by fibrosis and increased collagen cross-linking, and a decrease in contractility, produced as a result of widespread changes in contractile signaling pathways, cardiac metabolism and oxidative stress.
Myocyte Apoptosis, Alterations in G-proteins, RhoA-Rho Kinase, PKC, Metabolic 'Switch', Disordered calcium handling, DECREASED CONTRACTILITY, Myocyte Altered Cardiac Remodelling/Hypertrophy, DECREASED COMPLIANCE, Collagen Cross Linking, Myocardial Fibrosis, DECREASED CONTRACTILITY, DIABETIC CARDIOMYOPATHY
factors account for this. Firstly, to maintain a fixed ATP production rate, fatty acid oxidation requires greater oxygen consumption. The ATP produced per unit oxygen is theoretically predicted to be 3.17 for glucose but only 2.80 for palmitate (48). Secondly, fatty acids allow protons to leak across the mitochondrial membrane, uncoupling oxidative phosphorylation and wasting oxygen (54). Indeed, fatty acids have recently been shown to activate mitochondrial uncoupling in models of type 2 diabetes (55; 56). Thirdly, fatty acids activate sarcolemmal calcium channels. Calcium pumps must increase their activity, and therefore their ATP utilization, to compensate for the resulting increase in calcium influx (57). For optimal cardiac efficiency, the normal heart maintains a balance of 60-80% fatty acid oxidation and 20-40% pyruvate oxidation; the heart derives approximately equal amounts of pyruvate from glycolysis and lactate metabolism (58; 59). Ketone utilization is concentration dependent; in situations where blood ketone levels rise (starvation, diabetes), ketones become a major cardiac fuel (60; 61).

The metabolism of glucose and fatty acids by the heart is summarized in scheme 2. Glucose-6-phosphate, the glycolytic substrate, is obtained from endogenous glycogen stores and from exogenous glucose taken up by the Glut-1 and Glut-4 glucose transporters. Glucose uptake is regulated by Glut-4 vesicle translocation, docking and fusion to the sarcolemma (stimulated by insulin and AMP-activated protein kinase) and is responsible for the majority of glucose uptake (59; 62; 63). Glycolysis comprises a series of reactions which convert glucose-6-phosphate to pyruvate. In the normal aerobic heart, the major fate of pyruvate is decarboxylation to acetyl CoA, catalysed by the pyruvate dehydrogenase complex (PDH) in the mitochondria (64). Lactate is metabolised to pyruvate, but pyruvate is also metabolized to lactate. Under aerobic conditions, the normal heart is a net consumer of lactate; the heart only becomes a net producer of lactate when the glycolytic flux exceeds the rate of pyruvate oxidation (59). A small proportion of pyruvate is metabolized to either
oxaloacetate or malate in order to replenish tricarboxylic acid cycle (TCA cycle) intermediates, a process known as anaplerosis (65).

Fatty acids are highly hydrophobic and are therefore transported as triglycerides in chylomicrons or very-low-density lipoproteins (VLDL), although a small proportion are also transported bound to serum albumin. Fatty acids are released from chylomicrons and VLDL by lipoprotein lipase (LPL) on the luminal surface and are then taken up into the cardiomyocyte by fatty acid transporters such as CD36 (66). Upon entry to the cytoplasm, fatty acids are bound by fatty acid binding protein (FABP) until they are esterified to fatty acyl CoA by fatty acyl CoA synthetase (FACS). Fatty acyl CoA has two major fates: it can enter the mitochondria to be oxidized, or it can be esterified to triglyceride and stored (24; 67). A small proportion of palmitoyl CoA's are also converted to ceramides (24).

Whereas short and medium-chain acyl CoA's can pass freely into the mitochondria to be oxidized, long-chain acyl CoA's cannot cross the mitochondrial membrane and must be transported. This function is carried out by a carnitine-dependent shuttle system. In the first step, carnitine palmitoyltransferase 1 (CPT-1) converts acyl CoA to acyl-carnitine. This is a major control step of overall fatty acid oxidation. Acyl-carnitine is then transported to the mitochondrial matrix by carnitine acyltransferase (CAT) in exchange for free carnitine. Finally, carnitine palmitoyltransferase 2 (CPT-2) reverses the initial reaction and regenerates acyl CoA (68). Acyl CoA enters the β-oxidation spiral, a series of four reactions which cleaves two carbons (one acetyl CoA molecule) from the acyl CoA molecule per cycle and generates NADH and FADH$_2$. There are specific enzymes for long-, medium- and short-chain acyl CoA's (69). Acetyl CoA generated by carbohydrate or fatty acid oxidation (as well as from other pathways) enters the TCA cycle to generate additional reducing equivalents which drive oxidative phosphorylation.
SCHEME 2

Summary of fatty acid and glucose metabolism. Glucose is taken up by Glut-1 and Glut-4 transporters and is converted by glycolysis to pyruvate. Pyruvate then enters the mitochondria to be oxidized, producing acetyl CoA. Fatty acids are liberated from lipoproteins by LPL, and are taken up by CD36 and FABP. LCAS converts the fatty acid to a CoA ester which is then taken up by the carnitine shuttle system to the mitochondria. The fatty acyl CoA undergoes β-oxidation, removing two carbons per turn of the cycle and generating acetyl CoA. Acetyl CoA, generated by either pathway, enters the TCA cycle to generate reducing equivalents (NADH). These pass electrons to the electron transport chain which creates an electrochemical proton gradient to drive ATP synthesis. ATP synthesis is exquisitely coupled to the systems which create the ATP demand.

(abbreviations: LPL = lipoprotein lipase, CD36= fatty acid translocase, FABP = fatty acid binding protein, FACS = fatty acyl CoA synthase, CPT = carnitine palmitoyltransferase, CAT = carnitine acyl transferase, CoA = coenzyme A, TCA cycle = tricarboxylic acid cycle, AGE = advanced glycosylation endproduct, PDH = pyruvate dehydrogenase, MCT = monocarboxylate transporter, PDH = pyruvate dehydrogenase, NADH = reduced nicotinamide adenine dinucleotide, ATP = adenosine triphosphate, ADP = adenosine monophosphate)
Glucose Oxidation | The Randle Cycle | Fatty Acid Oxidation

Glucose

Lactate

GLUT-1

GLUT-4

Hexosamine Pathway
Polyol Pathway
AGE

GLYCOLYSIS

Glucose-6-phosphate

Glycogen

Fatty Acid Oxidation

Lipoproteins

LPL

Fatty Acids

CD36

FABP

FACS

Cytosol

Contractile Work
Calcium Uptake
Ion Homeostasis

Triglycerides

Fatty Acyl CoA

Ceramide

Carnitine

Fatty Acyl Carnitine

CPT-1

CPT-2

CAT

Pyruvate Transporter

Pyruvate

PDH

ACETYL CoA

TCA CYCLE

ACETYL CoA

β-Oxidation

Spiral

MCT

Lactate

Pyruvate

H^+

ADP

ATP

H^+

ADP

ATP

Fatty Acyl CoA

Carnitine

MITOCHONDRIUM
The rate of fatty acid oxidation is determined by the plasma concentration of fatty acids, the work performed by the heart, the entry of fatty acids into the cytoplasm, the entry of fatty acids into the mitochondrion, and the activity of the enzymes involved in the β-oxidation spiral (62; 70). High rates of fatty acid oxidation increase the ratios of NADH/ NAD+ and acetyl CoA/ free CoA, both of which feed back and inhibit glucose oxidation by decreasing flux through PDH (62). High rates of fatty acid oxidation also increase citrate production. Citrate inhibits glycolysis by inhibiting the key glycolytic enzyme phosphofructokinase (PFK). This is known as the Randle cycle. Conversely, high rates of glycolysis and glucose oxidation can feed back to inhibit fatty acid oxidation.

At the level of CPT-1, fatty acid oxidation is controlled by malonyl CoA, a potent inhibitor which binds CPT-1 on the cytosolic side (68); by altering malonyl CoA levels in the cytosolic microdomain adjacent to the site of fatty acyl-CoA uptake, fatty acid oxidation can be controlled; tonic inhibition of CPT-1 by malonyl CoA is always present, but a rise in malonyl CoA levels inhibits fatty acid oxidation while a fall relieves inhibition (71; 72). Two isoforms of CPT-1 are expressed in the heart: CPT-1A (the isoform which predominates in the liver) and CPT-1B (the isoform which predominates in the heart). The sensitivity of CPT-1B to malonyl CoA is 30 times greater than that of CPT-1A (71; 72). The turnover of malonyl CoA in the heart is rapid. Malonyl CoA is synthesized from acetyl CoA by acetyl CoA carboxylase (ACC) and is broken down to acetyl CoA by malonyl CoA decarboxylase (MCD) (73; 74). The activity of ACC is inhibited by phosphorylation; the major kinase responsible is AMPK, although PKA mediates β-adrenoceptor induced ACC phosphorylation (73). The mechanisms by which MCD is regulated are still unknown; it is possible that MCD is activated by AMPK (75). Malonyl CoA levels can be increased either by stimulating ACC or by inhibiting MCD (Scheme 3). MCD is a transcriptional target of the peroxisome proliferator activated receptor-α, whose actions are discussed below.
In the long term, regulation of the expression of the enzymes, transporters and kinases that comprise the metabolic machinery enables the heart to adapt more permanently when the stimulus to do so is sustained. The mitochondria contain DNA which codes for 13 electron transport chain subunits (for complexes I, III, IV, and V), but all other components of the metabolic machinery are coded for in the nuclear DNA (76). There are several important inter-related nuclear transcriptional regulators of metabolism genes. The first are the peroxisome proliferator activated receptors (PPAR). PPARs form heterodimers with retinoid X receptors (RXR), and formation of an active PPAR/ RXR complex requires binding of 9-cis-retinoic acid to the RXR and binding of long chain fatty acids or an exogenous PPAR ligand to the PPAR. Upon activation, the complex translocates to the nucleus and binds to PPAR response elements (PPREs) within the promoter regions of its target genes. In the heart, the major isoform is PPAR-α, and its target genes encompass the full pathway of fatty acid metabolism from fatty acid uptake to the β-oxidation spiral, as well as pyruvate dehydrogenase kinase-4 (PDK-4), the major inhibitory kinase of PDH (77).

The second important regulator is PPAR-γ coactivator-1α (PGC-1α). PGC-1α regulates the capacity of the cell to generate ATP so that, when ATP demand increases, the reserve of the metabolic machinery can meet the demand (78; 79). To this end, the major role of PGC-1α in cardiac muscle is to increase mitochondrial biogenesis and the capacity of each mitochondrion for oxidative phosphorylation. PGC-1α binds and enhances the action of other transcription factors including PPAR-α (fatty acid oxidation control), myocyte-enhancer factor 2A (MEF-2A, contractile machinery control) and orphan nuclear receptor estrogen-related receptor α (ERR-α, carbohydrate and fatty acid oxidation control, contractile machinery control, oxidative phosphorylation genes) (80; 81).
Regulation of malonyl CoA levels. Malonyl CoA is synthesised from cytosolic acetyl CoA by acetyl CoA carboxylase (ACC), and is broken down to acetyl CoA by malonyl CoA decarboxylase (MCD). MCD is regulated through transcription, but ACC undergoes acute regulation by inhibitory phosphorylation. PKA and AMPK both phosphorylate and inhibit ACC, decreasing malonyl CoA levels and relieving inhibition of CPT-1. This is the major mechanism by which CPT-1 is regulated in the heart (abbreviations: CPT-1 = carnitine palmitoyltransferase-1, PKA = protein kinase A, AMPK = adenosine monophosphate-activated protein kinase).
Malonyl CoA Decarboxylase

Acetyl CoA \[\text{Malonyl CoA} \]

Acetyl CoA Carboxylase

PKA  AMPK

CPT-1

FATTY ACID OXIDATION
III: Modulation of Cardiac Metabolism as a Therapeutic Strategy

Inhibition of fatty acid uptake and oxidation, or stimulation of glucose oxidation, would be expected to be beneficial to the diabetic heart for three reasons: improvement in myocardial efficiency, amelioration of lipotoxicity and amelioration of glucotoxicity. Most experimental and clinical data demonstrating the effectiveness of this therapeutic strategy pertain to non-diabetic heart failure, although a small body of data also exists for diabetic animal models and patients. Intravenous infusion of dichloroacetate (DCA), a drug which inhibits PDK, produces a rapid improvement in left ventricular performance in patients with heart failure (82). This suggests that decreased flux through PDH contributes significantly to the cardiac dysfunction of heart failure. We have previously shown that the administration of DCA also produces a marked improvement in left ventricular function in the diabetic heart (83). Increasing plasma insulin levels can also stimulate glucose oxidation both directly by increasing glucose uptake, and indirectly by decreasing fatty acid delivery to the heart, thereby relieving Randle cycle-mediated inhibition of glucose oxidation (84). Infusion either of insulin in heart failure patients (85) or the insulinotropic peptide glucagon-like peptide 1 (GLP-1) in dogs with pacing-induced heart failure improved left ventricular function (86). In the Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) study, stimulation of glucose oxidation reduced mortality following myocardial infarction in diabetic patients (87). Taken together, these data suggest that acute stimulation of glucose oxidation may be beneficial to the failing heart, but more definitive clinical studies are required to confirm this. Furthermore, there are, at present, no pharmacological agents which act as chronic glucose oxidation stimulators.

A number of agents have been developed which inhibit fatty acid oxidation, thereby stimulating glucose oxidation indirectly through the Randle
cycle. Fatty acid oxidation inhibitors have been classified according to whether they produce reversible (partial fatty acid oxidation inhibitors) or irreversible inhibition. Etomoxir is an irreversible CPT-1 inhibitor. It has been shown to improve cardiac function in rats with left ventricular hypertrophy (88; 89) and diabetic cardiomyopathy (90-92). In experimental studies, etomoxir treatment improved SERCA-2 expression and calcium handling, indicating that CPT-1 inhibition produces improvements in calcium handling (89; 93). Inhibition of CPT-1 without concomitant inhibition of fatty acid delivery and uptake results in an accumulation of long-chain fatty acyl CoA's in the cytosol and activation of the PPAR-α/RXR complex. Rupp et al speculated that the increased SERCA-2 expression induced by etomoxir is mediated by PPAR-α, noting that the regulatory region of the SERCA-2 gene contains a sequence similar to the PPRE (89). A small preliminary trial of etomoxir in patients demonstrated an improvement in left ventricular function (94). However, etomoxir produced an unacceptable rate of side-effects in the ‘etomoxir for the recovery of glucose oxidation’ (ERGO-1) Phase II clinical trial, and the trial was terminated early (95). Etomoxir has also been shown to induce mild myocardial hypertrophy which was prevented by feeding rats a medium-chain fatty acid diet, suggesting that the effect is attributable to CPT-1 inhibition (96; 97). Etomoxir has also been shown to cause oxidative stress in HepG2 cells (98). The fact that etomoxir is a potent irreversible inhibitor of CPT-1 may explain its tendency to produce adverse effects. It is not clear whether etomoxir-associated hypertrophy is related to excessive PPAR-α activation, a mild lipotoxicity or the fact that chronic CPT-1 inhibition mimics a growth-promoting anabolic state.

Perhexiline and oxfenicine are CPT-1 inhibitors which are considered to be partial fatty acid oxidation inhibitors. No cases of cardiac hypertrophy arising from either the experimental or clinical use of these agents have been reported. Perhexiline fell into disfavor when it was found to induce hepatotoxicity and neuropathy in a subset of patients; this toxicity is now known to be due to slow cytochrome P450 metabolism of the drug and can be avoided by appropriate
dose titration (99). In a small randomized-control trial, perhexiline was shown to improve ejection fraction, myocardial efficiency and symptoms (100). Trimetazidine is a partial fatty acid oxidation inhibitor which may act by inhibiting 3-ketoacyl CoA thiolase (3-KAT), the final step of the β-oxidation spiral (101). Several small clinical trials, including one randomized control trial, have shown that trimetazidine improves ejection fraction and symptoms in patients receiving optimal treatment for their heart failure (102). Trimetazidine was tested in diabetic patients with ischemic heart disease and was found to significantly improve heart function (103-105). Taken together, these data indicate that inhibition of fatty acid oxidation is beneficial to the failing heart and is a mechanism which can produce meaningful improvements in function.

IV: The Benefits of β-Adrenergic Blockade

Heart failure is associated with activation of the sympathetic nervous system. The sympathetic drive to a failing resting heart is equivalent to the maximum drive a normal heart is subjected to during severe exercise; spillover of catecholamines increases as much as 50-fold, producing marked elevation of cardiac and systemic catecholamine levels (106-109). This large response is initiated in an effort to maintain systemic perfusion, but sympathetic activation is harmful to the failing heart, regardless of the cause of the failure, and correlates inversely with survival (110). Conversely, the β-blocking agents bisoprolol, carvedilol and metoprolol have been shown in large-scale randomized controlled trials to reduce heart failure mortality by a third or more (111). β-blocking drugs produce negative chronotropic and inotropic responses when administered acutely. For this reason, they were contraindicated in heart failure for many years. However, in the 1970's, β-blockers were pioneered as heart failure treatments (112), and they are now among the agents of choice for the treatment of heart failure (111).
There have been no clinical or experimental studies examining whether β-blocking agents are beneficial in diabetic cardiomyopathy. However, a number of clinical studies have examined the effects of these agents in the context of ischemia. In patients taking β-blocker therapy post-myocardial infarction, the improvement in survival produced by treatment is greater in diabetic patients than non-diabetic patients (113). The DIGAMI study showed that diabetic patients receiving a β-blocker had a 50% reduction in mortality (87), and carvedilol produced a 29% reduction in mortality in the Carvedilol Post-Infarction Survival Control in Left Ventricular Dysfunction (CAPRICORN) trial (114). Similar improvements in survival are also seen in the presence of asymptomatic coronary artery disease (115).

Looking at heart failure patients as a whole, more than 20 clinical trials have been published covering more than 10,000 patients whose symptoms ranged from mild to severe; these studies consistently found that β-blocker therapy reduces all cause mortality by more than a third, a similar improvement to that seen following β-blocker treatment of myocardial infarction or ischemia (116-120). However, the decrease in mortality is greater for sudden deaths than other causes. When diabetes-associated ischemic heart failure is assessed, β-blocker therapy produces a large improvement in heart function and survival. In the Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF) trial, metoprolol produced a greater functional improvement in heart failure patients without diabetes than those with diabetes (119). In the Carvedilol Prospective Randomised Cumulative Survival Trial, survival was improved by more than a third in both diabetic and non-diabetic patients with severe heart failure (116). Taken together, these data indicate that the ability of β-blocker therapy to improve survival in heart failure patients is greater in the non-diabetic heart than the diabetic heart. However, the ability of β-blocker therapy to improve survival following myocardial infarction is greater in the diabetic heart than the non-diabetic heart. This raises the possibility that β-blockers could have a mechanism of action that is particularly beneficial to the diabetic cardiomyopathy...
component of the cardiotoxic triad in terms of protecting against acute injury. In the context of ischemic heart failure, however, this special benefit is lost. It should be noted that, in all heart failure clinical trials to date, β-blocker therapy had been instigated following the onset of systolic failure in accordance with existing evidence and guidelines, and the diabetic patients could therefore have had more severe heart failure to begin with. The crucial difference may be the timing of the intervention. It is not known whether β-blocker therapy can reverse the diabetic cardiomyopathy component at a much earlier stage because this possibility has not been investigated either experimentally or clinically.

Putative mechanisms for the chronic effect of β-blockers include antiarrhythmic effects, amelioration of cardiomyocyte hypertrophy, necrosis and apoptosis, reversal of the fetal gene program (thereby improving calcium handling and force of contraction), increases in cardiac receptor density (for some β-blockers including metoprolol), anti-inflammatory effects (β-blockers lower serum C-reactive protein levels) and partial restoration of cardiac glucose oxidation (37; 121). Metoprolol (122), carvedilol (123) and bucindolol (124) have all been shown to induce a switch from fatty acid to glucose oxidation in non-diabetic patients with heart failure. Furthermore, metoprolol was shown to increase lactate uptake in heart failure patients, an effect which is consistent with an increase in carbohydrate oxidation (125). A study in dogs with microembolism-induced heart failure revealed a potential mechanism for this effect: CPT-1 was inhibited by chronic treatment with metoprolol (126). Considering the hypothesis that the switch from glucose to fatty acid oxidation plays an important role in diabetic cardiomyopathy, the ability of β-blockers to partially reverse this switch could be especially beneficial to the diabetic heart.

**V: β-Adrenoceptor Signalling**

In 1948, Ahlquist first demonstrated the existence of two broad subtypes of adrenoceptors: α-adrenoceptors and β-adrenoceptors (127). Two subtypes of
β-adrenoceptors, β1 and β2, were identified and characterized in the late 1960’s (128), while a third, β3, was isolated and cloned in 1989 (129). All three subtypes are expressed in the heart, but the major subtypes are β1 and β2, the ratio of β1: β2 being approximately 60-70%: 40-30 %, with very low β3 expression (130). The effects of the putative β4 adrenoceptor are now believed to be mediated by a low-affinity state of the β1 adrenoceptor (131; 132). The receptor reserve is low because the absolute expression levels are in the femtomolar range (50-70 fmol/ mg protein for the β1 adrenoceptor) (37). The affinities of these receptors for their ligands differ: β1 (adrenaline = 4 μM, noradrenaline = 4 μM, isoproterenol= 0.2 μM), β2 (adrenaline = 0.7 μM, noradrenaline = 26 μM, isoproterenol = 0.5 μM), β3 (adrenaline = 130 μM, noradrenaline = 4 μM, isoproterenol = 2 μM) (133).

β-adrenoceptor signaling pathways are summarized in scheme 4. β-adrenoceptors are G-protein coupled receptors. In the classical β-adrenoceptor pathway, β1 and β2 adrenoceptors, acting via Gs, produce an acute positive inotopic response mediated by increased cAMP levels and stimulation of protein kinase A (PKA). PKA then phosphorylates several key proteins involved in calcium handling and calcium sensitivity of myofilaments. Phosphorylation and activation of L-type calcium channels and ryanodine receptors increases calcium uptake and release, while phosphorylation of phospholamban relieves inhibition of SERCA, thereby increasing sarcoplasmic reticulum calcium uptake (134-136). Finally, PKA modulates calcium sensitivity of myofilaments through phosphorylation of troponin I and myosin binding protein B (137; 138). PKA also activates protein phosphatase inhibitor-1, sustaining its effects by preventing dephosphorylation of its targets (139).

Recently, a major paradigm shift has occurred in adrenoceptor biology. The β-adrenoceptors are now known to form complex ‘signalomes’ which are temporally and spatially organized. A signalome can be defined as all genes,
β-adrenergic signaling pathways. β1-adrenergic receptors activate PKA, which regulates calcium sensitivity and calcium handling. Prolonged activation of this receptor activates a harmful CAMK-II pathway which is pro-apoptotic and induces pathological remodeling. β2-adrenergic receptors also activate PKA, but prolonged activation causes a switch to Gi signaling which activates PDE4, inhibiting cAMP formation, and activates the cardioprotective PI3K/ Akt pathway. Desensitization of β2-adrenergic receptors by β-arrestin can recruit p38 and ERK, which protect the cell from apoptosis. β3-adrenergic receptors produce a negative inotopic effect which is mediated by NO produced via the PI3K/ Akt pathway.
β1
Gs
→
cAMP → PDE4 → PKA → CAMK-II
↓
Calcium Handling
Calcium Sensitivity

β2
Gs
→
cAMP → PDE4 → PI3K
↓
Akt → Pathological Remodeling
↓
eNOS
↓
Apoptosis

β3
Gi
→
PI3K
↓

β2
βArr
→
ERK + p38
phosphorylation, mediated by β-arrestins acting together with G protein-coupled receptor kinases or PKA itself (140-142). In addition to receptor desensitization, proteins and ligands which are involved in the transduction and response to a biological signal. With regard to temporal organization, it is well-established that β-adrenoceptors, and most particularly the β2-adrenoceptor, desensitize by uncoupling from their G-proteins. This dissociation is stimulated by receptor β-adrenoceptors change their coupling to downstream signaling pathways. Prolonged activation of β1 adrenoceptors causes a switch from PKA to calcium/ calmodulin dependent protein kinase-II (CAMK II) – dependent signaling, leading to CAMK-II mediated apoptosis and pathological hypertrophy (143). In contrast, prolonged activation of β2-adrenoceptors switches G-protein coupling from Gs to Gi, which is cardioprotective (144).

Whereas β1 adrenoceptor signaling is widely disseminated throughout the cell, β2 adrenoceptor signaling is compartmentalized, and the positive inotopic effect elicited by β2/Gs signaling is therefore smaller (145; 146). β2 adrenoceptor compartmentalization is partly achieved by the selective enrichment of β2 adrenoceptors in caveolae (147; 148). It has been suggested that translocation of β2 adrenoceptors out of caveolae following sustained stimulation causes the switch from Gs to Gi association (149). β2 adrenoceptor-Gi signaling activates the phosphoinositol-3 kinase (PI3K) - protein kinase B (Akt) pathway and phosphodiesterase 4 (145). Phosphodiesterase 4 increases the breakdown of cAMP generated by β1-adrenoceptor-Gs stimulation, enabling the β2-adrenoceptor-Gi pathway to functionally antagonize the β1-adrenoceptor-Gs pathway. The PI3K-Akt pathway protects the cardiomyocyte against apoptosis (145). Recently, a role for the extracellular-signal-regulated kinase (ERK) 1/2 in mediating β2-adrenoceptor-Gi cardioprotection has been suggested (150). Taken together, these data indicate that the coupling of β-adrenoceptors to downstream signaling pathways is compartmentalized and time-dependent.
Sustained activation of β1 adrenoceptors is harmful, whereas sustained activation of β2 adrenoceptors could be cardioprotective.

Another consequence of PI3K/Akt activation is stimulation of nitric oxide (NO) production. NO is synthesized from the terminal guanidine nitrogen atom of the amino acid L-arginine and molecular oxygen by nitric oxide synthase (NOS). This process requires tetrahydrobiopterin (BH₄) as a cofactor; without BH₄, eNOS becomes 'uncoupled', and produces reactive oxygen species, including peroxynitrite, instead of NO. Endothelial nitric oxide synthase (eNOS) is constitutively expressed in adult cardiomyocytes, producing physiological NO signaling in the nanomolar range. Inducible nitric oxide synthase (iNOS) is expressed in response to inflammatory stimuli (151; 152) and produces higher levels of NO, mediating pathophysiological effects (153; 154). NO and related reactive nitrogen species (e.g. peroxynitrite) covalently modify target proteins in one of three ways: nitrosylation, oxidation or nitration. Binding of NO to a protein, termed nitrosylation, is a reversible reaction and the modification produced is labile. Oxidation (e.g. glutathiolation of cysteine residues) or nitration of a protein (on tyrosine residues) produces more stable covalent modifications (155). Tyrosine nitration, nitrosylation and oxidation can be stimulatory or inhibitory depending on the target protein and residue affected. Nitrosylation of the heme moiety of soluble guanylyl cyclase by NO activates the enzyme, stimulating the production of cyclic 3', 5'guanosine monophosphate (cGMP) from guanosine triphosphate (156). Just as cAMP activates PKA, cGMP activates protein kinase G (PKG) isoforms. The NO/cGMP signaling pathway induces a negative inotropic effect in the heart (151). β3-adrenoceptors always couple to Gi, activating the PI3K/Akt pathway. β3-adrenoceptors produce a negative inotropic effect which is mediated by NO. Therefore, β2 adrenoceptor-Gi signaling and β3 adrenoceptor-Gi signaling both stimulate NO production (157; 158).

The effects of diabetes on cardiac β-adrenergic responsiveness have been studied for many years, but the results obtained have been conflicting.
Vadlamudi and McNeill (159) showed a decrease in the cardiac relaxant effects without an effect on heart rate or contractility. Zola et al (160) showed a decrease in the chronotropic response in rabbit heart in vivo. Foy and Lucas (161) demonstrated an increased chronotropic response and a decreased inotropic response in atria. Most recent studies report decreased sensitivity to β-adrenergic stimulation in cardiac tissues (162; 163). The effects of diabetes on β-receptor expression and downstream signalling are also controversial (163-167). 14 weeks but not 8 weeks of diabetes blunted the chronotropic response to noradrenaline, but the response to fenoterol, a selective β2 agonist, was preserved (168). This suggests that β1-mediated responses are selectively blunted in the diabetic heart. The expression of β1 is markedly decreased and that of β2 adrenoceptors modestly decreased in the diabetic heart, whereas the expression of β3 adrenoceptors is increased twofold (163). A similar increase in β3 adrenoceptor expression has been reported in failing human hearts (169). The significance of this shift in receptor subtypes towards β3 adrenoceptors remains to be determined; it is possible that this shift contributes to cardiac dysfunction by promoting a negative inotropic effect; on the other hand, a cardioprotective effect may also result if β3 adrenoceptor-mediated activation of the PI3K/ Akt pathway also prevents apoptosis.

VI: Potential Links Between β-Adrenoceptors and Cardiac Metabolism

Mechanisms linking β-adrenergic signalling with cardiac metabolism have not been investigated in great detail. We therefore employed a combination of ‘bottom-up’ (known rate-limiting enzymes) and ‘top-down’ (known β-adrenoceptor pathways) approaches to unravel the pathways involved. As discussed above, a previous study in microembolism-induced heart failure demonstrated that chronic metoprolol treatment decreased the activity of CPT-1 (126). In the heart, the major mechanism by which CPT-1 is regulated is through modulation of malonyl CoA levels. Isoproterenol has previously been shown to
lower malonyl CoA levels by increasing PKA-mediated phosphorylation of ACC (73). It is therefore possible that β-adrenergic blockade could have the opposite effect, preventing ACC phosphorylation and increasing malonyl CoA levels. Recently, a study in isolated cardiomyocytes using activators and inhibitors of cAMP revealed that stimulation of fatty acid oxidation by contraction is PKA-dependent (170).

Chronic β-adrenergic blockade could also decrease the expression of CPT-1. The expression of CPT-1 is controlled by PPAR-α, but the PPAR-α/ RXR complex produces only modest induction of CPT-1 when acting alone (26; 171; 172). PGC1α greatly enhances CPT-1 induction by PPAR-α, but can also induce CPT-1 independently by binding to MEF-2A (173). PGC1α-mediated expression of CPT-1 has been shown to be repressed in isolated cardiomyocytes by upstream stimulatory factor (USF)-2. Upstream stimulatory factors are transcription factors of the basic helix-loop-helix leucine zipper family which bind to the E-box consensus sequence CANNTG (174). In the heart, USF’s are involved in excitation-transcription coupling, responding to sustained increases in electrical stimulation by increasing the expression of sarcomeric genes such as sarcomeric mitochondrial creatine kinase and MHC (175; 176). β-blockers, by improving function and thereby indirectly increasing electrical stimulation, could activate USF’s with the secondary effect that USF-2 represses PGC1α-mediated CPT-1 expression. Alternatively, if β-blockers are acute fatty acid oxidation inhibitors, the activation of PPAR-α and its binding to coactivators could be altered as a result of changes in cytoplasmic long chain fatty acid levels.

In the study by Panchal et al (126), a decrease in CPT-1 activity was detected using the in vitro assay; allosteric effects are typically lost during sample preparation, so the observed decrease could be due to decreased CPT-1 expression, or alternatively to a covalent modification of CPT-1 itself. Few studies have examined whether covalent modifications of CPT-1 occur. Phosphorylation of CPT-1A has been demonstrated in vitro (177), and the stimulation of CPT-1 by
okadaic acid in hepatocytes was prevented by a specific inhibitor of CAMK II, indicating that CAMK II is involved in stimulation of CPT-1A activity (178). Phosphorylation of CPT-1B in the heart has never been demonstrated. However, activation of the sympathetic nervous system centrally by cerulinin was found to stimulate CPT-1B activity in soleus muscle within 3 hours (179). This effect must have been mediated by an as-yet unidentified covalent modification of CPT-1B; the modification in question could conceivably be phosphorylation. It is possible that phosphorylation of CPT-1 requires the presence of other proteins present on or recruited to the outer mitochondrial membrane. Compartmentalization of PKA signaling in the cardiomyocyte is achieved in part by the action of A-kinase anchoring proteins (AKAPs), a group of proteins which bind to PKA targets in order to regulate PKA-dependent phosphorylation of those targets (180). Three mitochondrial AKAP’s have been identified - AKAP121, D-AKAP-1 and AKAP149 - but functional studies of their role in the heart are awaited (180). It is possible that mitochondrial AKAP’s mediate, and are even essential for, CPT-1 phosphorylation. This possibility has never been investigated.

Tyrosine nitration of CPT-1 by peroxynitrite has been shown to inhibit CPT-1 activity following endotoxemia (181). Furthermore, superoxide, NO and peroxynitrite were all shown to inhibit CPT-1 activity in vitro when CPT-1 was co-incubated with systems which continuously generated these reactive oxygen and nitrogen species (182). This suggests that CPT-1 can be regulated by covalent modifications mediated by nitrogen species. It is likely that, in addition to tyrosine nitration, cysteine nitrosylation and glutathiolation also occur. Indeed, cysteine-scanning mutagenesis of the muscle isoform of CPT-1 revealed that cysteine 305 was important for catalysis; nitrosylation or glutathiolation of this residue could conceivably increase or decrease the catalytic activity of the enzyme.

β-blockers could conceivably modulate CPT-1 activity through nitrogen species generated as a result of β2 adrenoceptor-Gi or β3 adrenoceptor-Gi
signaling. However, NO is known to have more extensive effects on cardiac metabolism which must be considered. Firstly, NO is well-known to inhibit overall oxygen utilization by irreversibly inhibiting mitochondrial respiration (183; 184). Secondly, NO can alter myocardial substrate selection. NO inhibits glycolysis by nitrosylating and inhibiting the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (185-187). In isolated hearts perfused without fatty acids, NO was found to inhibit Glut-4-mediated glucose uptake and glycolytic flux (188). In the same study, 8Br-cGMP, a stable analogue of cGMP, was found to double the activity of ACC (188). Treatment of dogs with NOS inhibitors stimulated glucose use and reduced fatty acid oxidation, and administration of a long-acting NO donor switched metabolism back to fatty acid use (189). These data suggest that NO is an inhibitor of glucose use acting primarily to inhibit glucose uptake and glycolytic flux. Modulation of NO levels by β-blockers could therefore affect glucose oxidation directly.

VII: Specific Research Problem and Research Strategy

There is no specific treatment strategy for diabetic cardiomyopathy despite its emerging importance as a cause of heart failure in diabetic patients and alarmingly high prevalence. β-blockers have been shown to improve function and survival in diabetic patients with ischemic heart disease. However, no experimental or clinical studies have investigated whether β-blockers also improve function in diabetic cardiomyopathy. Considering the hypothesis that the reliance of the diabetic heart on fatty acid oxidation is a causative injury of diabetic cardiomyopathy, the putative ability of β-blockers to inhibit fatty acid oxidation could be especially beneficial to the diabetic heart. Data obtained using fatty acid oxidation inhibitors support the idea that this is a mechanism which can produce meaningful improvements in cardiac function. β-blockers are proposed to inhibit fatty acid oxidation by inhibiting long chain acyl CoA uptake into the mitochondria, catalysed by CPT-1. They could do this by several mechanisms: increasing malonyl CoA levels, decreasing CPT-1 expression or decreasing
CPT-1 activity through covalent modifications (phosphorylation, nitrosylation, glutathiolation, nitration). We therefore undertook the present study to determine whether β-blocker treatment ameliorates diabetic cardiomyopathy by inhibiting fatty acid oxidation, and to determine the mechanism of the effect.

Three β-blockers have been shown to exert effects on cardiac metabolism: metoprolol, carvedilol and bucindolol. Carvedilol and metoprolol are used clinically in the treatment of heart failure. Carvedilol is a non-selective β-blocker which also blocks the α1-adrenergic receptor and, at high doses, calcium channels. Metoprolol is a second generation β-blocker which is selective for the β1 receptor and is an inverse agonist at this receptor. We chose to use metoprolol in the present study as it does not produce α1-adrenergic, calcium channel blocking or antioxidant effects which would complicate interpretation of the results. Although metoprolol is classically regarded as being highly β1-selective, its selectivity in intact cells is less than was previously supposed; the selectivity ratio of metoprolol for β1 and β2 adrenoceptors was recently shown to be 2.3 (190; 191). Metoprolol will therefore block both β1 and β2 adrenoceptors at clinical doses. The dose of metoprolol used in our studies was 75 mg/ kg/ day by intraperitoneal injection. This dose is equivalent to a human dose of approximately 100 mg per day after correction for inter-species differences in surface area (the surface area of 150 mg rat is 0.025 m² whereas the surface area of a 60 kg human is 1.6 m² (192) ). Furthermore, this dose was well tolerated by the rats in our preliminary studies, and produced a demonstrable improvement in cardiac function (see results).

The streptozotocin (STZ) diabetic rat is a model of poorly controlled type 1 diabetes which is associated with a marked decrease in insulin levels. STZ is an antibiotic synthesised by the bacterium Streptomyces achromogenes which selectively targets and destroys the insulin-secreting β-cells of the pancreas (193; 194). The mechanism of this cytotoxicity is incompletely understood. It is
believed that STZ induces DNA strand breaks through the generation of oxygen free radicals or carbonium ions (195; 196). As part of the repair process, poly (ADP-ribose) polymerase (PARP) is activated. PARP uses large amounts of ATP and NAD\(^+\), and the resulting depletion of these molecules impairs the synthesis and secretion of insulin and, if sufficiently severe, triggers cell death (197-199). The induction of oxidative stress may be partly related to the ability of NO to inhibit oxidative phosphorylation; ADP is shunted into degradation pathways which produce xanthine, which via xanthine oxidase generates oxygen free radicals and uric acid (200).

Intravenous (IV) or intraperitoneal (IP) injection of STZ (>40mg/ kg) produces stable insulin-deficient diabetes, but, at low doses, sufficient insulin secretion is preserved to enable the animals to survive without exogenous insulin. 2-4 hours following injection of STZ, insulin secretion is inhibited and glucose levels rise. Over the next 2-8 hours, hypoglycemia ensues, finally giving way to sustained hyperglycemia 24 hours following the injection (201). In our laboratory, we routinely use a dose of 60 mg/ kg. STZ diabetic rats develop both the symptoms (hyperphagia, polyuria, polydipsia, weight loss), and complications (diabetic retinopathy, nephropathy and cardiomyopathy) of clinical diabetes (202-204). The diabetic cardiomyopathy of the STZ rat closely resembles that which is seen clinically, and appears 6 weeks following STZ injection (202-204). STZ rats do not develop atherosclerosis or hypertension, thereby enabling diabetic cardiomyopathy to be studied in the absence of ischemic or hypertensive heart disease.

**VIII: Working Hypotheses**

It was hypothesized that:
1. Chronic treatment with metoprolol would improve cardiac function in the diabetic heart by directly inhibiting fatty acid oxidation and indirectly stimulating glucose oxidation through the Randle Cycle.

2. Acute perfusion with metoprolol directly inhibits fatty acid oxidation and indirectly stimulates glucose oxidation through the Randle Cycle.

3. Acute perfusion and chronic treatment with metoprolol decrease phosphorylation of ACC, leading to an increase in malonyl CoA levels.

4. Acute perfusion and chronic treatment with metoprolol decrease the activity of CPT-1 without affecting the sensitivity of CPT-1 to malonyl CoA.

5. Chronic treatment with metoprolol decreases the expression of CPT-1 by increasing the expression, activity and PGC1α-binding of USF-2.

6. Chronic treatment with metoprolol increases the expression of all three β-adrenoceptor subtypes without affecting G-protein association. Acute perfusion or chronic treatment with metoprolol decrease β1- and β2-adrenoceptor signaling, but accentuate β3-adrenoceptor signaling.

7. Phosphorylation of CPT-1 by PKA and CAMK II is demonstrable in isolated mitochondria and modulated by acute metoprolol perfusion in whole hearts.

8. PKA, AKAP 149 and CAMK II bind to and co-immunoprecipitate with CPT-1. AKAP 149 mediates the binding of PKA to CPT-1.

9. Nitrosylation, glutathiolation and nitration of CPT-1 is demonstrable in isolated mitochondria and modulated by acute metoprolol perfusion in whole hearts.

IX: Objectives

The first objective was to determine whether metoprolol improves cardiac function in the diabetic heart and whether the functional improvement is associated with direct inhibition of fatty acid oxidation. The second objective was to determine whether metoprolol directly inhibits fatty acid oxidation during short-term perfusion. In a series of studies, glucose oxidation and fatty acid oxidation
were measured in the presence or absence of insulin. To characterise the metabolic effects more fully, measures of glucose and fatty acid disposal (lactate production, glycogen levels, triglyceride levels) and myocardial energetics (tissue adenine nucleotide levels, AMPK activity) were obtained. The third objective was to determine the effect of short term metoprolol perfusion and long term metoprolol treatment on CPT-1 activity and regulation by malonyl CoA, as well as on the activities of other key fatty acid oxidation enzymes (acyl CoA dehydrogenase, citrate synthase). We measured CPT-1 activity, CPT-1 sensitivity to malonyl CoA and tissue malonyl CoA levels. To investigate the regulation of malonyl CoA levels, we measured MCD and ACC expression, as well as ACC phosphorylation by AMPK and PKA.

The fourth objective was to determine whether metoprolol treatment controls CPT-1 expression by increasing the expression, activation and PGC1α-binding of USF-2. We also investigated whether metoprolol decreases the binding of PGC1α to its coactivators. The fifth objective was to characterise the acute and chronic effects of metoprolol on the expression and G-protein coupling of β-adrenoceptors and the activation of downstream second messengers (PKA, CAMK II, PI3K/ Akt, NO).

The sixth objective was to determine whether short-term metoprolol perfusion regulates CPT-1 activity and malonyl CoA sensitivity through direct covalent modifications of CPT-1. We investigated whether PKA and CAMKII directly bind to CPT-1, whether AKAP149 binds to CPT-1 and mediates PKA-binding and whether CPT-1 is phosphorylated. We also measured the cysteine nitrosylation, glutathiolation and tyrosine nitration of CPT-1. In order to determine whether these changes were sustained, the measurements were also carried out following chronic metoprolol treatment. The seventh objective was to measure the effects of kinase phosphorylation and peroxynitrite-mediated nitrosylation, glutathiolation and nitration on CPT-1 activity and CPT-1 sensitivity. The final
objective was to use a mass spectroscopy approach to identify phosphorylation sites on CPT-1.
MATERIALS AND METHODS

I: Measurement of ex vivo Left Ventricular Function

(a) Animal Treatments

Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Male Wistar Rats (weight matched 200-220g) were purchased from Charles River Laboratories and allowed to acclimatize for 1 week prior to the beginning of the study. Rats were allowed ad libitum access to standard rat chow and water for the duration of the study. Rats were randomly divided into four groups: control (C), control treated (CT), diabetic (D) and diabetic treated (DT). Diabetes was induced by the injection of 60mg/kg streptozotocin (STZ) into the caudal vein. One week following the induction of diabetes, treatment was commenced. The treated groups received 75 mg/kg/day metoprolol by intraperitoneal injection while untreated groups received an equivalent volume of vehicle (normal saline). Six weeks following the induction of diabetes, the animals were euthanized. 5-hour fasting blood samples were taken one week following STZ-injection and immediately prior to termination. For perfused groups, metoprolol was added to the perfusate in the isolated working heart preparation as described below.

(b) Measurement of Plasma Parameters

Five-hour fasting plasma samples were collected one week following STZ injection and immediately prior to termination. Plasma glucose concentration was determined using the Beckmann Glucose analyser. Plasma insulin was measured using the radioimmunoassay kit available from Millipore/LINCO (Billerica, Massachusetts). Plasma free fatty acids, cholesterol and triglycerides were determined by colorimetric assay kits available from Roche (Basel,
(c) **Direct Measurement of Left Ventricular Pressure**

Six weeks after STZ injection, the rats were euthanized by an intraperitoneal injection of 60 mg/kg sodium pentobarbital. The hearts were removed, and mounted on the working heart apparatus by cannulation of the aorta. The hearts were first perfused in Langendorff mode with warm oxygenated (95% O₂, 5% CO₂) Chenoweth-Koelle buffer (composition: 120 mM NaCl; 5.6 mM KCl, 2.18 mM CaCl₂, 2.1 mM MgCl₂, 19.2 mM NaHCO₃, 10 mM glucose, Temp 37°C). Following cannulation of the pulmonary vein, the apparatus was switched to working heart mode so that the heart was being perfused via the pulmonary vein. The afterload was set by column of H₂O (height=19cm). The heart was paced at 300 beats per minute. A 20-gauge needle was inserted into the left ventricle to measure left ventricular pressure via a Stratham pressure transducer. Following equilibration for 10 minutes, the hearts were subjected to atrial filling pressures from 3 to 11 mm Hg. The left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP), rate of contraction (+dP/dT) and rate of relaxation (-dP/dT) were calculated for each atrial filling pressure by a microcomputer as it collected the data.

**II: Measurement of ex vivo Cardiac Metabolism**

(a) **Animal Treatments and Measurement of Plasma Parameters**

For preliminary studies in which glucose oxidation and glycolysis were measured, rats were divided into four groups (C, CT, D, DT). For the main studies in which glucose and fatty acid oxidation were measured, rats were randomly divided into six groups: control (C), control + acute metoprolol perfusion
(CP), control + chronic metoprolol treatment (CT), diabetic (D), diabetic + acute metoprolol perfusion (DP) and diabetic + chronic metoprolol treatment (DT). The treatment protocol was the same as described in section I (a). The treated groups received 75 mg/ kg/ day metoprolol by intraperitoneal injection, while the remaining groups received an equivalent volume of vehicle (normal saline). In the acute metoprolol perfusion groups, isolated hearts were perfused with metoprolol ex vivo as described below. Plasma parameters were measured as described in section I (b) for all groups. Animals were terminated six weeks following STZ injection. Tissue analyses were only undertaken in samples that were perfused with insulin.

To improve clarity, the acute and chronic effects of metoprolol on function and metabolism are presented separately. However, acute and chronic effects were always investigated together in the same experiment and the controls are the same.

(b) Measurement of Cardiac Metabolism

In a preliminary study, the effects of chronic metoprolol treatment on glucose oxidation and glycolysis were measured to confirm whether metoprolol improves glucose use by the heart. Activities of key enzymes involved in fatty acid oxidation (CPT-1, acyl CoA dehydrogenase, citrate synthase) were measured. For the main studies, the effects of acute metoprolol perfusion and chronic metoprolol treatment on glucose oxidation and palmitate oxidation were measured. In a series of studies, perfusions were carried out in the presence or absence of insulin to determine whether inhibition of fatty acid oxidation by metoprolol is direct or mediated by the Randle cycle in response to direct stimulation of glucose oxidation. Based on the findings of the preliminary study, we did not measure acyl CoA dehydrogenase or citrate synthase activities in the main studies.
Measurement of cardiac metabolism was carried out as previously described (20; 205-209). The hearts were perfused in working heart mode with Krebs-Henseleit buffer (composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 5.5 mM glucose, 0.5 mM lactate, 100 or 0 μunits insulin, 0.8 mM palmitate bound to 3% BSA) in an aerobic perfusion for 60 minutes. The preload was set at 11.5 mm Hg and the afterload set at 80 mm Hg. The palmitate concentration was selected based on the plasma lipid profile. A physiological glucose concentration was selected. For simultaneous measurement of glucose and palmitate oxidation, the production of $^{14}$CO$_2$ and $^3$H$_2$O from $^{14}$C glucose and $^3$H palmitate was measured. For simultaneous measurement of glucose oxidation and glycolysis, the production of $^{14}$CO$_2$ and $^3$H$_2$O from $^{14}$C glucose and $^3$H glucose was measured. Cardiac output, aortic and pulmonary flow were measured by probes positioned upstream of the pulmonary cannula and downstream of the aortic cannula. Pressure was measured by a pressure transducer positioned downstream of the aortic cannula. For perfused groups (CP, DP), 2000ng/ml (4.8 μM) metoprolol was added to the perfusate after 30 minutes. For other groups (C, CT, D, DT), an equivalent volume of vehicle was added. Lactate production was measured in the perfusate using the colorimetric lactate assay kit from BioVision. Following completion of the perfusion, tissues were immediately flash-frozen in liquid nitrogen, weighed and stored at -70°C for further assay.

(c) Measurement of Tissue Glycogen and Triglyceride Levels

Glycogen levels were determined by measurement of glycogen-derived glucose following extraction of glycogen in KOH and hydrolysis in H$_2$SO$_4$ as previously described (210). Tissue triglyceride levels were measured by performing a chloroform-methanol extraction and redissolving the lipid pellet in phosphate-buffered saline (PBS) containing 1% Triton X. Triglyceride levels in the PBS Triton X solution were assayed using the colorimetric assay from Roche (211).
(d) **Measurement of Tissue Malonyl CoA and Adenine Nucleotide Levels**

Rat heart samples that had been snap frozen in liquid nitrogen following the isolated working heart perfusions were extracted in 0.4M perchloric acid containing 0.5mM ethylene-glycol-bis(β-aminoethyl ether)tetraacetic acid (EGTA) in a ratio of 100 mg/ ml. HPLC assays for malonyl CoA and adenine nucleotides were carried out on the perchloric acid extracts.

Tissue adenine nucleotide levels were measured by gradient ion pair reversed-phase HPLC as a measure of cardiac energetics. The HPLC procedure has been described previously (212) and can be summarized as follows. Samples of acid extracts were applied to a C18 reverse-phase column via a precolumn cartridge at a flow rate of 0.5ml/min. Buffer A (25mM KH$_2$PO$_4$, 6mM tetrabutylammonium hydrogensulphate, pH 6.0, 125mM EDTA) and buffer B (1:1 v/v mixture of buffer A and HPLC-grade acetonitrile), were filtered through a 0.2 μm membrane filter and helium de-gassed. After 10 minutes of isocratic elution with 98% A and 2% B, Waters curvilinear program no 3 was used, ending with a gradient of 45%A and 55% B after 10 minutes. This gradient was be maintained at a flow rate of 1.5 ml/min for a further 5 minutes. The column was re-equilibrated with 98% A and 2% B.

The HPLC procedure for measurement of malonyl CoA levels was as follows (213). Samples of acid extracts were be applied to a C18 reverse-phase column via a precolumn cartridge at a flow rate of 0.5ml/min. The applied gradient was as follows: Buffer A, 0.2M NaH$_2$PO$_4$, Buffer B, 0.25M NaH$_2$PO$_4$ and acetonitrile (20% v/v). At the time of sample application, the buffer composition was 20% B. The gradient rose linearly to 57% at 16.7 minutes, remained at 57% until 18 minutes, rose to 90% B by 22 minutes, and fell back to 20% B by 30 minutes. CoA and CoA ester elution were detected by a flow through a monitor set at 254 nm.
(e) **Measurement of Tissue Nitrate/ Nitrite Levels**

NO is rapidly scavenged and has a half life of 4 seconds in biological fluids. It is converted, by chemical reactions, to nitrate and nitrite. The sum of nitrate and nitrite levels provides an indirect index of total NO production. Tissue nitrate and nitrite levels were measured using the colorimetric assay available from Cayman chemicals (Ann Arbor, Michigan). In the first step of the assay, nitrate reductase is added to convert nitrate to nitrite. In the second step, the addition of the Griess reagents converts nitrite to a purple azo product whose absorbance is read at 540 nm.

**III: Measurement of Kinase and Biochemical Enzyme Activities**

(a) **AMPK, PKA and CAMK Activities**

Protein kinase and enzyme activities were only measured in samples that had been perfused with insulin.

The activities of AMPK, PKA and CAMK were assayed using kits available from Upstate Biotechnology/ Millipore (Billerica, Massachussets) (214). The assays are based on the rate of incorporation of $^{32}$P from $[\lambda^{32}\text{P}]$ ATP into synthetic peptides containing specific consensus sequences for the kinase of interest. (AMPK: AMARA peptide, sequence = AMARAASAAALARRR; PKA: kemptide, sequence = LRRASLG; CAMK: Autocamtide-2 KKLRRQETVDAL; bold indicates phosphorylated residue). Prior to AMPK assay, samples were purified by immunoprecipitation with antibodies specific for the $\alpha 1$ or $\alpha 2$ AMPK subunits, or with an antibody specific for both $\alpha$-1 and $\alpha$-2 AMPK subunits ($\alpha$-pan). Prior to PKA and CAMK assays, samples were purified by immunoprecipitation with antibodies specific for PKA or CAMK. II. The immunoprecipitation protocol is described in section IVd.
(b) CPT-1 Assay

CPT-1 activity was estimated by measuring the production of \(^{14}\)C-palmitoylcarnitine by the reaction:

\[
\text{Palmitoyl CoA} + ^{14}\text{C-Carnitine} \Rightarrow ^{14}\text{C- Palmitoylcarnitine} + \text{CoA-SH}
\]

Under the conditions of this assay, the equilibrium favours production of \(^{14}\)C- palmitoylcarnitine by both CPT-1 and CPT-2. However, only CPT-1 is inhibited by malonyl CoA. CPT-1 activity was defined as the activity which is inhibited by 200\(\mu\)M malonyl CoA.

The heart tissue was homogenised in 15mM KCl/ 0.5mM Tris, pH 7.2 by two 10s bursts using a Polytron. Total protein concentration was determined by the BioRad Protein Assay. Assay buffer composition was as follows: 105mM Tris-Cl (pH 7.2), 50\(\mu\)M palmitoyl CoA, 500\(\mu\)M carnitine, 0.25\(\mu\)Ci/ml \[^{14}\text{C}\]-carnitine, 1\% BSA, 4mM ATP, 0.25mM glutathione, 40ug/ml rotenone and 4mM KCN. The reaction was started by the addition of 100ul homogenate and allowed to proceed for 5 minutes at 30°C. The reaction was terminated by the addition of 500ul concentrated HCl. 500ul of 1-butanol was added and the samples vortexed for 1 minute prior to centrifugation at 3000g for 8 minutes. 300ul of the butanol layer was be taken, added to 1ml butanol-saturated water, vortexed for 30 seconds and centrifuged in a microcentrifuge for 2 minutes. 100ul of the washed butanol layer was taken and counted in a scintillation counter. CPT-1 activity was calculated as CPT (total) – CPT (activity in the presence of 200\(\mu\)M malonyl CoA). Data were expressed as nmol/ min/ mg protein.

To measure the sensitivity of CPT-1 to malonyl CoA, CPT-1 activity was assayed in the presence of 0, 10, 20, 50, 100, 150 \(\mu\)M malonyl CoA. The dose-response data were subjected to non-linear regression analysis and converged
to sigmoidal dose-response curves using GraphPad Prism 5.0 curve fitting software. The IC$_{50}$ value was calculated.

To determine whether metoprolol is a pharmacological inhibitor of CPT-1, CPT-1 activity was assayed in whole heart homogenates from 5 control hearts in the presence of increasing concentrations of metoprolol (ranging from 2-50 µg/ml) and 0, 50 or 100 µM malonyl CoA.

(c) Acyl CoA Dehydrogenase Assay

Acyl CoA Dehydrogenase activity was measured by following the reaction:

$$\text{Acyl CoA} + [\text{Fc}]^+ \rightarrow \text{Trans-} \Delta^2\text{-Enoyl CoA} + [\text{Fc}]$$

In this assay, the ferricenium ion ([Fc]$^+$) replaced FAD$^+$ as the electron acceptor, and the reaction was followed by measuring the rate of reduction of [Fc]$^+$. Reduction of [Fc]$^+$ to [Fc] is associated with a decrease in absorption at 300 nm. Octanoyl CoA was used as the substrate as it can pass freely through the mitochondrial membrane (215).

Ferricenium hexafluorophosphate (Fc$^+$PF$_6$) is not available commercially and was synthesized in our laboratory as previously described (215). Heart tissue was homogenised in 100mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6 by two 10s bursts using a Polytron. Total protein concentration was determined by the BioRad Protein Assay. The assay buffer composition was as follows: 200µM Fc$^+$PF$_6$, 100mM HEPES, 190ml homogenate. The reaction was started by the addition of 25ul 5mM octanoyl CoA, and the absorption at 300nm was measured every 5 seconds for 5 minutes. Linear rates of reaction were obtained by this protocol. The data were collected and a zero-order rate constant calculated using Enzyme
Kinetics Pro (SynexChem, Fairfield, California). Data were expressed as μmol/min/mg protein.

(d) Citrate Synthase Assay

Citrate synthase activity was measured by following the reaction:

\[ \text{Acetyl CoA} + \text{Oxaloacetate} + \text{H}_2\text{O} \rightarrow \text{CoA-SH} + \text{Citrate} + \text{H}^+ + \text{H}_2\text{O} \]

The reaction was followed for one minute by measuring the rate of appearance of CoA-SH spectrophotometrically using dithio-bis (2-nitrobenzoic acid) (DTNB). The thiol (SH) group of CoA-SH releases TNB from DTNB, causing an increase in absorption at 412nm (216).

Heart tissue was homogenised in 0.1M tris (hydroxymethyl) aminomethane hydrochloride (tris-CI), pH 8.1 by two 10s bursts using a Polytron. Total protein concentration was determined by the BioRad Protein Assay. The assay buffer composition was as follows: 0.2mM DTNB, 0.3mM acetyl CoA, 0.1mM Tris-CI, 0.05 ml homogenate. The reaction was started by the addition of 0.05ml 10mM oxaloacetate, and the absorption at 412nm was measured every 5 seconds for 1 minute. Linear rates of reaction were obtained by this protocol. The data were collected and a zero-order rate constant calculated using the Enzyme Kinetics Pro software (SynexChem, Fairfield, California). Data were expressed as μmol/min/mg protein.
IV: Immunoprecipitation and Measurement of Protein Expression by Western Blotting

(a) Overview of Experimental Design

Measurements were only carried out on samples that had been perfused with insulin. Whenever possible, phosphorylation of specific residues on the protein of interest was measured using phospho-specific antibodies. The blots were stripped and re-blotted for total expression of the protein of interest. However, if phospho-specific antibodies were not available to probe the protein of interest, we measured the co-immunoprecipitation (co-IP) of the protein with pan-specific phosphoserine and phosphothreonine antibodies, either individually or in combination, (Upstate Biotechnology/ Millipore, Billerica, Massachusetts) as an index of the total phosphorylation state.

To obtain a measure of ACC and MCD activities, we measured the expression of ACC (Upstate Biotechnology/ Millipore, Billerica, Massachusetts) and MCD (a generous gift from Dr. G.D Lopaschuk and Dr. J. Dyck, University of Alberta), AMPK-mediated phosphorylation of Serine 79 on ACC and the total serine phosphorylation state of ACC. SERCA-2 expression was measured as an index of calcium handling and fetal gene program effects.

To investigate changes in CPT-1 expression, we measured total CPT-1 expression with a pan-specific CPT-1 antibody, as well as with antibodies specific for CPT-1A and CPT-1B (Santa-Cruz Biotechnology, Santa-Cruz, California). We measured the total protein expression of PGC1α, USF-1 and USF-2 (all from Upstate Biotechnology/ Millipore, Billerica, Massachusetts), and PPAR-α and PDK-4 (both from Santa-Cruz Biotechnology, Santa Cruz, California). α-myosin heavy chain (α-MHC) expression (Santa-Cruz Biotechnology, Santa Cruz, California) was measured as an index of USF
activity, and PDK-4 expression (Upstate Biotechnology/ Millipore, Billerica, Massachusetts) was measured as an index of PPAR-α activity. PPAR-α was purified by IP prior to Western blotting. The co-IP of PGC1α with MEF2A (Santa-Cruz Biotechnology, Santa-Cruz, California) and PPAR-α and to its repressor USF-2 was measured as an index of the association between these proteins. Finally, to determine whether PPAR-α binds the PGC1α. MEF2A functional complex, we measured the co-IP of PPAR-α with MEF2A and USF-2.

The expression of β1, β2 and β3 adrenoceptors (all from Santa-Cruz Biotechnology, Santa-Cruz, California) was measured. β2 association with Gs or Gi was measured by co-IP. PKA and CAMK II activities were measured using radioisotopic assays as described in section IIIa. PI3K/ Akt activation was determined by measuring the phosphorylation of Akt. Efforts to measure NOS activity in our laboratory were unsuccessful. We therefore measured the expression and PKA/ PI3K-mediated phosphorylation of eNOS, and the expression of iNOS. Tissue nitrate/ nitrite levels were measured as an indirect index of NO levels. Total protein glutathiolation and tyrosine nitration were measured by dot blotting as biomarkers of reactive nitrogen species.

The total phosphorylation states of CPT-1 and AKAP149 (Santa-Cruz Biotechnology, Santa-Cruz, California) were measured using phosphoserine and phosphothreonine antibodies in combination. The nitrosylation, glutathiolation and nitration of CPT-1 were determined by measuring the co-immunoprecipitation of CPT-1 with pan-specific anti-nitrotyrosine antibodies (Upstate Biotechnology/ Millipore, Billerica, Massachusetts), pan-specific anti-glutathione antibodies (Santa-Cruz Biotechnology, Santa-Cruz, California) and pan-specific anti- nitrosocysteine antibodies (AG Scientific, San Diego, California). To determine the relationship between AKAP 149 binding and PKA binding to CPT-1, co-immunoprecipitation of PKA with CPT-1 was measured. The blots were then stripped and rebotted for AKAP149. Co-immunoprecipitation of CAMK-II with CPT-1 was also measured.
(b) **Sample Preparation**

10-30mg of heart tissue was homogenized in total protein extraction buffer containing 20 mM HEPES, 1mM ethylenediamine tetraacetic acid (EDTA), 250 mM sucrose, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM sodium fluoride and 4μl/ml protease inhibitor cocktail (Sigma-Aldich, Saint-Louis, Missouri) at pH 7.4. The total extraction buffer was found to interfere with the BioRad protein assay. Protein concentration was therefore determined using the Pierce bicinechonic acid (BCA) protein assay (Pierce Biotechnology, Rockford, Illinois). To prepare samples for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 50 μg of protein was diluted with water and a reducing buffer (6% SDS, 185mM Tris pH 6.8, 30% glycerol, 14% mercaptoethanol, 0.7% bromophenol blue) to a total volume of 20 μl. The samples were boiled for 5 minutes and stored on ice prior to loading. Dot blotting samples were boiled for 5 minutes, but, to ensure adherence of the protein to the nitrocellulose membrane, an equivalent volume of water rather than reducing buffer was added. For immunoprecipitates, 15 μl of the immunoprecipitate was diluted with 5 μl sample buffer and boiled for 5 minutes.

(c) **SDS-PAGE, Western Blotting and Dot Blotting**

Samples were loaded onto an acrylamide/ bis acrylamide gel of the appropriate percentage (5, 7.5 or 10%) and subjected to SDS-PAGE as previously described (210). The protein bands were transferred to a nitrocellulose membrane and stained with Ponceau Red to measure total protein. The membrane was blocked in 5% bovine serum albumin in Tris-buffered saline containing 0.1% polyoxyethylene sorbitan monopalmitate (TWEEN). The membrane was subsequently incubated with primary antibody overnight at 4°C, washed three times with tris-buffered saline and incubated in secondary antibody at room temperature for 1 hour. Following three further washes with tris-buffered
saline, the blots were visualised using chemiluminescent detection. Due to the low abundance of some proteins of interest, blots were developed using the Super Signal ® West Femto Maximum Sensitivity substrate (Pierce Biotechnology, Rockford, Illinois). The blots were imaged using a ChemigeniusQ Image Analyser (Geneflow, Alexandria, Virginia) so the images obtained are inverted. Band intensity was quantified using ImageJ software available from the National Institutes of Health. For most analyses, 3 or 4 samples from each group were run on a single gel. However, blots were repeated to ensure that the observed patterns were consistent for all samples.

(d) Immunoprecipitation Protocol

500 µg protein was diluted into 500 µl total protein extraction buffer to create a 1 µg/µl protein solution. 2 µl of antibody was added and the samples incubated on a rotator overnight at 4°C. For combined phosphoserine and phosphothreonine IP, 1 µl of each antibody was added. 20 µl of protein A sepharose slurry was added and the samples incubated on the rotator at 4°C for a further hour. Samples were centrifuged to pellet the immunoprecipitate and the supernatant was discarded. The pellet was washed three times in total protein extraction buffer. Finally, the pellet was resuspended in suspension buffer (0.1 M Tris base, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10% glycerol, 0.02% sodium azide, pH 7.5).

(e) Dot Blotting

5 µg of protein was loaded directly onto a dry nitrocellulose membrane. Total protein was determined by Ponceau Red staining. The membrane was then blotted according to the Western blotting protocol. Pan-specific anti-nitrotyrosine or glutathione antibodies were used as the primary antibody.
V: Functional Effects of CPT-1 Covalent Modifications in Isolated Mitochondria

(a) Isolation of Mitochondria

Left ventricular tissue from control hearts was homogenized in 10ml of mitochondrial homogenisation buffer containing 20 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS), 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 2.5 mM reduced glutathione and 3% BSA at pH 7.2. The samples were centrifuged at 1000g for 5 minutes to remove the nuclei and incompletely disrupted tissue, and the supernatant was centrifuged at 10,000g for 10 minutes. The supernatant was discarded and the pellet resuspended in mitochondrial homogenisation buffer without BSA prior to further centrifugation at 10,000g for 10 minutes. The final pellet was resuspended in 0.6 ml of mitochondrial homogenisation buffer without BSA. A 50μl aliquot of the mitochondrial fraction was taken for protein quantification using the BioRad assay. The mitochondrial preparations were used on the day of isolation.

(b) Phosphorylation of Proteins in Isolated Mitochondria

Purified preparations of active PKA, CAMK II and Akt were purchased from Upstate Biotechnology/ Millipore. Phosphorylation of isolated mitochondria was achieved by incubating 100 μl (0.4 - 0.7 mg protein) of the mitochondrial isolate with the active kinase. For the control reaction, 100 μl of the same mitochondrial isolate was incubated without the active kinase. The reaction conditions were the same as those used to measure kinase activity: 20 mM MOPS, 25 mM β-glycerophosphate, 5 mM EGTA, 1mM sodium orthovanadate, 1 mM dithiothreitol, 12.5 mM magnesium chloride, 83 μM ATP, 1.7 μM cAMP (for PKA reactions only), 1mM calcium chloride (for CAMK reactions only) and 0.83 μg/ ml active kinase at pH 7.2. The final reaction volume was 600 μl. The
samples were incubated at 30°C for 10 minutes and the reaction terminated by flash freezing in liquid nitrogen. Separate reactions were carried out to generate samples for CPT-1 assay or measurement of total CPT-1 phosphorylation and co-immunoprecipitation of CPT-1 with the kinase.

(c) Peroxynitrite Dose Response in Isolated Mitochondria

Peroxynitrite has a half life of less than 5 seconds at physiological pH; 80% of the peroxynitrite loaded at pH 7.4 is degraded by protonation within 12 seconds (217). However, this timeframe is sufficient for peroxynitrite to produce measurable covalent modifications of target proteins (218). In order to examine the effects of tyrosine nitration, glutathiolation and cysteine nitrosylation on CPT-1, we incubated mitochondrial isolates with increasing concentrations of peroxynitrite. Peroxynitrite is stable at high pH as the equilibrium does not favour protonation. Peroxynitrite stock solutions were made in 0.3M NaOH.

Mitochondrial isolates from four control hearts were pooled. 100 μl (0.4 - 0.7 mg protein) of the pooled mitochondrial isolate was incubated with 0, 0.1, 1, 10, 100, 500 and 1000 μM peroxynitrite in mitochondrial homogenisation buffer without BSA. The final reaction volume was 600 μl. The samples were incubated at room temperature for 5 minutes. The addition of the 0.3M NaOH vehicle raised the pH of the buffer from 7.2 to 8. Separate reactions were carried out to generate samples for CPT-1 assay or measurement of total CPT-1 phosphorylation and co-immunoprecipitation of CPT-1 with the kinase. Immunoprecipitation was started immediately following the incubation with peroxynitrite. CPT-1 activity samples were frozen in liquid nitrogen and stored at -70°C until the day of assay.
(d) Measurement of CPT-1 Activity, Phosphorylation and Kinase Binding

CPT-1 activity and sensitivity to malonyl CoA was measured as described in section III (b) with two modifications: 50 µl of the sample was added and the reaction was allowed to proceed for 10 minutes rather than 5. Addition of the pH 8 mitochondrial samples had no effect on the final pH of the CPT-1 reaction mixture.

VI: Identification of CPT-1 Phosphorylation Sites by LC MS/ MS

The rat CPT-1B primary sequence was searched for consensus sites of the following kinases: PKA, PKC, CAMK I/ II, AMPK and Akt. Putative phosphorylation sites for PKA and CAMK I/ II were identified. To maximize our chances of finding phosphorylation, CPT-1B was purified by IP and phosphorylation enrichment was performed on the tryptic digests of the CPT-1 bands.

CPT-1 in whole cell homogenates was purified by IP with specific CPT-1B antibodies. The immunoprecipitates were subjected to SDS-PAGE and stained with Coumassie Blue. The bands corresponding to CPT-1 (88kDa) were excised and stored at -70ºC until the day of assay.

Tryptic digests of the CPT-1 bands were subjected to titanium TopTip phosphopeptide enrichment at the UBC MSL/LMB Proteomics Core Facility. The digests were applied to titanium tips for 30 minutes to allow binding of phosphopeptides. The tips were then washed with binding solution (0.1% trifluoroacetic acid, 10% acetonitrile) to remove unphosphorylated peptides. Phosphorylated peptides were eluted from the tips by a series of 50 µl washes with increasing concentrations of ammonium bicarbonate (10, 20, 50 and 100 mM), which release low to moderately phosphorylated peptides, and a final 50 µl wash with ammonium hydroxide, which releases highly phosphorylated peptides.
The ammonium bicarbonate eluents for each sample were pooled, and the ammonium bicarbonate and ammonium hydroxide eluents for each sample were subjected to LC/MS/MS separately.

Phosphorylation detection was carried out using Liquid Chromatography/Mass Spectroscopy/Mass Spectroscopy (LC/MS/MS) on an API Q STAR PULSARi Hybrid LC/MS/MS at the UBC MSL/LMB Proteomics Core Facility. Tryptic digests of CPT-1 bands underwent reversed-phase HPLC. The column eluent was subjected to MS/MS as it eluted from the column. In this procedure, atmospheric pressure ionization (API) was applied to generate a spectrum of mass-to-charge ratio (m/z) peaks. As each amino acid produces a characteristic m/z peak, the sequence of the eluted peptide can be determined and searched in the MASCOT protein database to confirm the identity of the protein. Ions with a change in m/z suggesting that phosphorylation had occurred (indicated by an increase of 80 Da) were selected as parent ions for MS/MS to confirm that the phosphorylation event was present and identify the affected residue.

VII: Data Analysis

Data are expressed as mean ± standard error of the mean (SEM). For statistical analysis, data were analysed using Number Cruncher Statistical Software (NCSS, Kaysville, Utah). Starling curves were analysed using GLM ANOVA with Neumann-Keuls post hoc test. All other data were analysed using One-Way ANOVA with Neumann-Keuls post hoc test. Comparisons between two groups were carried out using an unpaired student's t-test. Acute perfusion and chronic treatment data are presented separately, although the control and diabetic groups for each are the same, and the data were subjected to composite analysis.
RESULTS

I: General Characteristics

We measured plasma glucose and insulin levels to confirm successful induction of diabetes. STZ successfully induced marked and sustained hyperglycemia which was associated with a decrease in insulin levels and a mild elevation of plasma lipids. As expected, metoprolol treatment had no effect on either glucose or insulin (Table 1). Metoprolol had no effect on plasma lipids, but ameliorated the increase in ketone levels in the diabetic group (Table 1). Body weights were lower in the diabetic groups. Metoprolol had no significant effect on body weight. However, metoprolol significantly lowered heart weight in both control and diabetic rats (Table 1).

II: Functional and Metabolic Effects of Chronic Metoprolol Treatment

To establish whether chronic metoprolol treatment improves function in the STZ-model, cardiac function was measured ex vivo by direct left ventricular pressure measurements in paced isolated working hearts following chronic treatment with metoprolol. Metoprolol treatment (75mg/kg/day IP) significantly improved contractile function in diabetic cardiomyopathy as measured ex vivo by LVDP, +dP/ dt and −dP/ dt from the left ventricle in paced hearts (Figure 1). During subsequent studies for the measurement of cardiac metabolism, in which the hearts were not paced and fatty acid was present in the perfusate, metoprolol also ameliorated the depression in rate-pressure product, cardiac output and hydraulic power in diabetic hearts (Figure 2).

Chronic treatment with metoprolol increased palmitate oxidation and decreased glucose oxidation in control hearts (Figure 3). Lactate production was unchanged, but glycogen levels were decreased (Table 2). In the diabetic hearts, there was a marked increase in palmitate oxidation relative to controls and
**TABLE 1**

**GENERAL CHARACTERISTICS AND PLASMA PARAMETERS AT TERMINATION**

<table>
<thead>
<tr>
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<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
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<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>486.4 ± 31.3</td>
<td>480.3 ± 41.0</td>
<td>387.8 ± 37.5*</td>
<td>351.6 ± 53.7*</td>
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<tr>
<td><strong>Heart Weight (g)</strong></td>
<td>1.82 ± 0.10</td>
<td>1.47 ± 0.07*</td>
<td>1.51 ± 0.10*</td>
<td>1.38 ± 0.07*</td>
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<tr>
<td><strong>Plasma Glucose (mmol/l)</strong></td>
<td>7.25 ± 0.26</td>
<td>7.35 ± 0.40</td>
<td>27.99 ± 1.1*</td>
<td>24.09 ± 5.41*</td>
</tr>
<tr>
<td><strong>Plasma Insulin (ng/ml)</strong></td>
<td>1.59 ± 0.41</td>
<td>1.78 ± 0.78</td>
<td>0.49 ± 0.3*</td>
<td>0.37 ± 0.12*</td>
</tr>
<tr>
<td><strong>Plasma Triglycerides (mmol/l)</strong></td>
<td>0.19 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.25 ± 0.05*</td>
<td>0.26 ± 0.05*</td>
</tr>
<tr>
<td><strong>Plasma Cholesterol (mmol/l)</strong></td>
<td>1.89±0.05</td>
<td>1.86±0.10</td>
<td>2.05±0.18*</td>
<td>2.10 ± 0.15*</td>
</tr>
<tr>
<td><strong>Plasma Free Fatty Acids (mmol/l)</strong></td>
<td>0.19 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td><strong>Plasma Ketones (mmol/l)</strong></td>
<td>0.76 ± 0.05</td>
<td>0.50 ± 0.04</td>
<td>2.43 ± 0.57*</td>
<td>1.36 ± 0.28*</td>
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</table>

Animals were fasted for 5 hours prior to blood collection. Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from C, † = significantly different from untreated group (p<0.05) (C=control, n=8; CT=control treated with metoprolol, n=8; D=diabetic, n=8; DT=diabetic treated with metoprolol, n=8).
FIGURE 1

Mechanical performance of Isolated Perfused Hearts: Left Ventricular Pressure Measurements. Hearts were excised and perfused in working heart mode with Chenoweth-Keuls buffer containing 5.5 mM glucose but no palmitate. The hearts were paced at 300 bpm and direct left ventricular pressure measurements were taken. A-C: Left ventricular developed pressure (LVDP), maximum rate of contraction (+dP/dt) and maximum rate of relaxation (-dP/ dt) from direct left ventricular pressure measurements. Data represent means ± SEM and were analysed using GLM ANOVA with Neumann Keuls post-hoc test. * = significantly different from C, CT, DT at the same filling pressure, # = significantly different from C and D at the same filling pressure (C=control, n=12; CT=control treated, n=12; D= diabetic, n=12; DT= diabetic treated, n=12).
A. LEFT ATRIAL FILLING PRESSURE (mmHg)

B. LEFT ATRIAL FILLING PRESSURE (mmHg)

C. LEFT ATRIAL FILLING PRESSURE (mmHg)
FIGURE 2

Mechanical performance of Isolated Perfused Hearts: Flow and Rate-Pressure Product Measurements. Cardiac function measurements obtained by aortic pressure and heart rate measurements, and pulmonary and aortic flow measurements. Unpaced hearts were perfused with Krebs-Henseleit buffer containing 5.5 mM glucose and 0.8 mmol palmitate at constant preload (11.5 mm Hg) and afterload (80 mm Hg). Heart rate, peak systolic pressure, rate-pressure product, cardiac output and hydraulic power. Data represent means ± SEM and were analysed using GLM ANOVA with Neumann Keuls post-hoc test. * = significantly different from D and DT at same timepoint, # = significantly different from C, CT, D at same timepoint, + = significantly different from C, CT and DT at same timepoint (p<0.05), (C=control, n=5; CT=control treated, n=5; D= diabetic, n=5; DT= diabetic treated, n=5).
Effects of Chronic In Vivo Metoprolol Treatment on Metabolism of Isolated Perfused Hearts. A-B Glucose and palmitate oxidation during a 60 minute aerobic perfusion. Perfusions were carried out in the presence (left panel) or absence (right panel) of insulin (100 µUnits/ml) as indicated. Data represent means ± SEM. Data were analysed using One-Way ANOVA with Neumann Keuls post-hoc test, * = significantly different from C of metoprolol, † = significantly different from corresponding untreated group (p<0.05),
### TABLE 2

**LACTATE PRODUCTION AND TISSUE GLYCOGEN, TRIGLYCERIDE AND MALONYL CoA LEVELS FOLLOWING CHRONIC IN VIVO METOPROLOL TREATMENT AND EX VIVO PERFUSION IN THE PRESENCE OF INSULIN**

<table>
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<th>CT</th>
<th>D</th>
<th>DT</th>
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</thead>
<tbody>
<tr>
<td><strong>Lactate Production</strong></td>
<td>10.2 ± 2.2</td>
<td>13.9 ± 4.1</td>
<td>10.6 ± 3.6</td>
<td>10.8 ± 4.0</td>
</tr>
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<td>(nmol/ min/ g dry weight)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Tissue Glycogen Levels</strong></td>
<td>180.4±31.2</td>
<td>95.9 ± 18.3</td>
<td>285.0±32.0</td>
<td>286.6±35.2</td>
</tr>
<tr>
<td>(µmol/ g dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tissue Triglyceride Levels</strong></td>
<td>41.1 ± 2.6</td>
<td>31.2 ± 1.5 *</td>
<td>59.1 ± 3.9 *</td>
<td>44.6 ± 2.7 *</td>
</tr>
<tr>
<td>(µmol/ g dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Malonyl CoA Levels</strong></td>
<td>17.1 ± 2.4</td>
<td>7.7 ± 1.4 *</td>
<td>20.7 ± 5.2</td>
<td>17.4 ± 3.8</td>
</tr>
<tr>
<td>(µmol/ g wet weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding unperfused group, + = significantly different from corresponding untreated group, (p<0.05) (C=control, n=5; CT=control treated, n=5; D=diabetic, n=5; DT=diabetic treated, n=5).
TABLE 3

MYOCARDIAL ENERGETICS AND AMPK ACTIVITY
FOLLOWING CHRONIC IN VIVO METOPROLOL TREATMENT AND EX VIVO
PERFUSION IN THE PRESENCE OF INSULIN

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (µmol/g wet weight)</td>
<td>6.3 ± 0.3</td>
<td>7.2 ± 1.7</td>
<td>6.0 ± 0.5</td>
<td>5.1 ± 1.2</td>
</tr>
<tr>
<td>ADP (µmol/g wet weight)</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>1.6 ± 0.5+</td>
</tr>
<tr>
<td>AMP (µmol/g wet weight)</td>
<td>0.74 ± 0.09</td>
<td>1.03 ± 0.08</td>
<td>0.83 ± 0.08</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td>ATP/ADP Ratio</td>
<td>2.9 ± 0.3</td>
<td>6.0 ± 1.4+</td>
<td>2.5 ± 0.2</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>α-1 AMPK Activity</td>
<td>2.7 ± 0.3</td>
<td>3.5 ± 0.6</td>
<td>3.1 ± 0.4</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>(pmol ATP incorporated/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-2 AMPK Activity</td>
<td>3.6 ± 0.4</td>
<td>3.6 ± 0.3</td>
<td>3.4 ± 0.6</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>(pmol ATP incorporated/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-pan AMPK Activity</td>
<td>4.0 ± 0.9</td>
<td>3.8 ± 0.3</td>
<td>3.6 ± 0.5</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>(pmol ATP incorporated/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding unperfused group, + = significantly different from corresponding untreated group (p<0.05) (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
glucose oxidation was negligible; in these hearts, chronic metoprolol treatment decreased palmitate oxidation and increased glucose oxidation (Figure 3). Glycogen levels were elevated in diabetic hearts as compared with controls; metoprolol treatment had no effect on either glycogen levels or lactate production (Table 2). Tissue triglyceride levels were significantly elevated in the diabetic group. In both control and diabetic hearts, metoprolol treatment significantly lowered tissue triglyceride levels (Table 2). Myocardial energetics, as determined by tissue levels of ATP, ADP and AMP, and AMPK activity, were not altered either by metoprolol or by diabetes (Table 3). When perfusions were repeated in the absence of insulin, the effect of metoprolol on glucose oxidation was obliterated, but the effect on palmitate oxidation was preserved (Figure 3). In a preliminary study, chronic metoprolol treatment had no effect on glycolysis in either control or diabetic heart (Table 4), but improved coupling between glycolysis and glucose oxidation in diabetic hearts by increasing glucose oxidation.

When control or diabetic hearts were perfused for 30 minutes with metoprolol, palmitate oxidation was inhibited and glucose oxidation markedly stimulated (Figure 4) producing an increase in tissue ATP levels and a decrease in AMP levels (Table 5). Stimulation of glucose oxidation was associated with a fall in lactate production without any change in glycogen levels (Table 5). In both control and diabetic hearts, acute metoprolol perfusion lowered tissue triglyceride levels (Table 5). In the absence of insulin, the pattern of changes observed for glucose oxidation was obliterated in diabetic, but not control, hearts, whereas the pattern of changes observed for palmitate oxidation was preserved (Figure 4).
**TABLE 4**

GLYCOLYSIS AND FATTY ACID OXIDATION
ENZYME ACTIVITIES FOLLOWING IN VIVO METOPROLOL TREATMENT
AND EX VIVO PERFUSION IN THE PRESENCE OF INSULIN

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CT</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis</td>
<td>3900 ± 539</td>
<td>4484 ± 1116</td>
<td>1335 ± 358*</td>
<td>1729 ± 313*</td>
</tr>
<tr>
<td>(nmol/ min/ g dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose Oxidation</td>
<td>2336 ± 564</td>
<td>3749 ± 1378</td>
<td>123 ± 52</td>
<td>854 ± 312*</td>
</tr>
<tr>
<td>(nmol/ min/ g dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Coupling of Glycolysis to Glucose Oxidation</td>
<td>72 ± 20</td>
<td>82 ± 19</td>
<td>11 ± 6</td>
<td>40 ± 18*</td>
</tr>
<tr>
<td>Acyl CoA Dehydrogenase Activity</td>
<td>5.0 ± 0.4</td>
<td>5.5 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>(mmol/ min/ mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate Synthase Activity</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>(mmol/ min/ mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glucose and fatty acid metabolism measurements from a preliminary study. Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from C and CT, # = significantly different from D (p<0.05) (Glycolysis and glucose oxidation, C=control, n=4; CT=control treated with metoprolol, n=4; D=diabetic, n=4; DT=diabetic treated with metoprolol, n=4; Acyl CoA dehydrogenase and citrate synthase activities, C=control, n=8; CT=control treated with metoprolol, n=8; D=diabetic, n=8; DT=diabetic treated with metoprolol, n=8).
FIGURE 4

Acute Effects of Metoprolol on Metabolism of Isolated Perfused Hearts. A-B: Glucose and palmitate oxidation during a 60 minute aerobic perfusion. Perfusion were carried out in the presence (left panel) or absence (right panel) of insulin (100 μM/ml) as indicated. Data represent means ± SEM. Data were analysed using One-Way ANOVA with Neumann Keuls post-hoc test, * = significantly different from C of metoprolol, + = significantly different from corresponding untreated group (p<0.05),
PALMITATE OXIDATION
100 μIU/ml Insulin Present
(nmol/min/g dry weight)

GLUCOSE OXIDATION
100 μIU/ml Insulin Present
(nmol/min/g dry weight)

PALMITATE OXIDATION
Insulin Absent
(nmol/min/g dry weight)

GLUCOSE OXIDATION
Insulin Absent
(nmol/min/g dry weight)
### TABLE 5

**LACTATE PRODUCTION AND TISSUE GLYCOGEN, TRIGLYCERIDE AND MALONYL CoA LEVELS FOLLOWING ACUTE METOPROLOL PERFUSION EX-VIVO IN THE PRESENCE OF INSULIN**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CP</th>
<th>D</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactate Production</strong>&lt;br&gt; (nmol/ min/ g dry weight)</td>
<td>10.2 ± 2.2</td>
<td>6.7 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.6 ± 3.6</td>
<td>2.4 ± 0.8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Tissue Glycogen Levels</strong>&lt;br&gt; (µmol/ g dry weight)</td>
<td>180.4± 31.2</td>
<td>160.6 ±18.1</td>
<td>285.0 ±32.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>237.3±35.7&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Tissue Triglyceride Levels</strong>&lt;br&gt; (µmol/ g dry weight)</td>
<td>41.1 ± 2.6</td>
<td>35.3 ± 3.0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>59.1 ± 3.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>31.5± 0.8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Malonyl CoA Levels</strong>&lt;br&gt; (µmol/ g wet weight)</td>
<td>17.1 ± 2.4</td>
<td>8.1 ± 1.1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>20.7 ± 5.2</td>
<td>22.1 ± 4.7</td>
</tr>
</tbody>
</table>

Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from corresponding unperfused group, + = significantly different from corresponding untreated group, (p<0.05) (C=control, n=5; CP = control perfused, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5).
**TABLE 6**

**MYOCARDIAL ENERGETICS AND AMPK ACTIVITY FOLLOWING ACUTE METOPROLOL PERFUSION EX-VIVO IN THE PRESENCE OF INSULIN**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CP</th>
<th>D</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (μmol/g wet weight)</td>
<td>6.3 ± 0.3</td>
<td>7.3 ± 0.4+</td>
<td>6.0 ± 0.5</td>
<td>9.7 ± 0.9+</td>
</tr>
<tr>
<td>ADP (μmol/g wet weight)</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td>3.3 ± 0.4+</td>
</tr>
<tr>
<td>AMP (μmol/g wet weight)</td>
<td>0.74 ± 0.03</td>
<td>0.22 ± 0.03+</td>
<td>0.83 ± 0.08</td>
<td>0.39 ± 0.10+</td>
</tr>
<tr>
<td>ATP/ADP Ratio</td>
<td>2.9 ± 0.3</td>
<td>3.2 ± 0.14</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>α-1 AMPK Activity (pmol [P] incorporated/ min/ mg protein)</td>
<td>2.7 ± 0.3</td>
<td>3.8 ± 0.1+</td>
<td>3.1 ± 0.4</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>α-2 AMPK Activity (pmol [P] incorporated/ min/ mg protein)</td>
<td>3.6 ± 0.4</td>
<td>3.0 ± 0.5</td>
<td>3.4 ± 0.6</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>α-pan AMPK Activity (pmol [P] incorporated/ min/ mg protein)</td>
<td>4.0 ± 0.9</td>
<td>4.0 ± 0.9</td>
<td>3.6 ± 0.5</td>
<td>3.3 ± 0.4</td>
</tr>
</tbody>
</table>

Myocardial Energetics and AMPK Activity. Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. + = significantly different from corresponding untreated group (p<0.05) (C=control, n=5; CP = control perfused with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol).
FIGURE 5

ACC and MCD Expression and Phosphorylation. A: Expression of ACC and MCD measured by Western blotting. (C=control; CT=control treated; D=diabetic; DT=diabetic treated). B: Phosphorylation of ACC measured by Western blotting. Band intensity was quantified using ImageJ software. *= significantly different, p<0.05. (C=control; CT=control treated; D=diabetic; DT=diabetic treated).
CPT-1 Activity and Malonyl CoA Sensitivity. A: CPT-1 Activity in whole tissue homogenates. Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. + = significantly different from corresponding untreated group, * = significantly different from all other groups (p<0.05) (C=control, n=5; CP = control perfused, n=5 (Conc of metoprolol?); CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5) B. Malonyl CoA IC\textsubscript{50} values calculated following curve-fitting analysis of CPT-1 dose-response curves. Data represent means ± SEM. Data were analysed using one-way ANOVA with Neumann-Keuls post-hoc test. * = significantly different from control, † = significantly different from corresponding untreated group, p<0.05. (C=control, n=5; CP = control perfused, n=5; CT=control treated, n=5; D=diabetic, n=5; DP = diabetic perfused, n=5; DT=diabetic treated, n=5).
A.

![Graphs showing CPT-1 activity across different treatment groups.

B.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C</th>
<th>CP</th>
<th>CT</th>
<th>D</th>
<th>DP</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ Malonyl CoA (µM)</td>
<td>29.5 ±</td>
<td>113.6 ±</td>
<td>31.7 ±</td>
<td>9.0 ±</td>
<td>63.0 ±</td>
<td>163.5 ±</td>
</tr>
<tr>
<td>0.17</td>
<td>22.9*</td>
<td>8.0</td>
<td>0.1*</td>
<td>6.3*</td>
<td>± 11.7*</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 7

Pharmacological Effects of Metoprolol on CPT-1 Activity. CPT-1 Activity following incubation of control tissue homogenates with increasing concentrations of metoprolol and in the presence of 0, 50 or 100μM malonyl CoA. Data represent means ± SEM (n=5).
The graph shows the CPT-1 activity (nmol/min/mg protein) measured for different concentrations of metoprolol and malonyl CoA. The concentrations range from 0 to 50000 ng/ml. The graph includes three lines representing different malonyl CoA concentrations:

- Black circles: 0 μm Malonyl CoA
- Open circles: 50 μmol Malonyl CoA
- Black triangles: 100 μmol Malonyl CoA

The x-axis represents the metoprolol concentration in ng/ml, while the y-axis shows the CPT-1 activity.
III: Malonyl CoA Levels

In vivo treatment with metoprolol lowered malonyl CoA levels in control hearts, as did acute treatment of control hearts with metoprolol during perfusion (Tables 2 and 5). Diabetes had no effect on malonyl CoA levels, and malonyl CoA levels in the diabetic heart remained unchanged by chronic metoprolol treatment and acute metoprolol perfusion. ACC and MCD expression in control and diabetic hearts was unchanged by chronic metoprolol treatment (Figure 5), and AMPK-mediated phosphorylation of ACC, assessed by phosphorylation of Ser 79 on ACC, was also unchanged either by metoprolol perfusion or chronic in vivo metoprolol treatment (Figure 5). Furthermore, metoprolol had no effect on the total phosphorylation state of ACC, assessed by reactivity with pan-specific anti-phosphoserine and anti-phosphothreonine antibodies. Overall, malonyl CoA levels did not correlate with the observed changes in the rate of fatty acid oxidation, and the observed decrease in malonyl CoA levels in control hearts could not be explained by changes in ACC or MCD.

IV: CPT-1 Activity and Malonyl CoA Sensitivity

In a preliminary study, we measured the activities of CPT-1 (entry of fatty acyl CoA to mitochondria), acyl-CoA dehydrogenase (a β-oxidation enzyme) and citrate synthase (a TCA cycle enzyme). Chronic metoprolol treatment decreased CPT-1 activity in both control and diabetic hearts but had no effect on the other enzymes (Table 4 and Figure 6). Acute metoprolol perfusion also decreased CPT-1 activity. To investigate whether metoprolol altered CPT-1 sensitivity, we assayed CPT-1 activity in the presence of increasing concentrations of malonyl CoA to obtain dose-response curves for each of the groups. The IC<sub>50</sub> of malonyl CoA was calculated (Figure 6). Chronic metoprolol treatment decreased the sensitivity of CPT-1 to malonyl CoA in diabetic, but not control, hearts. Acute metoprolol perfusion decreased the sensitivity in both control and diabetic hearts.
The rightward shift in the dose-response curve was preserved when absolute activity data were plotted (data not shown).

To test whether metoprolol is a direct pharmacological inhibitor of CPT-1, or whether it can directly interfere with malonyl CoA inhibition of CPT-1, we incubated CPT-1 with increasing concentrations of metoprolol in the presence of 0, 50 and 100\(\mu\)M malonyl CoA. Metoprolol did not inhibit CPT-1 activity directly, and did not decrease or enhance inhibition of CPT-1 by malonyl CoA (Figure 7).

V: Regulation of CPT-1 Expression

Chronic metoprolol treatment decreased total CPT-1 expression in control and diabetic hearts (Figure 8). Metoprolol decreased CPT-1B expression, but did not alter CPT-1A expression, which was present at low levels (Figures 9 and 10). Changes in CPT-1 sensitivity cannot, therefore, be attributed to a shift in CPT-1 isoform expression.

Metoprolol did not alter the total expression of PPAR-\(\alpha\), PGC1\(\alpha\) or PDK-4 in either control or diabetic hearts (Figures 11 and 12). In control hearts, metoprolol increased the expression of USF-1 but the expression of \(\alpha\)-MHC was not significantly changed. The expression of both USF-1 and USF-2 was decreased in the diabetic heart, as was \(\alpha\)-MHC. Metoprolol increased the expression of USF-2 in control and diabetic hearts, but this was only associated with an increase in MHC expression in diabetic hearts; indeed, although the \(\alpha\)-MHC band in control treated hearts was larger and more diffuse, the band was less intense in densitometric analysis, indicating that \(\alpha\)-MHC expression may actually have decreased (Figures 13-15). SERCA expression was markedly depressed in diabetic hearts, and metoprolol restored SERCA expression.

In control hearts, metoprolol decreased the association of the coactivator PGC1\(\alpha\) with the transcription factors PPAR-\(\alpha\) and MEF-2A without changing the
FIGURE 8

Total CPT-1 expression. Data represent means ± SEM. Data were analysed using an unpaired student’s t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 9

CPT-1B (muscle isoform) expression. Data represent means ± SEM. Data were analysed using an unpaired student's t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
CPT-1 MUSCLE ISOFORM EXPRESSION (Normalised to Total Protein)

TREATMENT GROUP

CPT-1A (Muscle)

C

CT

D

DT

CPT-1B (Muscle)

C

CT

D

DT
FIGURE 10

CPT-1A (liver isoform) expression. Data represent means ± SEM. Data were analysed using an unpaired student's t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
CPT-1 LIVER ISOFORM EXPRESSION (Normalised to Total Protein)

TREATMENT GROUP

C

CT

D

DT

CPT-A (Liver)

Ponceau

C

CT

D

DT

C

D
FIGURE 11

PPAR-α, PGC1α and PDK-4, expression following chronic treatment with metoprolol. Densitometric analyses of these data are presented in Figure 12. (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
CT

PPAR-α
PGC1α
PDK-4
Ponceau

DT

PPAR-α
PGC1α
PDK-4
Ponceau

D

PPAR-α
PGC1α
PDK-4
Ponceau
FIGURE 12

Densitometric analysis of PPAR-α, PGC1α and PDK-4, expression following chronic treatment with metoprolol. Data represent means ± SEM. Data were analysed using an unpaired student's t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 13

USF-1, USF-2, MHC and SERCA-2 expression following chronic treatment with metoprolol. Densitometric analyses of these data are presented in Figures 14 and 15. (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 14

Densitometric analysis of USF-1 and USF-2 expression following chronic treatment with metoprolol. Data represent means ± SEM. Data were analysed using an unpaired student's t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
USF-2 EXPRESSION (Normalised to Total Protein)

USF-1 EXPRESSION (Normalised to Total Protein)

TREATMENT GROUP

C

CT

D

DT

Control

Treated
FIGURE 15

Densitometric analysis of MHC and SERCA expression following chronic treatment with metoprolol. Data represent means ± SEM. Data were analysed using an unpaired student's t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 16

Binding of PPAR-α, MEF-2A and USF-2 to PGC1α, and of MEF-2A and USF-2 to PPAR-α measured by immunoprecipitation. Samples underwent immunoprecipitation with anti- PGC1α antibodies as indicated, and were subsequently subjected to immunoblotting for PPAR-α, MEF-2A or USF-2. Samples also underwent immunoprecipitation with anti-PPAR-α antibodies and immunoblotting with anti- MEF-2A and USF-2 antibodies as indicated. Densitometric analyses of these data are presented in Figure 17. (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
IP: PGC1α
Blot: PPAR-α

IP: PGC1α
Blot: MEF-2A

IP: PGC1α
Blot: USF-2

IP: PPAR-α
Blot: MEF-2A

IP: PPAR-α
Blot: USF-2
FIGURE 17

Densitometric analysis of PGC1α binding. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from untreated group, # = significantly different from all groups, + = significantly different from C and D, (p<0.05). (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
binding of USF-2. In the diabetic heart, the association of PGC1α with PPAR-α was enhanced but its associations with MEF-2A and USF-2 were abolished. Metoprolol decreased the association between PGC1α and PPAR-α in the diabetic heart but the association between PGC1α and MEF-2A increased. However, in the diabetic heart, metoprolol markedly increased the binding of USF-2 to PGC1α (Figures 16 and 17).

VI: β-Adrenoceptor Signalling

Chronic treatment with metoprolol increased β1 receptor expression in control hearts. In the diabetic heart, β1 receptor levels were decreased, and chronic metoprolol treatment increased β1 receptor levels (Figures 18 and 19). β2 receptor levels were increased in both control and diabetic hearts, but diabetes itself did not alter β2 receptor expression (Figures 18 and 19). Both metoprolol treatment and diabetes induced marked increases in the expression of the β3 receptor (Figure 14). Overall, metoprolol caused a shift in β-receptor expression towards β2 and β3 in control hearts, and increased the expression of all three receptor subtypes in the diabetic heart. There was no clear shift in the association of the β2 adrenoceptor with Gs or Gi in any of the groups (Figure 20).

The activity of PKA, as expected, was decreased by metoprolol (Table 7). By contrast, the activity of the PI3K/ Akt pathway was increased by metoprolol (Figure 21), whereas CAMK activity was not significantly altered (Table 7).

Attempts to measure tissue peroxynitrite levels and eNOS and iNOS
Expression of β-Adrenoceptor subtypes following chronic metoprolol treatment. Densitometric analyses of these data are presented in Figure 19. (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 19

Densitometric analysis of expression of β-Adrenoceptor subtypes following chronic metoprolol treatment. Data represent means ± SEM. Data were analysed using an unpaired student’s t-test. * = significantly different, p<0.05 (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
B3 ADRENOCEPTOR EXPRESSION (Normalised to Total Protein)

B2 ADRENOCEPTOR EXPRESSION (Normalised to Total Protein)

B1 ADRENOCEPTOR EXPRESSION (Normalised to Total Protein)
FIGURE 20

Binding of Gs and Gi to β2-Adrenoceptors. Densitometric analysis was carried out and data analyzed using an one-factor ANOVA with Neumann-Keuls post hoc test. (data not shown). There were no significant differences between groups. (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
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<td>Blot: Gi</td>
<td>Blot: β2 AR</td>
<td>Blot: β2 AR</td>
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</tbody>
</table>

The images show gel blots for different conditions and blots, indicating the presence of specific proteins or patterns.
FIGURE 21

A. Akt phosphorylation. B. Densitometric analysis of Akt phosphorylation. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from C and CP, p<0.05. (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
TABLE 7

TISSUE ACTIVITIES OF PKA AND CAMK

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>CP</th>
<th>CT</th>
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<th>DP</th>
<th>DT</th>
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<tr>
<td>PKA Activity</td>
<td>828 ±</td>
<td>52 ±</td>
<td>214 ±</td>
<td>792 ±</td>
<td>276 ±</td>
<td>454 ±</td>
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<tr>
<td>(pmol ATP incorporated/ min/mg protein)</td>
<td>279</td>
<td>8*</td>
<td>42*</td>
<td>281</td>
<td>36*</td>
<td>143</td>
</tr>
<tr>
<td>CAMK Activity</td>
<td>611 ±</td>
<td>563 ±</td>
<td>714 ±</td>
<td>426 ±</td>
<td>388 ±</td>
<td>325 ±</td>
</tr>
<tr>
<td>(pmol ATP incorporated/ min/mg protein)</td>
<td>90</td>
<td>94</td>
<td>90*</td>
<td>118</td>
<td>102</td>
<td>96</td>
</tr>
</tbody>
</table>

PKA and CAMK-II were purified by immunoprecipitation prior to assay. Data represent means ± SEM. Data were analysed using One-Way ANOVA with Neumann Keuls post-hoc test, * = significantly different from C, + = significantly different from D (p<0.05). (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
activity were unsuccessful. We therefore used a combination of western blotting, biomarkers and indirect measures of NOS activity to determine how metoprolol influences the generation of peroxynitrite (Figures 22-24).

Metoprolol decreased the expression of eNOS in control hearts (Figure 22). Acute metoprolol perfusion decreased Akt- and PKA-mediated phosphorylation of eNOS in control hearts at Ser 1177 and Thr 495 respectively, but the decrease was not sustained by chronic treatment (Figure 23). Functionally, NO production, as indicated by nitrate/ nitrite levels, was increased by metoprolol despite the decrease in eNOS expression (Figure 24). In diabetic hearts, eNOS expression was very low but iNOS was induced. Metoprolol prevented the induction of iNOS without restoring eNOS (Figure 22).

Akt-mediated phosphorylation of eNOS was not detected in diabetic hearts. PKA-mediated phosphorylation of eNOS was high, and was decreased only by chronic metoprolol treatment (Figure 23). NO production, as indicated by nitrate/ nitrite levels, was low in the diabetic heart and was not altered by metoprolol (Figure 24).

Total protein glutathiolation, a biomarker of NO and reactive nitrogen species, was increased by acute and chronic metoprolol treatment in control hearts. In diabetic hearts, total protein glutathiolation was low, and was decreased further by chronic metoprolol treatment. Total protein tyrosine nitration, a biomarker of peroxynitrite, was unchanged by metoprolol in control hearts, and was unchanged in diabetic hearts as compared to control. In diabetic hearts, chronic metoprolol treatment produced a marked decrease in tyrosine nitration (Figure 24).
A. Expression of eNOS and iNOS. B. Densitometric analysis of eNOS expression. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. + = significantly different from C, D and DT, * = significantly different from C and CT, (p<0.05). (C=control, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DT=diabetic treated with metoprolol, n=5).
A. 

![Western blot analysis](image)

B. 

![Graph showing eNOS expression](image)
Phosphorylation of eNOS at Ser 1177 and Thr 495. Data represent means ± SEM. Ser 1177 phosphorylation was not detected in diabetic hearts (data not shown). Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from other groups, (p<0.05). (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
Biomarkers of NO and RNS. A: Tissue nitrate and nitrite levels. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from C groups, # = significantly different from C, (p<0.05). B: Total protein glutathiolation measured by Dot Blotting. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from all groups, # = significantly different from C, (p<0.05). C: Total protein tyrosine nitration measured by Dot Blotting. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from all groups, (p<0.05). (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
TOTAL PROTEIN
TYROSINE NITRATION
(Normalised to Total Protein)

TOTAL PROTEIN
GLUTATHIOlation
(Normalised to Total Protein)

NITRATE + NITRITE LEVELS
(µmol/ mg protein)
VII: CPT-1 Covalent Modifications

Phosphorylation of CPT-1 was detected by immunoprecipitation, and the total phosphorylation state of CPT-1 was increased by acute metoprolol perfusion in control and diabetic hearts. Cysteine nitrosylation, glutathiolation and tyrosine nitration were all detected. Acute metoprolol perfusion increased nitrosylation and glutathiolation but abolished tyrosine nitration (Figures 25 and 26).

Having confirmed that phosphorylation of CPT-1 is detectable by immunoprecipitation, we next investigated whether kinases are physically associated with CPT-1. Both PKA and CAMK were found to co-immunoprecipitate with CPT-1 (Figures 27-29). Increased phosphorylation of AKAP-149 was associated with increased binding of PKA to CPT-1 and a corresponding decrease in AKAP-149 binding to CPT-1. Conversely, as AKAP-149 phosphorylation decreased, PKA binding to CPT-1 decreased and the association between CPT-1 and AKAP-149 increased.

In control hearts, acute metoprolol perfusion increased the phosphorylation of AKAP-149 and the binding of PKA to CPT-1, while AKAP-149 binding to CPT-1 was decreased. These changes persisted with chronic treatment. CAMK binding to CPT-1 was not decreased acutely, but chronic treatment with metoprolol did produce a modest decrease in CAMK binding to CPT-1. In diabetic hearts, a different pattern is observed. AKAP-149 binding to CPT-1 was low in diabetic hearts, and metoprolol increased its binding. Metoprolol modestly decreased CAMK binding acutely in diabetic hearts. This decrease persisted and became more marked following chronic metoprolol treatment.

When PKA and CAMK were incubated with isolated mitochondria, both kinases bound to and phosphorylated CPT-1. By contrast, Akt neither bound nor
Covalent modifications of CPT-1 measured by immunoprecipitation. Samples underwent immunoprecipitation with combined pan-specific anti-phosphoserine and anti-phosphothreonine antibodies, anti-glutathione antibodies or anti-nitrosocysteine antibodies, followed by immunoblotting with pan-specific CPT-1 antibodies. Densitometric analyses of these data are presented in Figure 26. (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
<table>
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<th>CP</th>
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<td><img src="image2" alt="Image" /></td>
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<table>
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<tr>
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<tr>
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<td><img src="image23" alt="Image" /></td>
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FIGURE 26

Densitometric analysis of CPT-1 covalent modifications. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from C or D (p<0.05). (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
FIGURE 27

Co-immunoprecipitation of PKA and AKAP-149 with CPT-1, and phosphorylation state of AKAP-149. (C=control, n=5; CP = control perfused with metoprolol, n=5; CT=control treated with metoprolol, n=5; D=diabetic, n=5; DP = diabetic perfused with metoprolol, n=5; DT=diabetic treated with metoprolol, n=5).
IP: CPT-1
Blot: AKAP 149
IP: Phosphoserine/threonine
Blot: AKAP 149
IP: CPT-1
Blot: PKA

C  CP  CT

IP: CPT-1
Blot: AKAP 149
IP: Phosphoserine/threonine
Blot: AKAP 149
IP: CPT-1
Blot: PKA

D  DP  DT
FIGURE 28

Densitometric analysis of the binding of PKA and AKAP-149 to CPT-1, and phosphorylation state of AKAP-149. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from C or D, # = significantly different from C and CP or D and DP (p<0.05).
FIGURE 29

Binding of CAMK-II to CPT-1. Data represent means ± SEM. Data were analyzed using one-factor ANOVA with Neumann-Keuls post hoc test. * = significantly different from C or D, # = significantly different from C and CP or D and DP (p<0.05).
FIGURE 30

Phosphorylation of CPT-1 by PKA in isolated mitochondria. Data represent means ± SEM. Phosphorylation, kinase binding, densitometric analysis of phosphorylation, malonyl CoA dose response and catalytic activity following incubation of isolated mitochondria with PKA. Data were analyzed using student's t-test. * = significantly different (p<0.05). n=4 for each intervention.
FIGURE 31

Phosphorylation of CPT-1 by CAMK in isolated mitochondria. Data represent means ± SEM. Phosphorylation, kinase binding, densitometric analysis of phosphorylation, malonyl CoA dose response and catalytic activity following incubation of isolated mitochondria with CAMK. Data were analyzed using student's t-test. * = significantly different (p<0.05). n=4 for each intervention.
CONTROL + CAMK

IP: Phosphoserine/threonine Blot: CPT-1

IP: CPT-1 Blot: CAMK

CPT-1 Ser/Thr PHOSPHORYLATION (Rabbit Anti-1:800)

<table>
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<tr>
<td>Phosphorylation</td>
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<td>120</td>
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TREATMENT GROUP

% MAXIMUM CPT-1 ACTIVITY

MALONYL CoA CONCENTRATION (μM)

<table>
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<th>Malonyl CoA Concentration</th>
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<th>+ CAMK</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
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<tr>
<td>150</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

CPT-1 ACTIVITY (nmol/min/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ CAMK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>1.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>
FIGURE 32

Phosphorylation of CPT-1 by Akt in isolated mitochondria. Data represent means ± SEM. Phosphorylation, kinase binding, densitometric analysis of phosphorylation, malonyl CoA dose response and catalytic activity following incubation of isolated mitochondria with CAMK. Data were analyzed using student's t-test. * = significantly different (p<0.05). n=4 for each intervention.
CONTROL + Akt
IP: Phosphoserine/threonine
Blot: CPT-1
IP: CPT-1
Blot: Akt

200
180
160
140
120
100
80
60
40
20
0
CPT-1 Ser/Thr
PHOSPHORYLATION
(Arbitrary Units)

MALONYL CoA CONCENTRATION (µM)

Control + Akt
TREATMENT GROUP

% MAXIMUM CPT-1 ACTIVITY

MALONYL CoA CONCENTRATION (µM)

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CPT-1 ACTIVITY
(nmol/min/mg protein)

Control + Akt
TREATMENT GROUP
FIGURE 33

Incubation of isolated mitochondria with peroxynitrite. CPT-1 nitrosylation, glutathiolation and tyrosine nitration, and CPT-1 activity following exposure to increasing concentrations of peroxynitrite. Three separate experiments were run using the same pooled mitochondrial isolate; the n-number is therefore 1. Data represent means.
phosphorylated CPT-1, and had no effect on CPT-1 activity or sensitivity. PKA-
mediated phosphorylation of CPT-1 decreased the sensitivity of CPT-1 to
malonyl CoA without affecting activity. CAMK-mediated phosphorylation of CPT-
1 increased the sensitivity of CPT-1 to malonyl CoA without affecting activity
(Figures 30-32).

Having confirmed that functionally-significant phosphorylation of CPT-1
does occur, we undertook a study to search for the specific phosphorylation
sites. CPT-1 was eluted in the ammonium hydroxide fraction. CPT-1 was
definitively identified by peptide mass fingerprinting and Mascot search.
However, coverage of the CPT-1B sequence was poor (< 10%) and only one
phosphorylation site of interest was covered by the search. Phosphorylation of
that site was not detected.

Incubation of isolated mitochondria from control hearts with increasing
concentrations of peroxynitrite revealed that basal levels of CPT-1 nitration and
 glutathiolation were very low (Figure 33). When 0.1-1 μM peroxynitrite was
added, a marked increase in glutathiolation occurred which was associated with
very modest inhibition of CPT-1 activity. In the range 10-500 μM, glutathiolation
was sustained and then decreased while tyrosine nitration increased, and a
dose-dependent increase in CPT-1 activity was observed. By contrast, basal
levels of cysteine nitrosylation were high, and peroxynitrite produced a dose-
dependent decrease in cysteine nitrosylation. Loss of cysteine nitrosylation was
associated with an increase in CPT-1 activity. 1mM peroxynitrite was toxic to the
enzyme.

Acute metoprolol perfusion increased glutathiolation in control and diabetic
hearts, and increased S-nitrosylation in diabetic hearts only. Acute metoprolol
perfusion also decreased tyrosine nitration of CPT-1. With the exception of
tyrosine nitration in control hearts, all changes produced by acute metoprolol
perfusion were sustained with chronic treatment (Figure 25).
DISCUSSION

1: Effects of Metoprolol on Cardiac Function and Metabolism

Our current knowledge of the benefits of β-blockers in diabetic heart failure is limited to patients with concurrent diabetes and ischemia. We have demonstrated, for the first time, that metoprolol also ameliorates the cardiac dysfunction produced by diabetic cardiomyopathy. This improvement was evident both from Starling curves generated by direct left ventricular pressure measurements and from measurements of cardiac output and hydraulic power taken over the course of an hour-long perfusion at constant preload and afterload.

Having confirmed that metoprolol improves cardiac function, we investigated whether inhibition of fatty acid oxidation could account for this effect. We began by investigating the effects of metoprolol on known cardiac fuels. As expected, metoprolol had no effects on plasma glucose levels. To account for the putative ability of β-blockers to suppress lipolysis, we measured circulating plasma lipids. Metoprolol had no effect on plasma free fatty acids, triglycerides or cholesterol. Surprisingly, however, metoprolol attenuated the observed increase in plasma ketone bodies in the diabetic rats. It is not clear why metoprolol would produce such an effect.

The rate of ketogenesis is determined by the rate of fatty acid delivery to the liver, the rate of fatty acid oxidation in the liver, and the activity of mitochondrial β-hydroxy-β-methylglutaryl-CoA synthase (HMG CoA synthase), which catalyses the first step in ketogenesis. Stimulation of HMG-CoA synthase is the underlying mechanism by which low insulin levels, starvation and low-carbohydrate/low protein/high fat diets increase ketogenesis (219). β-adrenergic stimulation is known to increase lipolysis in adipocytes. The effect is mediated partly by hormone-sensitive lipase and partly by a poorly understood alternative
pathway (220). β-blockers, acting on the same pathway, may decrease lipolysis, thereby decreasing the delivery of fatty acids to the heart. The fact that metoprolol treatment had no effect on circulating plasma lipids argues against a major effect on lipolysis. However, there are two components to lipolysis: a pulsatile release under the control of the sympathetic nervous system and a basal release which is independent of the sympathetic nervous system (221); attenuation of the pulsatile release could be sufficient to reduce ketogenesis without affecting cholesterol synthesis by the liver. Alternatively, the effect could be mediated by inhibition of CPT-1 in the liver. It is also possible that metoprolol increases the peripheral utilization of ketones. However, present evidence suggests that ketone utilization by peripheral tissues is largely unregulated, being determined solely by ketone body supply (222). It is therefore difficult to postulate a mechanism by which metoprolol could directly increase the peripheral utilization of ketones. Further studies are needed to investigate the mechanism of this intriguing effect of metoprolol on plasma ketones.

We next measured ex vivo fatty acid and glucose metabolism in the heart to explore the acute and chronic effects of metoprolol. By studying the effects of chronic metoprolol treatment, we hoped to gain insights into the sustained changes in cardiac metabolism that accompany the improvement in cardiac function. By studying the rapid effects of acute metoprolol perfusion, we hoped to gain insights into positive events that could occur in vivo immediately following the commencement of treatment, preceding the improvements in function. The pattern of changes we observed in our studies was complex and depended on the disease state and the duration of metoprolol exposure.

The heavy reliance of the diabetic heart on fatty acid oxidation observed in our studies was expected and agrees with previous experimental findings in isolated perfused hearts; palmitate oxidation was markedly increased, glycolysis was decreased by 50% and glucose oxidation was negligible (27; 47; 60; 223-226). Surprisingly, however, myocardial energetics, as indicated by tissue
adenine nucleotide levels, were not altered. Chronic diabetes is known to be associated with a fall in cardiac ATP production (227). It is possible that six weeks of diabetes is too soon to observe a fall in ATP levels as this is the timepoint at which cardiac dysfunction first appears. Furthermore, we did not observe any activation of AMPK in the diabetic heart. This is consistent with previous reports (74; 228), and a recent study suggested that AMPK activation is prevented by high circulating and tissue lipids (228). However, circulating fatty acids were only mildly elevated in our studies.

Contrary to expectations, we observed that chronic metoprolol treatment increased palmitate oxidation and decreased glucose oxidation in control hearts. However, in diabetic hearts, chronic metoprolol treatment had the expected effect of lowering fatty acid oxidation and increasing glucose oxidation. Before attempting to resolve this apparent paradox, it was important to establish whether the main target of metoprolol was in the fatty acid or the glucose oxidation pathway. In a preliminary study, we found that chronic metoprolol treatment had no effect on glycolysis, but increased coupling between glucose oxidation and glycolysis in the diabetic heart by increasing glucose oxidation. To determine whether the observed changes in glucose oxidation were direct, or mediated through the Randle Cycle by direct changes in fatty acid oxidation, we repeated the perfusions in the absence of insulin to reduce glucose uptake and utilization to low levels. When this was done, the effect of metoprolol on glucose oxidation was abolished while the effect on palmitate oxidation was preserved. This strongly suggests that fatty acid oxidation is the direct target of metoprolol. It also indicates that the effect of metoprolol is independent of insulin.

Short term perfusion with metoprolol inhibited fatty acid oxidation and produced marked stimulation of glucose oxidation in both control and diabetic hearts which was associated with a decrease in lactate production, reflecting a marked improvement in glycolytic/glucose oxidation coupling, and an increase in tissue ATP levels. When the perfusions were repeated in the absence of insulin,
the effect of metoprolol on glucose oxidation was attenuated in control hearts and abolished in diabetic hearts. However, the effect on palmitate oxidation was preserved. Once again, this suggests that fatty acid oxidation is the direct target of metoprolol, and inhibition of fatty acid oxidation occurs immediately following exposure to the drug.

Intriguingly, we found that acute metoprolol perfusion and chronic metoprolol treatment lowered tissue triglyceride levels regardless of whether fatty acid oxidation was increased or decreased. This effect cannot be explained on the basis of fatty acid oxidation changes alone. Indeed, inhibition of CPT-1 by metoprolol in dogs produced an increase in tissue triglycerides (126); treatment of rats with CPT-1 inhibitors also increased tissue triglyceride levels (229). However, our tissue triglyceride measurements were carried out in hearts that had been perfused ex vivo. The heart is known to utilize its endogenous triglyceride pool over the course of an ex vivo perfusion, which may partly account for the difference. Nevertheless, inhibition of CPT-1 would be expected to decrease the utilization of fatty acids from all sources, so the decrease in tissue triglyceride levels following metoprolol treatment is unlikely to be attributable to increased triglyceride utilization. It is possible that metoprolol decreases the uptake of fatty acids into the cytoplasm. Uptake of long chain fatty acids into the cytoplasm is known to be stimulated by contraction, but the effect is likely to be mediated by AMPK (which was unaffected in our studies) and PKC isoforms (230). Triglyceride levels can be decreased by the secretion of lipoproteins by the heart itself. Indeed, overexpression of apolipoprotein B (apo B) prevents triglyceride accumulation in the diabetic heart (231; 232). However, there is presently no evidence to suggest that β-adrenoceptors regulate this process.

The β-blocker propanolol was reported to induce an increase in CPT-1 activity in normal Sprague-Dawley rats (233). Metoprolol, by contrast, was reported to decrease CPT-1 activity in conscious dogs with micro-embolism-
induced heart failure (126). In dogs with pacing-induced heart failure, glucose uptake was improved by carvedilol but not metoprolol (234). However, in clinical studies, metoprolol, carvedilol and bucindolol (122-124) have all been shown to inhibit fatty acid oxidation. The wide variation in responses reported in the literature reflects the complexity observed in our own studies in which the effect of metoprolol on fatty acid oxidation varied according to the length of exposure to the drug and the disease state.

An increase in diastolic filling increases cardiac work and oxygen consumption in direct proportion via the Frank-Starling mechanism. However, in the normal heart, ATP supply is maintained at a steady level regardless of cardiac work or oxygen consumption. This means that cardiac metabolism is driven by cardiac function (51). What is less clear, however, is how cardiac function influences cardiac energy substrate selection. It is possible that some of the beneficial effects of metoprolol on cardiac metabolism may be attributable to, rather than responsible for, its effects on cardiac function. When palmitate and glucose oxidation rates were normalized to cardiac function, the pattern of changes observed was preserved, and, in the case of palmitate oxidation, even accentuated. However, to fully account for effects of function on metabolism, future studies are needed to investigate whether the effect of metoprolol is preserved in isolated cardiomyocytes, in which the effects of cardiac function and the Frank-Starling mechanism do not apply.

To identify the second-messenger signalling pathways involved in this response, we employed both ‘top down’ (known β-adrenoceptor signalling pathways) and ‘bottom up’ (known regulatory enzymes of cardiac metabolism) approaches. We had established that metoprolol acts directly on fatty acid oxidation. In a preliminary study, we found that neither diabetes nor chronic metoprolol treatment had any effect on the activities of acyl-CoA dehydrogenase or citrate synthase. Based on these findings, and previous reports of the effects
of β-blockers on CPT-1, we investigated whether the observed effects of metoprolol on fatty acid oxidation are mediated by CPT-1.

**II: CPT-1 Activity and Regulation by Malonyl CoA**

We hypothesized that metoprolol would increase malonyl CoA levels by decreasing the phosphorylation of ACC. However, malonyl CoA levels were decreased by metoprolol in control hearts and were unchanged in diabetic hearts. The mechanism of this effect is unclear, because ACC and MCD expression were unchanged, and we found no evidence of changes in AMPK or PKA-mediated phosphorylation of ACC. Dobutamine, a non-selective β-agonist, was previously found to decrease malonyl CoA levels without an effect on AMPK, ACC or MCD (235; 236). In addition to the activities of ACC and MCD, malonyl CoA levels are also dependent on the cytosolic supply of acetyl CoA (236). Most of the acetyl CoA in the cardiomyocyte is present in the mitochondria (237), and cytosolic acetyl CoA is derived from peroxisomal β-oxidation, citrate and acetylcarnitine (238). Intriguingly, acute inhibition of CPT-1 has been shown to produce a fall in malonyl CoA levels independent of ACC and MCD (238). The fall in malonyl CoA levels observed in control hearts could, therefore, have been secondary to the inhibition of CPT-1. It is unclear why such a mechanism would only lower malonyl CoA levels in control hearts. One possibility is that fatty acid oxidation rates, and therefore the acetyl CoA/CoA ratio, are higher in the diabetic heart, and the fall in cytosolic acetyl CoA levels produced by CPT-1 inhibition in this context may not be sufficient to decrease malonyl CoA levels. Metoprolol tended to decrease tissue acetyl CoA levels in our studies, but measurements of the cytosolic and mitochondrial acetyl CoA pools would be required to confirm these speculations. Overall, however, malonyl CoA levels did not correlate with the observed changes in fatty acid oxidation. The action of metoprolol, therefore, could not be explained solely on the basis of malonyl CoA regulation.
We next investigated the effects of metoprolol on CPT-1 itself. Metoprolol decreased the maximum capacity of CPT-1 activity as measured in vitro. This effect was observed following both short-term perfusion with metoprolol and chronic metoprolol treatment, and was seen in both control and diabetic hearts. Since allosteric effects are lost during sample preparation, these effects could only be explained by a decrease in CPT-1 expression or a covalent modification. Surprisingly, metoprolol also decreased the sensitivity of CPT-1 to malonyl CoA. To our knowledge, this is the first study to demonstrate that regulation of CPT-1 sensitivity occurs in the heart. Long-term changes in CPT-1 catalytic activity and malonyl CoA sensitivity were previously believed to occur only in the liver (239; 240).

Taken together, the time and disease-dependent changes in fatty acid oxidation can be described as follows. In control hearts, acute metoprolol perfusion causes malonyl CoA levels to fall. The sensitivity of CPT-1 to malonyl CoA decreases, and the activity of CPT-1 is markedly decreased. With chronic treatment, malonyl CoA levels remain low but the sensitivity of CPT-1 to malonyl CoA is restored and the inhibition of CPT-1 activity is less marked. Fatty acid oxidation is therefore inhibited following acute exposure to the drug, but this effect is lost with time. In diabetic hearts, acute metoprolol perfusion markedly reduces CPT-1 activity. With chronic treatment, this reduction is sustained and produces inhibition of fatty acid oxidation despite a concomitant decrease in malonyl CoA sensitivity. The major determinants of the fatty acid oxidation rate are CPT-1 activity and malonyl CoA levels. Using metabolic control analysis, it has been shown that CPT-1 only becomes rate-limiting when its activity is inhibited by approximately 50% (241). Consistent with this observation, in our studies, fatty acid oxidation was always inhibited if CPT-1 activity was inhibited by approximately 50%. The observed changes in CPT-1 sensitivity would be expected to increase flux through CPT-1; however, they may represent a fine tuning mechanism of the system since at no point do they hold sway over the overall fatty acid oxidation rate.
Both CPT-1A and CPT-1B are present in the heart (242; 243). The net IC$_{50}$ of malonyl CoA in the heart is intermediate between the high sensitivity of CPT-1B and the low sensitivity of CPT-1A; in our studies, the IC$_{50}$ of control hearts was approximately 30μM malonyl CoA. Catalytic activity and malonyl CoA sensitivity could change for several reasons. Firstly, total CPT-1 expression could be altered. Secondly, isoform switching between CPT-1A and CPT-1B could alter sensitivity; the fetal heart expresses CPT-1A, and CPT-1B expression is asserted during development, eventually becoming the major isoform (243). However, CPT-1 isoform switches in the adult heart have not been reported. Finally, two splicing variants of CPT-1 have been identified in the heart which are predicted to be malonyl CoA-insensitive (244; 245).

The N- and C- termini of CPT-1 both face the cytosol, separated by a loop region inserted into the outer mitochondrial membrane which contains two membrane spanning domains. The C-terminus is the catalytic region, and residues which regulate malonyl CoA sensitivity have been found within the C-terminus, the N-terminus and the loop region (246-249). In the liver, regulation of CPT-1A sensitivity is more important than regulation of malonyl CoA levels, and has been attributed to regulation by cytoskeletal elements (250), changes in the membrane environment (251) and direct phosphorylation of CPT-1 (177). Peroxynitrite-mediated nitration of CPT-1B has been shown to decrease CPT-1B catalytic activity following endotoxemia in the heart (181). However, no other covalent modifications of CPT-1B have been identified. We therefore pursued two lines of enquiry. Firstly, we investigated the effects of chronic metoprolol treatment on CPT-1A and CPT-1B expression. Secondly, we investigated the effects of short-term metoprolol perfusion and chronic metoprolol treatment on CPT-1B covalent modifications.
III: Regulation of CPT-1 Expression

Chronic metoprolol treatment decreased total CPT-1 expression in the heart, and this was attributable to a decrease in CPT-1B expression. The decrease was only seen in diabetic hearts. CPT-1A was detected at low levels, but its expression was not altered either by diabetes or by metoprolol. The protein expression of PPAR-α and PGC1α remained unchanged, as did the expression of the PPAR-α target, PDK-4. However, when we investigated the binding of PGC1α to the transcription factors it coactivates and to USF-2, we uncovered some intriguing associative changes which suggest an explanation for the changes in CPT-1B expression.

The data obtained to date only establish associative effects. Based on these data, we propose the following model. In control hearts, USF-2 maintains a constant level of tonic repression of CPT-1 expression, and CPT-1 expression is modulated through the activation of PGC1α and PPAR-α. This produces modest changes in CPT-1 expression. Even though USF-1 and 2 expression are increased by metoprolol in control hearts, MHC expression is unaffected, indicating that USF activity is unchanged. In the diabetic heart, USF expression, and USF activity as indicated by MHC expression, are both decreased in the diabetic heart, and metoprolol increases USF expression and activity. The result is that tonic repression of PGC1α by USF-2 is lost in the diabetic heart, and restoration of USF-2 repression produces marked changes in CPT-1 expression. These effects are summarized in scheme 5. The role of USF-2 could be tested in transgenic mice with USF-2 knockout targeted to the heart, or alternatively in a conditional knockout model. Alternatively, USF-2 could be silenced using an interfering RNA approach. If USF-2 mediates repression of CPT-1 by metoprolol, the effect would be attenuated or lost following USF-2 knockout and mimicked by USF-2 overexpression. Furthermore, we would expect USF-2 knockout to be associated with an increase in CPT-1 expression. We were able to demonstrate
that both USF-2 and MEF-2A co-immunoprecipitate with PPAR-α, suggesting that PGC1α, PPAR-α, MEF-2A and USF-2 could form a single transcriptional complex.

In a recent study, Moore et al demonstrated that PGC1α/ MEF2A-dependent induction of CPT-1 was repressed by USF-2 in isolated cardiomyocytes (173). We have demonstrated that binding of USF-2 to PGC1α occurs in the heart with the native proteins, and that this is associated with functionally significant repression of CPT-1 expression. Our results suggest that binding of USF-2 can be induced by the transcriptional activation of USF-2 itself, since USF-2 binding to PGC1α always changed in the same direction as USF activity. USF is activated by increases in electrical stimulation (176). It is therefore likely that activation of USF by metoprolol is mediated by the increase in electrical stimulation that accompanies the improvement in function; this explains why the effect is only seen in the diabetic heart. Function did not change in control hearts. However, the more global regulation of the PGC1α transcriptional complex observed in our studies is not explicable solely on the basis of USF binding. The decrease in PGC1α association with PPAR-α and MEF2A could be an indirect effect of the acute changes in fatty acid metabolism. However, it is more likely that active regulation of the complex is occurring. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) increases both PGC1α/ PPAR-α coactivation and downstream signaling to PGC1α and PPAR-α targets (252-255). It has been suggested that phosphorylation by p38 MAPK may serve to integrate and coordinate contractile and metabolic gene expression (173).

Activation of β2-adrenoceptors in the heart has been shown to increase signaling through the p38 MAPK pathway (256). It is therefore possible that metoprolol decreases p38 phosphorylation by blocking β2-adrenoceptors, leading to a decrease in the association of PGC1α with its coactivators. Further
**SCHEME 5**

Proposed mechanism of action of metoprolol: chronic metoprolol treatment decreases the activation of PGC1α, possibly by preventing p38 activation, and increases the repression of PGC1α by USF-2 (abbreviations: PPAR-α: peroxisome proliferator activated receptor -α; PGC 1α: PPAR-γ coactivator 1α; MEF = myocyte enhancer factor; USF = upstream stimulatory factor, CPT-1 = carnitine palmitoyltransferase-1.)
METOPROLOL

β2 Adrenoceptor

p38

PGC1α

Δ Function

USF-2

MEF-2A

PGC1α

PPAR-α

CPT-1
studies are required to investigate the role of stress-kinase signaling in the regulation of the PGC1α transcriptional complex.

α-MHC expression is decreased as part of the fetal gene program induction. In the diabetic heart, a fall in both α-MHC and SERCA expression was observed, both of which were improved by metoprolol. This improvement in fetal gene program expression is consistent with what is known about the mechanism of action of β-blockers. α-MHC is regulated by USF’s, while SERCA has been shown to be induced by MEF-2A (257) and is proposed to be induced by PPAR-α (89). It is therefore possible that the PGC1α/PPARα/MEF2A/USF complex may be able to prevent or reverse the induction of at least some components of the fetal gene program. In other words, improvement of gene expression and modulation of cardiac metabolism could occur in parallel as a result of modulation by the same transcriptional complex. It is not clear whether DNA binding of PPAR-α or MEF-2A was altered by metoprolol treatment; further experiments are required to measure occupancy of PPRE and MEF-2A binding sites.

IV: β-Adrenoceptor Signalling Pathways: Modulation of Kinases and eNOS

Consistent with previous reports, diabetes produced a decrease in β1-adrenoceptor expression and a marked increase in β3-adrenoceptor-expression. Metoprolol increased the expression of all 3 adrenoceptor subtypes. PKA activity was decreased by both acute metoprolol perfusion and chronic metoprolol treatment, whereas PI3K activity, as indicated by Akt phosphorylation, was increased by metoprolol only following chronic treatment. CAMK activity was not significantly affected by metoprolol. There was no clear shift in β2-adrenoceptor association with Gs or Gi; association with both G-proteins was detected. These results indicate that, in the whole heart, the major acute effect of metoprolol is to decrease classical cAMP/ PKA signaling. Chronic treatment with metoprolol, in
addition, increases PI3K/ Akt signaling, and we speculate that this is primarily due to the marked increase in β3-adrenoceptor-expression.

eNOS is regulated by two main mechanisms; phosphorylation of Ser 1177, mediated by the PI3K/ Akt pathway, was shown to increase eNOS activity in transfected COS cells (258; 259); phosphorylation of Thr 495, mediated by PKA, partially blocks the phosphorylation of Ser 1177 in bovine aortic endothelial cells (260). Calcium-dependent translocation of eNOS from caveolae in the plasma membrane to calmodulin in the cytosol is also associated with an increase in eNOS activity (261; 262). Activation of eNOS by β3-adrenoceptors has been shown to be due both to an increase in Ser 1177 phosphorylation and to translocation from caveolae, but the importance of these mechanisms is region-specific; in atria, translocation is the predominant mechanism whereas, in the left ventricle, phosphorylation is the predominant mechanism (263). Intriguingly, β3-adrenoceptor stimulation has also been shown to uncouple eNOS and increase oxygen free radical formation (263).

Our efforts to measure NOS activity in the heart were unsuccessful. We therefore measured nitrate/ nitrite levels and total protein glutathiolation as biomarkers of NO and physiological reactive nitrogen species (RNS) production respectively, and correlated these with changes in the phosphorylation and expression of NOS isoforms. The pattern of changes produced for both markers was the same, with the exception of the DT group, and can be interpreted as follows. In control hearts, acute metoprolol perfusion increased NO/ RNS production by decreasing the inhibitory phosphorylation of Thr 486, a PKA-site. Stimulatory phosphorylation of Ser 1177 by Akt was also decreased, but we speculate that the decrease in PKA-mediated phosphorylation exerted a greater effect on activity. Following chronic treatment with metoprolol in control hearts, NO production remained high despite a surprising decrease in eNOS expression and a loss of any effect on eNOS phosphorylation. We speculate that this
increase in activity could be due to increased eNOS translocation from caveolae to the cytosol.

In the diabetic heart, NO production is reduced and is dependent on iNOS rather than eNOS. iNOS is not regulated by β-adrenoceptors acutely, so acute perfusion with metoprolol has no effect on NO production. Chronic metoprolol treatment prevented the induction of iNOS without restoring eNOS expression. The net result was that chronic treatment with metoprolol had no effect on NO production. However, as indicated by the fall in glutathiolation, prevention of iNOS induction by chronic metoprolol treatment did decrease RNS production. The changes produced by metoprolol on the phosphorylation of eNOS by Akt did not correlate with the changes in Akt-phosphorylation produced by the same treatment; this suggests that the elevated Akt signal was compartmentalized, and that eNOS was not its primary target. It is important to note that several cell types would have been present in the whole heart homogenate, including cardiomyocytes and endothelial cells; we did not differentiate between endothelial and cardiomyocyte NO signaling in our studies.

When tyrosine nitration, a marker of peroxynitrite, was measured, levels of total protein tyrosine nitration remained constant as long as either eNOS or iNOS were present. When eNOS expression was low and iNOS was absent, total protein tyrosine nitration fell. These data indicate that nitrosative stress was not significantly increased in diabetic hearts, although eNOS expression was low and NO levels had fallen. The fact that prevention of iNOS induction had a marked effect on RNS production but no effect on NO production suggests that iNOS was producing predominantly RNS. As discussed above, NO has been reported to inhibit glucose utilisation predominantly through inhibition of glycolysis (264). However, chronic metoprolol treatment had no effect on glycolysis in control hearts despite the fact that it increased NO production.
In recent years, there has been increasing interest in the ability of NO and its associated RNS to directly regulate protein function in a similar manner to phosphorylation. Residues that are targeted by NO and RNS are cysteine, methionine and tyrosine (265; 266). The unique redox chemistry of protein thiol groups confers specificity and reversibility to thiol covalent modifications. The attachment of NO to thiol groups on critical cysteine residues within a protein, termed S-nitrosylation, is a major mechanism by which NO acts as a signaling molecule. Intriguingly, there is a consensus sequence, analogous to kinase consensus sequences, which confers site specificity on NO-mediated thiol modifications (267). Furthermore, S-nitrosylation is a reversible reaction, and a number of enzymatic and non-enzymatic reactions have been identified which can remove NO from cysteine thiols (268-270). S-nitrosylation activates guanylate cyclase, the classical NO target. The list of targets proposed to be regulated by S-nitrosylation is growing, and, in the heart, includes GAPDH and SERCA (271).

Reversible oxidation or nitrosation of thiol groups is mediated by physiological levels of NO and RNS, and typically produces the following reversible modifications: S-nitrosylation (addition of NO), glutathiolation (formation of mixed disulphides between the thiol group and glutathione) or oxidation from thiol to sulfenate. Any of these modifications can regulate protein function, but glutathiolation and S-nitrosylation have been most frequently implicated in the regulation of enzyme activity (265). Higher levels of RNS induce further oxidation of the sulfenate (one oxygen) to sulfinate (two oxygens) and sulfonate (three oxygens). This is toxic, causing irreversible loss of function. Glutathiolation, by committing the thiol to an alternate reaction pathway, protects critical thiol residues against irreversible oxidation (265). These effects are summarized in scheme 7.
Tyrosine nitration is classically regarded as an inhibitory modification. However, some proteins are activated by tyrosine nitration including cytochrome C, fibrinogen and PKC (272-275). As with thiol-modification, tyrosine nitration also exhibits site-specificity (276). Tyrosine nitration is frequently used as a biomarker of peroxynitrite (272), and we also used it as such. Previous studies have demonstrated that incubation of CPT-1 with continuous peroxynitrite, NO or hydrogen peroxide producing systems produces a decrease in CPT-1 activity which is associated with tyrosine nitration (182). Furthermore, endotoxemia produced inhibition and nitration of CPT-1 in suckling rats (181).

Cysteine-scanning mutagenesis of CPT-1 revealed that cysteine 305 is critical for catalytic activity of the enzyme (277). We therefore tested whether nitrosylation or glutathiolation of cysteine residues, or nitration of tyrosine residues, inhibits CPT-1 activity. To test the effects of the modifications per se on CPT-1 activity, we incubated isolated mitochondria with increasing concentrations of peroxynitrite ranging from 100 nM to 1 mM. At neutral pH, peroxynitrite is rapidly degraded, but even brief exposure to peroxynitrite is sufficient for it to induce the full range of its target modifications. Because of the large amount of mitochondrial isolate required for these measurements, three duplicate experiments were run on samples taken from a single mitochondrial pool, meaning that the n-number was 1. Although the results were consistent, further experiments are required to enable a statistical analysis to be carried out.

Peroxynitrite induced glutathiolation at a lower concentration than tyrosine nitration, and caused a decrease in S-nitrosylation. Dose-dependent loss of S-nitrosylation and gain of tyrosine nitration was associated with a dose-dependent increase in CPT-1 activity. This suggests that s-nitrosylation is inhibitory and tyrosine nitration stimulatory of CPT-1 activity. 100 nM peroxynitrite produced a marked increase in glutathiolation but only a slight decrease in CPT-1 activity which did not prevent activation of the enzyme by higher concentrations. We therefore speculate that glutathiolation of CPT-1 serves as a protective
SCHEME 6

NO and RNS-mediated modifications of thiol residues. Thiol (SH) residues undergo a series of reversible modifications in response to changes in the redox potential or exposure to physiological levels of reactive nitrogen species or nitric oxide. Oxidation of the thiol to the corresponding sulphenide or the formation of a disulphide bond between the thiol and glutathione (glutathiolation) are reversible either by changes in the equilibrium, or enzymatic restoration of the thiol group by thiol transferases. Further oxidation of a glutathiolated residue is not possible, so glutathiolation confers protection against oxidative damage for as long as it persists. However, exposure of the thiol group or the sulphenide to pathological levels of reactive nitrogen or oxygen species results in the formation of sulfinate and then sulfonate; these are irreversible modifications which result in protein damage and loss of activity. Modified from Figure 2, Klatt and Lamas, 2000 (265).
Glutathiolation

R-SSG

R-SNO ↔ R-SH ↔ R-SOH

S-Nitrosylation Unmodified Oxidation to
Thiol Group Sulfinide

R-SO$_2$H ➔ R-SO$_3$H

Oxidation to Oxidation to
Sulfinate Sulfonate

Physiological RNS Levels Pathological RNS Levels
Signal Transduction Protein damage
Protection Against Irreversible Oxidation Irreversible Loss of Activity
mechanism against sulfonation, whereas S-nitrosylation and tyrosine nitration regulate the activity in opposite directions. Even a concentration of 500 μM peroxynitrite, which can be toxic to some enzymes, stimulated CPT-1 activity, indicating that this enzyme is well-protected against oxidative damage.

It was surprising, though convenient, that peroxynitrite decreased S-nitrosylation of CPT-1. As discussed above, increasing concentrations of peroxynitrite promote the glutathiolation of nitrosylated thiol groups, and this is the most likely mechanism of the decrease we observed. The possibility that tyrosine nitration of CPT-1 could be stimulatory is surprising, considering that nitration of CPT-1 was associated with inhibition of activity following endotoxemia (181). However, the authors of that study did not measure cysteine oxidation, so it is possible that sulfination or sulfonation, rather than tyrosine nitration, produced inhibition of CPT-1 activity. In our studies, tyrosine nitration of CPT-1 appeared following physiological doses of peroxynitrite, whereas loss of activity did not appear at 1mM. It is noteworthy that one or more of these covalent modifications was present in every treatment group, and at every peroxynitrite concentration. It is therefore possible that all the modifications were inhibitory, but to different degrees. This possibility could be tested by incubating isolated mitochondria with a reducing agent such as dithiothreitol and assaying CPT-1 activity. Although we did not measure cysteine sulfination or sulfonation, it is likely that this kind of severe oxidation, rather than tyrosine nitration, caused the loss of CPT-1 activity observed at 1 mM peroxynitrite. Similarly, incubation with NO and peroxynitrite-producing systems would produce continuous nitrosylation and, in the latter case, possibly more severe oxidation which a single brief exposure to peroxynitrite would not produce; this could explain why continuous NO or peroxynitrite exposure is always inhibitory whereas a single brief exposure to peroxynitrite produces a more complex dose-response. The physiological relevance of both types of response would depend on the temporal regulation of CPT-1 exposure to NO and RNS.
We successfully detected cysteine-nitrosylation, glutathiolation and nitration of CPT-1 in whole heart homogenates. Acute metoprolol perfusion increased nitrosylation and glutathiolation in diabetic hearts, but decreased tyrosine nitration in both control and diabetic hearts. In control hearts, nitrosylation was low and glutathiolation increased only following chronic treatment. Taking into account the fact that nitrosylation appears to be inhibitory, and nitration stimulatory, these data suggest that metoprolol acutely inhibits CPT-1 activity by increasing cysteine nitrosylation and removing tyrosine nitration of CPT-1. However, the mechanism of these effects is not explicable on the basis of cell-wide changes in NO and RNS production, because the observed patterns in systemic NO/RNS and CPT-1 covalent modifications did not match. There is a mitochondrial isoform of NOS (mtNOS), but NO and peroxynitrite produced by mtNOS affect targets within the mitochondrial matrix and the inner mitochondrial membrane (278). CPT-1 predominantly faces the cytosol, so it is likely that regulation of CPT-1 by NO/ RNS is mediated by eNOS and possibly iNOS. eNOS has been proposed to translocate to the mitochondria (279; 280); mitochondrial eNOS translocation could therefore be a major determinant of NO/ RNS mediated effects on CPT-1.

VI: Phosphorylation of CPT-1

Both acute metoprolol perfusion and chronic metoprolol treatment increased the total phosphorylation state of CPT-1. However, when PKA and CAMK-II-interactions with CPT-1 were examined in greater detail, an intriguing pattern emerged. Firstly, we found, for the first time, that PKA and CAMK-II physically associate with CPT-1. Furthermore, we also found that AKAP-149 also physically associates with CPT-1 and appears to mediate PKA binding. AKAP’s bind the regulatory subunit of PKA, and activation of PKA occurs following release of the catalytic subunit. The PKA antibodies we used in our studies recognize the catalytic subunit of PKA. Phosphorylation of AKAP-149 was always associated with a decrease in AKAP-149/ CPT-1 association and an increase in
PKA/ CPT-1 binding. Conversely, loss of AKAP-149 phosphorylation was always associated with an increase AKAP-149/ CPT-1 binding and a decrease in PKA/ CPT-1 binding. Based on these findings, we propose the following scheme. PKA is targeted to the mitochondria by AKAP-149. Phosphorylation of AKAP-149 by PKA causes AKAP-149 to disassociate from CPT-1, enabling PKA to bind and phosphorylate CPT-1. CAMK-II binding followed a different pattern, indicating that it is regulated by another, as-yet unidentified mechanism. There are other mitochondrial AKAPs which could mediate similar effects as AKAP-149: for example, AKAP-121 has also been shown to target PKA to mitochondria (281). CAMK association proteins (KAPs), by contrast, are not localized to mitochondria, but are found only in the sarcoplasmic reticulum and the nucleus (282). It is therefore unclear how CAMK-II binding to CPT-1 would be mediated. It is conceivable that CAMK-II binds calmodulin associated with other proteins which translocate to the mitochondria. For example, CAMK-II is known to associate with eNOS (283). Alternatively, there may be mitochondrial CAMK-II association proteins which have not been identified.

Phosphorylation of CPT-1B has never been reported, and we speculate that this is because the kinases involved require other mediators such as AKAP-149 to be present in order to bind their targets. This was our rationale for using isolated mitochondria rather than purified enzyme preparations which are usually used for investigating enzyme phosphorylation. When PKA was incubated with isolated mitochondria, it bound and phosphorylated CPT-1; the functional effect was a decrease in CPT-1 sensitivity without any effect on catalytic activity. When CAMK-II was incubated with isolated mitochondria, it also bound and phosphorylated CPT-1; however, the functional effect in this case was an increase in CPT-1 sensitivity without any effect on catalytic activity. By contrast, Akt did not bind or phosphorylate CPT-1, and had no effect on the activity or sensitivity of the enzyme.
The effects of metoprolol, and the basal state of the system, differed between control and diabetic hearts. In control hearts, acute metoprolol perfusion and chronic metoprolol treatment increased PKA binding to CPT-1. Chronic metoprolol treatment also modestly decreased CAMK-II binding to CPT-1. In diabetic hearts, PKA-binding to CPT-1 was decreased by metoprolol, but CAMK-II binding was also decreased, and, following chronic treatment, the decrease in CAMK-II binding was marked. Taken together with our findings in isolated mitochondria, these data explain the sensitivity changes produced by metoprolol; Metoprolol increases PKA-mediated desensitization in control hearts, and decreases CAMK-II-mediated sensitization in diabetic hearts. It is noteworthy that the binding of PKA and CAMK-II to CPT-1 bears no relation to the overall activities of these kinases measured in the whole heart. It is the translocation of the kinases to the mitochondria which is crucial. The mechanisms by which β-adrenoceptors might regulate such a translocation process are unknown and require further investigation.

Having confirmed that functionally significant phosphorylation of CPT-1 occurs, we attempted to identify specific phosphorylation sites on CPT-1. Consensus sites were identified for PKA, CAMK I and CAMK II, so we proceeded to use LC MS MS to examine phosphorylation of these sites. Identifying phosphorylation events is challenging due to the labile nature of the modification and the overwhelming number of peptides generated by tryptic digestion. In order to maximize our chances of finding the phosphorylation sites, we performed two purification steps. Firstly, we purified CPT-1 by immunoprecipitation. Secondly, we performed phosphorylation enrichment on the tryptic digest. CPT-1 was always found in the highly phosphorylated fraction. However, this was due to the high concentration of acidic residues in the peptides; peptides rich in acidic residues are also retained by the titanium tips. Although coverage of CPT-1 was sufficient to identify the presence of the protein, it was not sufficient to examine the phosphorylation sites of interest; all the sites except one were missed. Several factors may account for this. CPT-1 abundance in the whole-cell
homogenate may have been too low. Also, there were relatively few trypsin cutting sites on CPT-1. Many of the CPT-1 peptides could have been too large to be eluted from the column during the LC MS MS procedure (peptides over 2500 Da in mass are retained). The yield of CPT-1 could be improved by using mitochondrial fractions, and coverage improved by the use of an alternative digestion enzyme. In addition, the chances of detecting a phosphorylation event would be greater if mitochondrial preparations which have been incubated with different kinases are used because the intensity of the signal is increased and the time between the phosphorylation event and sample collection can be controlled more easily.

**VII: Significance of the Present Studies**

These studies have unraveled several mechanisms by which metoprolol can modulate fatty acid oxidation in the heart. Metoprolol is able to decrease malonyl CoA levels in control hearts independent of ACC and MCD; this effect may be related to cytosolic acetyl CoA availability. Acutely, metoprolol decreases CPT-1 activity by increasing S-nitrosylation and decreasing tyrosine nitration, and decreases CPT-1 malonyl CoA sensitivity by increasing PKA-mediated or decreasing CAMK-ll-mediated phosphorylation. Following chronic treatment, these covalent modifications are sustained, but CPT-1 expression is also decreased. In control hearts, metoprolol decreases CPT-1 expression by decreasing the association of PGC1α with its coactivators. In diabetic hearts, metoprolol decreases CPT-1 expression by increasing the binding of the repressor USF-2 to PGC1α. The increase in USF-2 binding is likely to be produced by the increase in electrical stimulation produced by the improvement in function, whereas the decrease in PGC1α binding to its coactivators might be related to p38 phosphorylation. We did not investigate p38 phosphorylation in the present study so future studies need to investigate this mechanism. When combined, these mechanisms result in a complex pattern of metabolic
modulation which is dependent on the duration of exposure to metoprolol and to the disease state.

Changes in CPT-1 covalent modifications did not correlate with tissue activity measurements and levels of the second messengers which produced them. A separate CPT-1 associated microdomain, consisting of AKAP-149, PKA, CAMK-II and possibly eNOS, could exist, and covalent modifications of CPT-1 may be regulated by translocation of the relevant kinases and eNOS into the microdomain. This CPT-1 microdomain is likely to contain other components. It is well established that ACC and MCD localize close to CPT-1. Recent studies suggest that the fatty acid transporter CD36 translocates from the plasma membrane to the mitochondria where it associates with CPT-1 (284). A picture is therefore emerging of a secondary mechanism for fatty acid oxidation control which can, as in the case of metoprolol treatment, be unmasked and produce meaningful changes in fatty acid oxidation rates.

We chose to investigate metoprolol because it had previously been found to inhibit fatty acid oxidation, is a clinically useful drug and because its known range of actions is narrower than that of carvedilol. Several other β-blockers have been reported to have effects on metabolism. However, several key questions remain to be answered. Is this effect a class effect or is it mediated only by certain β-blockers? Are the effects mediated by β-adrenoceptors, and if so, what is the contribution of each receptor? Comparative studies of a wider range of β-blockers, as well as studies in which β-adrenoceptor expression is silenced, must be carried out in order to answer these questions.

There has been considerable interest in the ability of β-blockers to act as antioxidants. Propanolol, pindolol, metoprolol, atenolol and sotalol were all found to inhibit membrane peroxidation, and the effect was attributed to chemical rather than pharmacological properties (285-287). Recently, carvedilol has been shown to be a potent antioxidant (288). In a recent study, the scavenging activities of a
range of β-blockers (atenolol, labetalol, metoprolol, pindolol, propanolol, sotalol, timolol and carvedilol) against reactive oxygen and nitrogen species were compared (289). In all cases, effective scavenging required higher concentrations than are achieved clinically. None of the β-blockers could scavenge oxygen free radicals, but all could scavenge peroxynitrite. Metoprolol exhibited weak antioxidant effects in this study; the IC$_{50}$ of metoprolol on peroxynitrite was greater than 5 mM. By contrast, the beneficial effects observed in our study were achieved at μM levels of the drug. It is therefore unlikely that direct antioxidant effects of metoprolol played a significant role in our studies.

A major limitation of the present studies was that the observed changes in CPT-1 and the factors that may regulate it were occurring simultaneously under the various treatment conditions, and this makes it challenging to disentangle the mechanisms which are of true regulatory importance. We attempted to rationalize which changes in CPT-1 activity, sensitivity and malonyl CoA levels were of greater importance based on the measured rates of fatty acid oxidation. The patterns of activation of nitric oxide synthases, PKA and CAMK were different from the patterns of phosphorylation, nitrosylation, glutathiolation and nitration observed for CPT-1. We hypothesized that translocation of kinases and eNOS to the mitochondria may be a more important determinant of CPT-1 post-translational modifications. Further studies are required to clarify this using eNOS knockout mice or pharmacological inhibitors of the relevant kinases. It will also be essential to determine whether the observed changes in CPT-1 activity and sensitivity produced in vitro by PKA, CAMK and peroxynitrite also occur following metoprolol treatment in vivo. To this end, it will be important to measure the post-translational modifications on CPT-1 directly using mass spectroscopy.

We have shown that metoprolol improves cardiac function in diabetic cardiomyopathy, raising the question as to whether the drug should be used earlier in diabetic patients. However, there are a number of concerns with the administration of β-blockers to diabetic patients which need to be weighed
against the benefits of introducing the drug so early. First and foremost are concerns about the effects of β-blockers on glycemic control. Coadministration of a β-blocker with a thiazide has been reported to worsen glycemic control since the 1980's when the effects of propanolol and hydrochlorothiazide were reported (290). Recently, however, the use of β-blockers as antihypertensive agents has been associated with an increased risk of new-onset diabetes, leading to concern about their use in this context (291). Hepatic glucose output is controlled by the β2 adrenoceptor, and blockade of this receptor, which does occur with the β1 selective agents, decreases hepatic glucose output and delays recovery from hypoglycemia (292; 293). Blockade of the symptoms of hypoglycemia by β-blockade is no longer considered to be a problem, because the symptoms of sweating and paresthesias are preserved, and patients can be educated to recognize these signs (293; 294).

Another concern with chronic β-blockade is the presence of sustained unopposed α1-adrenoceptor stimulation. This is problematic in two situations. Firstly, activation of the sympathetic nervous system by hypoglycemia increases unopposed α1-adrenoceptor stimulation to the point where a hypertensive crisis can be precipitated (293). Secondly, unopposed α1-adrenoceptor stimulation produces peripheral vasoconstriction which could worsen peripheral vascular disease and, by decreasing muscle flow, increase insulin resistance (121; 295). Indeed, use of β-blockers in diabetic patients increases glucose levels and triglycerides and lowers high density lipoprotein cholesterol levels by decreasing insulin sensitivity (121). None of these concerns are considered great enough to deny β-blockers to patients with systolic heart failure because these drugs are lifesaving in this context. However, the risks and benefits of earlier β-blocker use will need to be weighed carefully and no evidence currently exists on which to base these considerations.
We studied diabetic cardiomyopathy in a model of type 1 diabetes. This was a useful model in which to identify potential mechanisms of action, and a model which develops the cardiomyopathy quickly was needed in order to widen the scope of the study. However, future studies should examine whether the beneficial effects of metoprolol are also observed in models of type 2 diabetes.

In conclusion, our studies demonstrate that metoprolol is a fatty acid oxidation inhibitor which ameliorates the cardiac dysfunction of diabetic cardiomyopathy. A role for β-blocker therapy earlier in this condition may be considered, but careful study and consideration of the risks and benefits will be required before such a recommendation can be made.

**VIII: CONCLUSIONS**

1. Chronic metoprolol treatment improves cardiac function in the diabetic heart by inhibiting fatty acid oxidation and, through the Randle cycle, increasing glucose oxidation.

2. In control hearts, chronic metoprolol treatment increases fatty acid oxidation and decreases glucose oxidation.

3. Chronic metoprolol treatment selectively decreases the expression of CPT-1B by decreasing the co-activation and increasing USF-2 mediated repression of PGC1α.

4. Metoprolol decreases malonyl CoA levels independent of ACC and MCD in control hearts only.

5. CPT-1 undergoes S-nitrosylation by NO and glutathiolation and tyrosine nitration by peroxynitrite. CPT-1 activity is inhibited by S-nitrosylation and glutathiolation, and stimulated by tyrosine nitration.
6. Acute metoprolol perfusion decreases the activity of CPT-1 by increasing CPT-1 S-nitrosylation and glutathiolation, and decreasing CPT-1 tyrosine nitration. With the exception of tyrosine nitration in control hearts, these changes persist with chronic treatment.

7. CPT-1 is phosphorylated by PKA, which decreases malonyl CoA sensitivity, and CAMK-II, which increases CPT-1 sensitivity. PKA-phosphorylation of CPT-1 is mediated by AKAP-149.

8. Acute metoprolol perfusion decreases the sensitivity of CPT-1 to malonyl CoA by increasing PKA-mediated phosphorylation of CPT-1 and decreasing CAMK-mediated phosphorylation of CPT-1. The changes persist with chronic treatment.
SCHEME 7

Summary of the acute effects of metoprolol on malonyl CoA levels, CPT-1 malonyl CoA sensitivity and CPT-1 activity. Metoprolol lowers malonyl CoA levels in control hearts only. Metoprolol decreases CPT-1 catalytic activity in both control and diabetic groups by increasing S-nitrosylation and glutathiolation of CPT-1 and reducing tyrosine nitration of CPT-1. Metoprolol decreases the sensitivity of CPT-1 to malonyl CoA by decreasing CAMK-II mediated phosphorylation of CPT-1 and, in control hearts, by increasing PKA-mediated phosphorylation of CPT-1 (abbreviations: PKA = protein kinase A, CAMK = calcium/calmodulin-dependent protein kinase, CPT-1 = carnitine palmitoyltransferase-1).
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