THE MYOCARDIAL METABOLIC AND HAEMODYNAMIC EFFECTS OF PERHEXILINE IN *IN VIVO* AND *IN VITRO* MODELS

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TABLE OF CONTENTS

Table of Contents ii

Thesis Summary vii

Declaration ix

Acknowledgments x

CHAPTER 1: A LITERATURE REVIEW OF METABOLIC APPROACHES TO MYOCARDIAL ISCHAEMIA 1

1.1 INTRODUCTION 2

1.2 CHRONIC MYOCARDIAL ISCHAEMIA 3

1.2.1 Epidemiology 3

1.2.2 Pathogenesis 4

1.3 CARDIAC METABOLISM 7

1.3.1 Overview of fatty acid metabolism 8

1.3.1.1 Uptake and transport within the aqueous cytoplasm 8

1.3.1.2 Transport into mitochondria 9

1.3.1.3 β-oxidation 10

1.3.1.4 TCA cycle and electron transport chain 10

1.3.2 Regulation of fatty acid metabolism 11

1.3.3 Overview of carbohydrate metabolism 13

1.3.4 Regulation of carbohydrate metabolism 14

1.3.5 Substrate utilisation by the heart: the cost in oxygen 16

1.3.6 Myocardial metabolism during ischaemia/reperfusion 18

1.3.7 The role of fatty acid metabolites 22

1.4 MANAGEMENT OF CHRONIC MYOCARDIAL ISCHAEMIA 24

1.4.1 Medical therapy 24

1.4.1.1 Nitrates 24

1.4.1.2 β-adrenoceptor antagonists 25

1.4.1.3 L-type calcium antagonists 25
1.4.2 Revascularisation 26
1.4.2.1 Percutaneous transluminal coronary angioplasty 26
1.4.2.2 Coronary artery bypass surgery 28
1.4.2.3 Transmyocardial laser revascularisation 29
1.4.3 Limitations of current strategies 30
1.5 METABOLIC APPROACHES TO MYOCARDIAL ISCHAEMIA 32
1.5.1 Increasing glucose supply to the heart 35
1.5.1.1 Glucose-insulin-potassium (GIK) 35
1.5.2 Decreasing fatty acid supply to the heart 38
1.5.2.1 Antilypolytic agents 38
1.5.2.2 β-adrenoceptor antagonists 38
1.5.3 Stimulation of glucose oxidation 39
1.5.3.1 Dichloroacetate 39
1.5.3.2 L-carnitine 40
1.5.4 Inhibition of fatty acid oxidation 41
1.5.4.1 CPT-1 inhibitors 41
1.5.4.2 Thiazolidinediones 44
1.5.4.3 Piperazine derivatives 45
1.5.5 Other pharmacologic approaches 48
1.5.5.1 Coenzyme Q_{10} 48
1.6 PERHEXILINE 49
1.6.1 Haemodynamic effects 50
1.6.1.1 Animal studies 50
1.6.1.2 Human studies 52
1.6.2 Clinical efficacy 54
1.6.3 Toxicity 56
1.6.3.1 Short-term toxicity 56
1.6.3.2 Interactions with hypoglycaemic agents 56
1.6.3.3 Long-term toxicity 57
1.6.3.4 Torsade de pointes 59
1.6.4 Pharmacokinetics 59

1.6.5 Circumvention of toxicity 61

1.6.6 Mechanisms of therapeutic action 62

1.6.7 The role of perhexiline in the 21st century 64

1.7 EXPERIMENTAL TECHNIQUES USED TO MEASURE CARDIAC ENERGY METABOLISM 66

1.7.1 Coronary sinus catheterisation 66

1.7.2 Biochemical tissue analysis 68

1.7.3 Nuclear magnetic resonance spectroscopy 69

1.7.4 ^{14}C- and ^{3}H- labelled substrates 71

1.7.5 Positron emission tomography 73

1.7.6 Nuclear imaging with radioiodinated fatty acids 76

1.8 SCOPE OF THE CURRENT STUDY 79

CHAPTER 2: THE HAEMODYNAMIC EFFECTS OF PERHEXILINE IN CONSCIOUS SHEEP 93

2.1 INTRODUCTION 94

2.2 METHODOLOGY 95

2.2.1 Animals and materials 95

2.2.2 Preliminary experiments using bolus doses of perhexiline 96

2.2.3 Set-up of chronically instrumented sheep model 97

2.2.4 Haemodynamic measurements 99

2.2.5 Perhexiline assay and estimation of myocardial drug content 102

2.2.6 Experimental protocol 104

2.2.7 Statistical analysis 105

2.3 RESULTS 106

2.3.1 Bolus perhexiline administration (preliminary experiments) 106

2.3.2 Perhexiline infusions 107

2.3.2.1 Perhexiline pharmacokinetics 107

2.3.2.2 Haemodynamic effects 108

2.3.2.3 Other effects 110

2.4 DISCUSSION 111

CHAPTER 3: THE EFFECT OF PERHEXILINE ON FATTY ACID OXIDATION RATES IN SHEEP 126

3.1 INTRODUCTION 127

3.2 METHODOLOGY 128

3.2.1 Animals and materials 128

3.2.2 Preliminary experiments 129

3.2.3 Preparation of IPPA 130

3.2.4 Experimental protocols 131

3.2.5 Imaging and data analysis 133

3.2.6 Statistical analysis 135

3.3 RESULTS 135

3.3.1 Short-term infusions 135

3.3.2 24 hour infusions 136

3.4 DISCUSSION 137

CHAPTER 4: THE EFFECTS OF PERHEXILINE ON CARDIAC METABOLISM, FUNCTION, AND EFFICIENCY IN THE ISOLATED WORKING RAT HEART 147

4.1 INTRODUCTION 148

4.2 METHODOLOGY 149

4.2.1 Background 149

4.2.1.1 Working rat heart models 148

4.2.1.2 Ischaemia/reperfusion models 154

4.2.1.3 Measurement of metabolism in the working rat heart 157

4.2.2 Preliminary experiments: problems and solutions 159

4.2.2.1 Pressure build-up in the closed system 159

4.2.2.2 BSA foaming 161

4.2.2.3 Bacterial contamination 161

4.2.2.4 Selection of albumin source 162

4.2.2.5 Ischaemia 162

4.2.2.6 Chronic dosing of rats with perhexiline 163

4.2.3 Experimental procedures 166

4.2.3.1 Animals and materials 166
4.2.3.2 Preparation of perfusate 167
4.2.3.3 Set-up of apparatus 170
4.2.3.4 Working heart perfusions 174
4.2.3.5 Haemodynamic measurements 177
4.2.3.6 Measurement of palmitate and glucose oxidation 178
4.2.3.7 Biochemical tissue analysis 180
4.2.3.8 Statistical analysis 185
4.3 RESULTS 185
4.3.1 Acute perhexiline exposure 185
4.3.1.1 Haemodynamics 186
4.3.1.2 Metabolic effects 187
4.3.2 24 Hour perhexiline exposure 189
4.3.2.1 Haemodynamics 190
4.3.2.2 Metabolic effects 191
4.4 DISCUSSION 193
4.4.1 Summary of results 193
4.4.2 Comparison with published studies using the working rat heart 195
4.4.3 Establishment of an ex vivo model 199
4.4.4 Changes in metabolic efficiency 203

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS 247
5.1 SUMMARY: MAJOR EXPERIMENTAL FINDINGS 248
5.2 ISSUES ARISING FROM MAJOR EXPERIMENTAL FINDINGS 249
5.3 FUTURE EXPERIMENTS 255

APPENDIX 260

BIBLIOGRAPHY 261

SUMMARY

Perhexiline is an effective anti-anginal agent without clinically significant haemodynamic effects which is postulated to have a primary "metabolic" mechanism of action. Recent in vitro work in isolated cardiac mitochondria suggested that inhibition of the key enzyme in the regulation of fatty acid catabolism, carnitine palmitoyltransferase (CPT-1), may play a key role in the action of perhexiline. The experiments described in this thesis are primarily aimed at investigating the postulated metabolic effects of perhexiline in the heart, and correlating these to changes in haemodynamics. Secondary aims are the assessment of the time-dependence of such changes, and the investigation of haemodynamic changes induced by parenteral perhexiline in a conscious animal model.

Haemodynamic effects of parenteral perhexiline

An experimental model of chronically catheterised sheep enabled the monitoring of haemodynamics during and after intravenous perhexiline administration in conscious animals. The major haemodynamic effects of brief intravenous infusions of perhexiline were vasoconstriction, followed by bradycardia. There was no evidence of negative inotropy to suggest any significant calcium channel antagonism at the dosages used.

Metabolic effects of perhexiline in vivo

Nuclear medicine imaging of the heart following injection of a radio-iodinated fatty acid (IPPA) enabled assessment of the effects of parenteral perhexiline administration

on myocardial fatty acid utilisation in an *in vivo* animal model. The use of a known CPT-1 inhibitor, etomoxir, resulted in a significant delay in myocardial clearance of IPPA, indicating inhibition of beta oxidation. However no significant changes in the uptake or clearance kinetics of IPPA were seen following either short-term or long-term (24 hours) perhexiline infusions.

Metabolic effects of perhexiline in vitro

The working rat heart model was used to assess both substrate utilisation and haemodynamics in response to perhexiline and the known CPT-1 inhibitors, etomoxir and oxfenicine. Although there were no significant effects of acute in vitro perhexiline exposure on myocardial energetics of efficiency, ex vivo experiments following 24 hours of transdermal perhexiline administration demonstrated an increase in cardiac work performed per unit of fatty acid consumption, associated with improved myocardial efficiency.

In conclusion, perhexiline's effects on cardiac metabolism are not as striking as those of oxfenicine *in vitro* and etomoxir *in vivo*. However it appears that any such effects of perhexiline are related to total duration of perhexiline exposure, and further work is required in more suitable models of chronic perhexiline administration.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopy.

Steven Unger

(September 2000)

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CHAPTER 1:

A LITERATURE REVIEW OF METABOLIC APPROACHES TO MYOCARDIAL ISCHAEMIA

1.1 INTRODUCTION

An imbalance between myocardial energy demands and coronary flow will give rise to the metabolic, electrophysiologic, and mechanical aberrations that culminate in ischaemia. The term "myocardial ischaemia" describes a condition that exists when the oxygen supply is not sufficient to meet the rate of mitochondrial oxidation (Ferrari, 1999). As a consequence, changes in metabolism and function occur, and lack of adequate wash-out causes abnormal accumulation of ions and metabolites. Once mitochondrial impairment is established, a series of typical metabolic alterations, such as intracellular acidosis, anaerobic metabolism and reduced production of high energy phosphates, occur (Ferrari, 1999). These all result in haemodynamic impairment, and may lead to arrhythmias or tissue necrosis. Ischaemia that lasts for a short period usually causes the clinical condition known as angina pectoris. When the ischaemia is prolonged enough to cause irreversible myocardial damage, the result is myocardial infarction.

Treatment of ischaemic heart disease has traditionally been aimed at either increasing oxygen supply to the heart, or reducing myocardial oxygen demand. Less attention has been given to directly addressing the metabolic derangements which occur during ischaemia. However there is a growing body of literature which suggests that a primary "metabolic approach" aimed at maintaining the metabolic capacities of the myocytes, has enormous potential in the treatment of myocardial ischaemia.

In this chapter, the epidemiology and pathogenesis of ischaemic heart disease are briefly presented, followed by a detailed review of cardiac metabolism during normal

flow and ischaemia. Current management options of chronic myocardial ischaemia and their potential inadequacies are discussed, followed by a review of alternate "metabolic" approaches.

Finally the history of the drug perhexiline, including a review of its clinical track record, toxicity, and potential clinical applicability in the 21st century, is presented in detail. Although the precise mechanism of action of this agent remains unclear, the available literature suggests a primary metabolic anti-ischaemic effect. The scope of this thesis is to further investigate the metabolic and haemodynamic effects of perhexiline both in *in vivo* and *in vitro* models.

1.2 CHRONIC MYOCARDIAL ISCHAEMIA

1.2.1 Epidemiology

The prevalence of angina pectoris appears roughly similar across most Caucasian populations (Cleland, 1996), with an overall prevalence in the population over 30 years of age of approximately 2.4% (Gandhi et al. 1992). The prevalence and severity of ischaemic heart disease increases dramatically with age in Western society, with a reported prevalence of approximately 30% by the age of 70 years (Lernfelt et al. 1990).

Despite an overall decline in mortality due to cardiovascular disease throughout Western society since the late 1960s (Braunwald, 1997), this remains the leading cause of death in the elderly. More than half of all deaths in people aged 65 years or

older are due to coronary disease, and about three quarters of all deaths from ischaemic heart disease occur in the elderly (Braunwald, 1997). An Australian community-based study reported that, in people over the age of 60 years, 46% of male deaths and 53% of female deaths were due to cardiovascular disease (Simons et al. 1996).

1.2.2 Pathogenesis of ischaemic heart disease

Angina pectoris is the clinical manifestation of myocardial ischaemia, which occurs when coronary blood flow is insufficient to meet the energy demands of the myocardium. The most common underlying pathology is atherosclerosis involving the coronary arteries, or "coronary artery disease" (CAD). However, myocardial ischaemia may also occur in the absence of CAD, as in "variant angina pectoris" (Prinzmetal's angina) due to coronary spasm, vasculitis such as syphilitic aortitis, aortic valve stenosis, hypertrophic cardiomyopathy, and less-well defined pathologies such as "syndrome X", in which true myocardial ischaemia may occur in the absence of angiographic coronary stenoses as a result or abnormal coronary flow reserve (Braunwald, 1997).

Atherosclerosis is a progressive disease that usually begins in the second or third decades, with flat, lipid-rich lesions consisting of macrophages and some smooth muscle cells known as "fatty streaks" (Ross & Glomset, 1976). Results of progressive atherosclerosis become manifest clinically as angina pectoris, myocardial infarction, or sudden death, in middle to late adulthood. This multi-factorial process

progresses more rapidly in the presence of predisposing conditions, or CAD "risk factors", such as hyperlipidaemia, diabetes mellitus, hypertension, renal failure, and smoking.

The form and content of the advanced lesions of atherosclerosis demonstrate the results of three fundamental biological processes: (i) proliferation of intimal smooth muscle cells, together with variable numbers of accumulated macrophages and T-lymphocytes; (ii) formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix, including collagen elastic fibres, and proteoglycans; and (iii) accumulation of lipid, principally in the form of cholesteryl esters and free cholesterol within the cells as well as in the surrounding connective tissues (Ross & Glomset, 1976).

Theories of the pathogenesis of the lesions of atherosclerosis date back to 1856, when Virchow hypothesised that a form of low-grade injury to the artery wall led to local inflammation allowing increased passage and accumulation of plasma constituents in the intima of the artery (Virchow, 1856). Knowledge of the changes in cellular and molecular biology of the artery wall led to the generally accepted "response to injury" hypothesis of atherosclerosis, in which injury to the lining endothelial cells at particular anatomical sites in the artery wall is the primary event in atherogenesis (Ross & Glomset, 1976). Endothelial injury can lead to interference with the permeability barrier role of the endothelium, alterations in the anti-thrombogenic

properties of the endothelium, and increased release of vasoactive and mitogenic substances.

At least two pathways may be involved in the formation of intimal-smooth muscle proliferative lesions of atherosclerosis, reviewed by Ross (Ross, 1986). One pathway, based on experimental studies of diet-induced hypercholesterolaemia in animals (Faggioto & Ross, 1984; Faggioto et al. 1981; Gerrity et al. 1979; Schaub et al. 1981), begins when injury to the endothelium induces secretion of growth factors. Monocytes attach to the endothelium, and subendothelial migration of these cells leads to fatty streak formation and release of platelet-derived growth factor (PDGF). Fatty streaks may convert to fibrous plaques through release of growth factors from macrophages, endothelial cells, or both. In some cases platelet attachment may occur when macrophages lose their endothelial cover, which provides three possible sources of growth factors: platelets, macrophages, and endothelium.

The second pathway involves direct stimulation of the endothelium, which may be injured but remains intact. Increased endothelial turnover may cause formation of growth factors by endothelial cells, which may stimulate migration of smooth muscle cells from the media into the intima. This is often accompanied by endogenous production of PDGF by smooth muscle cells as well as growth factor secretion from the "injured" endothelial cells. These interactions may lead to fibrous plaque formation and further lesion progression. This pathway may be important in diabetes,

hypertension, cigarette smoking, or other circumstances with increased incidence of atherosclerosis (Ross, 1986).

Injury to the endothelium may be manifested as several forms of dysfunction, e.g. interference with permeability, or vasodilator dysfunction. Endothelial vasodilator dysfunction precedes the clinical development of atherosclerosis (Vanhoutte, 1997), and is strongly associated with risk factors for coronary disease, such as smoking, hypertension, and hypercholesterolaemia (Vita, et al., 1990). Treatment of these risk factors restores endothelial function (Celermajer, 1997) and decreases cardiovascular mortality (Scandanavian Simvastatin Survivial Study Group, 1994; The West of Scotland Coronary Prevention Study Group, 1995; Kannel, 1978). These observations confirm the strong relationship between endothelial dysfunction and risk factors for atherosclerosis, although the pathophysiological mechanisms for endothelial dysfunction have not been fully elucidated.

1.3 CARDIAC METABOLISM

As alterations in cardiac metabolism are integral to the pathogenesis of myocardial ischaemia, an insight into regulation of the primary metabolic pathways is necessary as a prelude to discussing possible metabolic approaches to the treatment of ischaemic heart disease. An overview of the major pathways of intermediary metabolism in the cardiomyocyte is shown in Figure 1.1

To meet the high energy demands of the contracting muscle, the heart must produce a constant and plentiful supply of ATP. This energy is produced by the metabolism of a variety of carbon substrates, which include carbohydrates, free fatty acids (FFA), and ketone bodies. FFA and carbohydrates are the major substrates for ATP production. The predominance of either source varies according to the physiological state of myocardial workload and relative oxygen supply. When catabolised to 2-carbon chain length acetyl molecules, they share a common pathway through the tricarboxylic acid (TCA) cycle, as illustrated in Figure 1.1.

The high energy requirements of the mammalian heart are met primarily by fatty acid oxidation. Under normal physiological conditions, FFA (predominantly palmitic and oleic acid) account for 60-70% of O_2 consumption. This increases to 80-90% in the fasting state, while postprandially carbohydrates (predominantly glucose and lactate) can contribute 60-100% of myocardial energy needs, due to increased insulin secretion, which both enhances myocardial glucose uptake and reduces plasma FFA concentrations via inhibition of lipolysis. Both fatty acid and carbohydrate metabolism are tightly coupled, and regulation of substrate supply is complex and differs from other tissues such as liver.

1.3.1 Overview of fatty acid metabolism

1.3.1.1 Fatty acid uptake and transport within the aqueous cytoplasm

The main source of fatty acids for the heart are long-chain free fatty acids bound to albumin, and fatty acids present in chylomicrons and very-low-density lipoproteins

(VLDL). Hydrolysis of VLDL and chylomicron triacylglycerols occurs via a lipoprotein lipase present on the luminal side of the endothelial cell (van der Vusse, et al. 1992). Fatty acid uptake into the myocytes occurs via a carrier dependent system mediated by a specific 40-kDa membrane fatty acid transporter (Stremmel, 1988). The fatty acids then bind to fatty acid-binding proteins (FABPs) within the aqueous cytoplasm and are transferred to the outer mitochondrial membrane, where they are converted to acyl-CoA esters by acyl-CoA synthetase (van der Vusse et al. 1988). The acyl groups on these long-chain acyl-CoAs are then targeted for either oxidation within the mitochondria, or incorporation into myocardial triacylglycerol stores. About 10% of fatty acids taken up by the heart are cycled through the triacylglycerol pool, the role of which is probably to ensure an adequate supply of fatty acids for oxidation when extracellular fatty acid levels are low (Lopaschuk et al. 1994).

1.3.1.2. Transport into mitochondria

The long-chain acyl-CoAs are transferred into the mitochondrial matrix by the combined efforts of three carnitine-dependent enzymes, reviewed by McGarry et al (McGarry, et al., 1991). The first, carnitine palmitoyltransferase 1 (CPT-1), located on the inner surface of the outer mitochondrial membrane, catalyses the conversion of long-chain acyl-CoA to long-chain acylcarnitine (LCAC). The second enzyme, carnitine:acylcarnitine translocase, transports the LCAC across the inner mitochondrial membrane. CPT-2, on the inner mitochondrial membrane, then catalyses the reverse formation of acyl-CoA and carnitine, the acyl-CoA entering the

mitochondrial β-oxidation pathway and the carnitine "shuttling" back across the membrane via the translocase (see Figure 1.2).

1.3.1.3. β-oxidation

The acyl-CoA molecules undergo a series of enzymatic steps within the mitochondrial matrix to produce the end-product acetyl-CoA. Each successive cycle of the of the β-oxidation pathway results in a 2 carbon shortening of the fatty acid and formation of 1 NADH and 1 $FADH_2$ molecule (Schulz, 1991). At sufficiently high levels of fatty acids, β-oxidation is primarily dependent on the energy demand of the tissue (Lopaschuk, 1994). Perfused heart studies have shown that the ratios of $NADH/NAD^+$ and acetyl-CoA/CoA decrease in response to a higher workload, and thus energy demand, imposed on the isolated heart (Neely & Morgan, 1974).

1.3.1.4. TCA cycle & electron transport chain

The acetyl CoA thus formed enters the tricarboxylic acid (TCA) cycle, resulting in the liberation of 2 CO_2, 3 NADH, and 1 $FADH_2$. The NADH derived from glycolysis, the pyruvate dehydrogenase complex (PDC; see below), the TCA cycle, and β-oxidation, as well as the $FADH_2$ from the TCA cycle and β-oxidation, then enter the electron transport chain. The hydrogen on NADH and $FADH_2$ is transferred to H_2O in the presence of O_2, and ADP is converted to ATP (Liedtke, 1981).

1.3.2 Regulation of FFA metabolism

While the regulatory processes involved in fatty acid uptake by the myocyte and transfer to the mitochondria have not been clearly delineated (van der Vusse et al. 1992), it is well-established that CPT-1 is a key regulatory point and rate-limiting step in fatty acid oxidation (McGarry et al. 1989). Molecular studies have recently proven the existence of tissue-specific isoforms of CPT-1. Rat liver and skeletal muscle express distinct isoforms of CPT-1 while the heart expresses both liver (L-CPT 1) and skeletal muscle (M-CPT 1) isoforms (Weis et al. 1994a; Weis et al. 1994b). This explains the difference in sensitivity of the cardiac and hepatic forms to various inhibitors. The cardiac form is more sensitive to inhibition by malonyl-CoA (Cook, 1984), and sensitivity to physiological inhibitors does not alter in response to dietary or pathological states, as is the case with hepatic CPT-1 (Cook & Lappi, 1992; Mynatt et al. 1992).

The main physiological regulator of cardiac CPT-1 appears to be malonyl-CoA. Only CPT-1, and not CPT-2, is inhibited by malonyl CoA. Cardiac CPT-1 is extremely sensitive to inhibition by malonyl-CoA (K_i + 50 nM) (Cook, 1984), and malonyl CoA is present in measurable quantities in the heart (10-15 nmol/g dry wt) (McGarry, et al. 1983). Malonyl CoA is synthesised in the aqueous cytoplasm from acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACC), which also appears to play an important role in the balance between carbohydrate and fatty acid metabolism. Saddick and Lopaschuk (Saddick & Lopaschuk, 1992) postulated that acetyl-CoA derived via the pyruvate dehydrogenase complex (PDH) during glucose oxidation can be transported

from the mitochondria to the aqueous cytoplasm where it can serve as a substrate for ACC, increasing malonyl-CoA production and decreasing fatty acid oxidation (see Figure 1.3). Further support for this hypothesis came from Lysiak et al (Lysiak et al. 1988), who showed that most of the acetyl-CoA generated from pyruvate by PDH is readily accessible to carnitine acetyltransferase (CAT), while that generated from β-oxidation is more available to the TCA cycle. This may explain the somewhat paradoxical effect of exogenous carnitine on cardiac metabolism (see later): carnitine stimulates the CAT pathway resulting in a decrease in the intramitochondrial acetyl-CoA/CoA ratio and a "paradoxical" decrease in fatty acid oxidation and a parallel increase in the rate of glucose oxidation (Broderick et al. 1993).

Recent molecular studies, summarised by McGarry and Brown (McGarry & Brown, 1997) have characterised the primary structures of both CPT-1 and CPT-2, and have allowed chromosomal mapping of their genes. This work has settled longstanding debates by unequivocally establishing two key points: (i) that the CPT-1 molecule consists of a single polypeptide containing both the inhibitor binding and catalytic domains; and (ii) that CPT-1 and CPT-2 are distinct entities.

Studies with protease treatments of mitochondria suggest that the malonyl-CoA binding site on CPT-1 lies on the cytoplasmic face of the mitochondrial membrane while the active (catalytic) site appears to face the inner mitochondrial membrane (Murthy & Pande, 1987; Kashfi & Cook, 1992) (see Figure 1.2). Other inhibitors of CPT-1, such as CoA, acetyl-CoA, and propionyl CoA appear to interact with the

active site since they are unaffected by protease treatment (Kashfi et al. 1994). With respect to pharmacologic agents in use as CPT-1 inhibitors (see below), acyl CoA derivatives such as etomoxiryl-CoA compete with palmitoyl CoA at the active site (McGarry & Brown, 1997), while 4-hydroxy phenyl glyoxylate (the active metabolite of oxfenacine) competes for the same binding site as malonyl CoA (Kashfi et al. 1994).

1.3.3 Overview of carbohydrate metabolism

The other major source of acetyl-CoA for the tricarboxylic acid cycle is oxidation of carbohydrates, particularly glucose and lactate (see Figure 1.1). Glucose transport into the myocyte is regulated both by the transmembrane glucose gradient and the concentration and activity of the plasma membrane glucose transporters (GLUT 4 and GLUT 1) (Stanley et al. 1997).

Once in the cell, glucose is phosphorylated by hexokinase to glucose-6-phosphate and then used primarily for either glycolysis or glycogen synthesis (Stanley et al. 1997). Two molecules of ATP are produced in the glycolysis of each G-6-P molecule, the end-product of this pathway being pyruvate. Under aerobic conditions, pyruvate is transported into the mitochondria where it is oxidised by the intramitochondrial multienzyme complex, PDH, to form acetyl CoA, which then enters the tricarboxylic acid cycle.

The pyruvate formed via glycolysis may also be converted to lactate by cytoplasmic lactate dehydrogenase, and then released from the heart (this process does not require the presence of oxygen). Extracellular lactate can also be taken up by the heart, where it can be converted to pyruvate and undergo mitochondrial oxidation. In fact, the lactate derived from glycolysis can be released from the heart at the same time as extracellular lactate is taken up (thus lactate release cannot be taken as an accurate measurement of glycolytic rates) (Lopaschuk, 1997).

1.3.4 Regulation of carbohydrate metabolism

The two major sites of regulation of glycolysis are glucose transport into the cell (which is carrier-mediated and non-energy-dependent) and the activity of phosphofructokinase (the rate-limiting enzyme in the glycolytic pathway) (Opie, 1992). Increased cardiac work, several hormones such as insulin, growth hormone and adrenaline, and anoxia all accelerate glucose transport into the cell, largely related to translocation of the glucose transporters GLUT-1 and GLUT-4 from an intracellular storage pool within microsomal vesicles to the plasma membrane (Liedtke, 1981; Stanley et al. 1997; Young et al., 1997).

Under normoxic conditions, myocardial glucose utilisation is inhibited when alternative oxidisable substrates are available. Fatty acid oxidation inhibits phosphofructo-kinase (Pogson & Randle, 1966) (as well as PDH, see below), explaining why fatty acids are used preferentially when available. The inhibition of

phosphofructokinase by fatty acid oxidation increases the levels of intracellular free glucose, which in turn decreases the rate of glucose uptake (Opie, 1992).

The activity of the PDH complex is the major determinant of carbohydrate oxidation *in vivo* (Randle, 1986), which in turn depends primarily on the availability of competing substrates such as fatty acids. Products of fatty acid oxidation (acetyl-CoA, NADH, and ATP) are potent inhibitors of PDH via the activation of PDH kinase, which phosphorylates PDH leading to its inactivation (Kerbey et al. 1985; Stanley et al. 1997) (see Figure 1.3). Hence the pathways of glucose and fatty acid oxidation are tightly coupled (known as the Randle glucose-fatty acid cycle) (Randle et al. 1963), two of the key regulators being PDH and CPT-1 respectively (see Figure 1.3). Inhibition of CPT-1 with etomoxir or oxfenicine (see later) results in greater glucose oxidation by lowering acetyl CoA levels and relieving tonic inhibition of PDH (Higgins, 1980; Lopaschuk & Spafford, 1989). On the other hand, stimulation of PDH activity with dichloroacetate, an inhibitor of PDH kinase, increases the levels of acetyl CoA and malonyl CoA, inhibiting fatty acid oxidation at the level of CPT-1 (Stanley, et al., 1996; Saddick et al. 1993). Preferential carbohydrate utilisation can also be stimulated by increased levels of cyclic AMP, via direct activation of phosphorylase, PDH, and phosphofructo-kinase resulting in a concerted stimulation of glucose transport, glycolysis, glycogen breakdown, and glucose oxidation (Depre et al. 1998).

In addition, there is continuous exchange of carbohydrate into and out of glycogen storage pools *in vivo*, with simultaneous glycogen synthase and glycogen phosphorylase activity. The glycogen content of the heart is highly dependent on diet and feeding state, and is quite variable between species (higher in human than rat) (Stanley et al. 1997). Studies in rats have shown that fasting, or high plasma FFA levels, results in higher cardiac glycogen levels, while ischaemia or increased myocardial work leads to a fall in glycogen concentration (Stanley et al. 1997; Liedtke, 1981).

1.3.5 Substrate utilisation by the heart: the cost in oxygen

This tight coupling between the major metabolic pathways means that the heart can shift rapidly from the use of one substrate to another to produce the required energy according to its needs. While heart muscle will use predominantly FFA under most conditions, cardiac efficiency (the ratio between ventricular work and oxygen consumption) is up to 40% greater with glucose utilisation (Kjekshus, 1981; Vik-Mo & Mjøs, 1981). Under conditions of normal coronary flow in dogs, the presence of high arterial concentrations of FFA increases myocardial oxygen consumption without any change in mechanical work (Mjøs, 1971), while in ischaemic hearts both *in vitro* (Henderson et al. 1970) and in vivo (Kjekshus & Mjøs, 1972) FFA in high concentrations also depresses myocardial contractile performance.

There are several possible reasons why ventricular efficiency is greater with glucose than with FFA utilisation. Firstly, fatty acids and carbohydrates are not equivalent in

terms of oxygen requirement for ATP production. One molecule of glucose requires 12 oxygen atoms (2 per carbon) to produce 38 ATP molecules (6.3 per carbon), while the oxidation of one molecule of palmitate produces 130 ATP molecules (8.2 per carbon) and requires 46 oxygen atoms (2.8 per carbon) (Grynberg & Demaison, 1996). That is, the number of moles of ATP produced per mole of carbon oxidised is approximately 29% higher for FFA relative to glucose, but the number of moles of ATP produced per mole of oxygen consumed is 12% higher for glucose than for FFA oxidation. Therefore, FFA appear to be the most efficient substrates for energy production in terms of fuel supply, but the worst in terms of oxygen consumption. Thus, when oxygen is abundant during normal perfusion conditions, it is more efficient for the aerobic myocardium to utilise and oxidise FFA, but during ischaemia glucose appears to be a better myocardial substrate than FFA. In addition, under conditions of low coronary flow, utilisation of glucose, but not FFA, supports anaerobic ATP generation via glycolysis.

However, the overall increase in myocardial oxygen consumption with a shift to fatty acid utilisation is even greater than the expected 10-15%, up to 40% in some studies (Vik-Mo & Mjøs, 1981). Some of the increased energy requirement associated with fatty acid metabolism may be caused by cycling of FFA into and out of the triglyceride pool, which is stimulated by ischaemic conditions (see below) (van Bilsen, et al., 1989; Vik-Mo & Mjøs, 1981). FFA utilisation may also cause uncoupling of oxidative phosphorylation, consuming oxygen that is not linked to the

generation of ATP, which may play a significant role in myocardial stunning and reperfusion injury (Burkhoff, et al., 1991).

1.3.6 Myocardial metabolism during ischaemia/reperfusion

Under physiological conditions, oxygen supply is not a limiting factor and the prime determinant of myocardial energy supply is substrate availability (e.g. postprandial vs. fasting, increased lactate utilisation during exercise). During hypoxia, the shift from FFA to carbohydrates may significantly increase the amount of ATP that can be produced from the available oxygen. This shift might enable the heart to preserve contractile function during mild to moderate ischaemia, and possibly protect the heart from irreversible cell death during more severe or prolonged ischaemia.

During an acute ischaemic episode, the release of catecholamines results in high plasma levels of free fatty acids (Vallori et al. 1967), which have been shown to increase the severity of ischaemic damage in a number of animal models, and have been linked to a depression of mechanical function following reperfusion (Lopaschuk, et al. 1990; Liedtke et al. 1978). Early clinical observations showed that high plasma FFA concentrations were strongly associated with an increase in the incidence of ventricular arrhythmias during acute myocardial infarction in dogs (Oliver et al. 1968), later confirmed in patients (Tansey & Opie, 1983). These findings, together with the known oxygen-wasting effects of FFA metabolism, led to views that provision of glucose is "good" and that a raised circulating FFA concentration is "bad" for the ischaemic myocardium (Oliver & Opie, 1994). Despite this knowledge,

metabolic management of acute ischaemia has been largely overlooked in the clinical setting.

Although fatty acids remain the major residual source of oxidative metabolism during low-flow ischaemia (Stanley et al. 1997), the increased ratio of NADH/NAD^+ due to the lack of O_2 leads to a suppression of β-oxidation (Liedtke, 1981). The depression of β-oxidation despite high levels of FFAs during ischaemia leads to an increase in cellular concentrations of fatty acids and in the intermediates of FA oxidation, long chain acyl-CoA and long-chain acylcarnitine (LCAC) (see below).

The main alternative fate of FFA is esterification to triglyceride, which is markedly stimulated in ischaemic myocardium (van Bilsen, et al., 1989; Vik-Mo & Mjøs, 1981). This ATP-dependent process could potentially further deplete the myocardial energy stores (van Bilsen, et al., 1989), and this triglyceride accumulation, shown both in experimental studies and in the margins of myocardial infarcts in humans, may be toxic to the myocardium (Vik-Mo & Mjøs, 1981). In addition, the lipolysis of endogenous triglycerides is enhanced in ischaemic perfused rat hearts, releasing FFA and glycerol locally within the ischaemic myocardium (Vik-Mo & Mjøs, 1981). This cycling of FFA into and out of the triglyceride pool may be a major mechanism for the energy wasting effects of FFA during myocardial ischaemia.

As mentioned above, glucose assumes a central role for energy production in the ischaemic heart, although the relative contribution to energy production is highly

dependent on the degree of ischaemia. In moderate ischaemia (reduction of coronary flow by 75%), glucose extraction increases and the metabolism of glucose is directed from oxidation to lactate production. The increased glucose uptake occurs via an increase in plasma membrane GLUT 1 and GLUT 4 which are recruited from intracellular stores (Liedtke, 1981; Stanley et al. 1997; Young, et al., 1997). If oxygen deprivation is prolonged, the transcription of these glucose transporters is also modified (Shetty et al. 1993). The majority of pyruvate formed via non-oxidative glycolysis is converted to lactate which accumulates in the ischaemic tissue. PDH activity is inhibited by increased NADH and acetyl CoA (Stanley et al. 1997), resulting in a decreased rate of glucose oxidation and a switch from net lactate uptake to net production. This becomes more pronounced during exercise or pacing-induced work in the presence of moderate coronary stenoses; metabolic activity may remain "normal" at rest unless tissue perfusion is very low ("hibernating" myocardium).

In severe ischaemia, myocardial glucose extraction is inversely related to coronary flow ("flow-metabolic mismatch"; see PET imaging below), until the degree of ischaemia becomes so severe that glycolysis becomes inhibited by the accumulation of its products. Under conditions of no myocardial perfusion, there is no residual ATP synthesis from oxidative phosphorylation, and thus there is total dependence on anaerobic metabolism with endogenous substrates. The sole source of glycolytic substrate becomes glycogen, as there is no blood flow to deliver glucose to the tissue. As there is no washout of lactate, intracellular pH decreases, and eventually a reduction in the rate of glycolysis occurs via H^+ inhibition of phosphofructokinase

activity and reduced flux through glyceraldehyde 3-phosphate dehydrogenase due to a low cytosolic NAD^+/NADH ratio (Rovetto et al. 1975). Irreversible ischaemic contracture occurs as glycogen stores run out.

Following reperfusion, the relationship between substrate utilisation and restoration of function in the previously ischaemic heart remains controversial. Reperfusion after transient ischaemia in the canine heart is accompanied by delayed clearance of the positron tracer [^{11}C]palmitate and increased accumulation of [^{18}F]deoxyglucose in non-necrotic myocytes (Schwaiger, et al., 1985). Conversely, other investigators have shown that the previously ischaemic myocardium rapidly regains the ability to oxidise fatty acids in both isolated rat heart (Saddick & Lopaschuk, 1992) and working swine heart (Renstrom et al. 1989) models of ischaemia, which may be partly due to the decreased sensitivity of CPT-1 to inhibition by malonyl-CoA which has been observed in isolated mitochondria following ischaemia (Pauly et al. 1991). In addition, a fall in malonyl CoA levels during post-ischaemic reperfusion, corresponding to a fall in acetyl CoA carboxylase activity, has been recently demonstrated in isolated rat hearts (Kudo et al. 1995). The resultant release of malonyl CoA inhibition on CPT-1 results in an "overshoot" in fatty acid oxidation and inhibition of pyruvate oxidation during the reperfusion period (Stanley et al. 1997).

However, in hearts reperfused with fatty acids, mechanical function is depressed compared to those reperfused with carbohydrates, without any significant decrease in

oxygen consumption (Liedtke & Nellis, 1978; Kjekshus & Mjøs, 1972; Opie et al. 1973). The uncoupling of O_2 consumption and substrate oxidation from mechanical function due to inefficient energy usage (partly due to the activation by FFA of futile metabolic cycles that waste ATP and therefore oxygen (van Bilsen, et al., 1989; Vik-Mo & Mjøs, 1981) may play an important role in reperfusion injury and myocardial stunning.

In addition, accumulation of fatty acid oxidation products leads to PDH inhibition, resulting in a marked imbalance between glycolysis and glucose oxidation. This leads to a net production of $2H^+$ from each glucose molecule undergoing glycolysis, leading to an increase in Na^+/H^+ exchange. The resultant increase in intracellular Na^+ in turn activates the sarcolemmal Na^+/Ca^{++} exchanger, which can lead to cellular Ca^{++} overload and contribute to cell death or post-ischaemic stunning (Stanley et al. 1997). Correcting the imbalance with the use of the PDH stimulator dichloroacetate improves post-ischaemic recovery of mechanical function and cardiac efficiency (Lopaschuk et al. 1993).

1.3.7 The role of FFA metabolites

The accumulation of myocardial long chain acylcarnitines (LCACs) and long chain acyl-CoA levels during ischaemia may also contribute to ischaemic and reperfusion injury. Studies have shown the rapid accumulation of LCACs in ischaemic conditions *in vivo* (DaTorre et al. 1991), while *in vitro* >100 fold increases of LCACs are seen in the sarcolemmal compartment of isolated myocytes within 10 minutes of

the onset of hypoxia (McHowat et al. 1993). These amphiphilic intermediates can interfere with a number of cellular processes, including membrane pump function and electrical activity of the heart (Liedtke, 1981; Katz et al. 1981). In addition, they have been shown to alter the functional characteristics of a number of integral membrane proteins by altering their lipid environment (Adams et al. 1979). This may explain the reported increase in intracellular Ca^{2+} concentrations, via a diminished ability of the sarcoplasmic reticulum to accumulate and store calcium (Huang et al. 1992), as well as a reduction in gap junctional conductance between myocytes (Wu et al. 1993). Such changes in membrane integrity favour the development of cardiac arrhythmias and cell damage.

However, all of the above work comes from *in vitro* studies, and the effects of LCACs on ischaemic injury remain controversial. Recent studies have dissociated the build up of these metabolites from the detrimental effects of FFA (Ichihara & Neely, 1985; Madden et al. 1995; Lopaschuk et al. 1988), which appear to be more closely related to the ability of the products of FFA oxidation to inhibit glucose metabolism via inhibition of PDH, an enzyme which appears critical for functional recovery following ischaemia via its direct effects on cellular phosphorylation potential (Mallet et al. 1990). In addition, PDH inhibition and the resultant decreased glucose oxidation could increase the production of H^+ from glycolytically derived ATP, possibly potentiating the development of Ca^{2+} overload during reperfusion. Lopaschuk et al (Lopaschuk et al. 1993) showed that the uncoupling of glucose oxidation from glycolysis is significant in the presence of high concentrations of FFA

1.4 MANAGEMENT OF CHRONIC MYOCARDIAL ISCHAEMIA

1.4.1 Medical therapy

Myocardial ischaemia results directly from an imbalance between myocardial energy demands and coronary blood flow. Pharmacological therapy has traditionally been aimed at either decreasing demand (cardiac contractility, heart rate, and wall stress) and/or increasing supply (perfusion pressure and coronary vascular resistance). The three cornerstones of prophylactic anti-anginal therapy have been the nitrates, β-adrenoceptor antagonists ('β-blockers') and L-channel calcium antagonists.

1.4.1.1 Nitrates:

Organic nitrates, such as nitroglycerine, isosorbide dinitrate, and isosorbide mononitrate are the most commonly used anti-anginal agents. These organic nitrates act as exogenous nitric oxide donors (Horowitz & Henry, 1987), and their effects are related to the action of nitric oxide on the vasculature and platelets.

The nitrates are unique as vasodilators in that they not only dilate the arteries and arterioles, but also selectively induce venous dilation. The effects on large arteries are most profound at the coronary vessels, especially at stenotic sites (Horowitz & Henry, 1987). Hence the nitrates decrease myocardial oxygen demand by reducing afterload and particularly preload, thus reducing cardiac work. In addition, they improve myocardial oxygen supply via coronary vasodilatation. Finally, several investigators

have reported significant anti-aggregatory effects (Chirkov et al. 1990), another potential role in the management of coronary disease.

1.4.1.2 β-adrenoceptor antagonists

β-adrenoceptor antagonists competitively inhibit binding of catecholamines to β-adrenoceptors. They reduce myocardial oxygen demand, particularly in response to sympathetically modulated stress, by reducing contractility, heart rate, and blood pressure (Gorlin, 1992). In addition, they may have a metabolic protective effect related to a reduction in the mobilisation of fatty acids from adipose tissue by catecholamines (Oliver & Opie, 1994) (see later).

1.4.1.3 L-type calcium antagonists

These agents, such as diltiazem, verapamil, and nifedipine, act via selective inhibition of the L-type calcium channel, thus interfering with the entry of calcium into (1) myocytes, producing negative inotropy, and (2) coronary and peripheral vascular smooth muscle, producing vasodilatation (Schwartz et al. 1985). Hence they have beneficial anti-ischaemic effects on oxygen demand via reductions in myocardial contractility and afterload and improved oxygen supply via coronary vasodilatation. As with nitrates, there is some evidence that they may also have beneficial effects on platelet aggregability (Wallen et al. 1995).

Importantly, not all calcium antagonists are identical with respect to their actions. Dihydropyridines such as nifedipine act mainly on the peripheral vasculature, and

have minimal negative inotropic effects at clinical doses (Opie, 1980). This can result in a reflex tachycardia, with a potential increase in myocardial oxygen demand. Their role as monotherapy is therefore limited, and there is some controversy as to their potential deleterious effects on long-term outcomes in patients with potential or actual myocardial ischaemia (Opie & Messerli, 1995).

On the other hand, verapamil and diltiazem have more marked negative chronotropic effects in addition to their vasodilator effects (Opie, 1980), thus avoiding potentially deleterious reflex tachycardia.

1.4.2 Revascularisation

Percutaneous transluminal angioplasty (with or without coronary stenting), coronary artery bypass surgery, and transmyocardial laser revascularisation are invasive strategies which may be employed, particularly if medical therapy is ineffective or is not tolerated.

1.4.2.1 Percutaneous transluminal angioplasty

Percutaneous transluminal angioplasty (PTCA) is playing an increasingly important role in the treatment of chronic stable angina. PTCA of optimal lesions (concentric subtotal stenoses situated in a straight portion of a single proximal coronary artery, without side-branches and easily accessible) has a primary success rate of over 90%, with success defined as a post-procedure luminal diameter that is narrowed by no more than 50% by visual estimation (Landau et al. 1995). However, PTCA in less

optimal lesions (eccentric, calcified, less accessible, or totally occluded) carries more risk and has a lesser chance of primary success.

Although PTCA is generally safe, risks include myocardial infarction or the need for emergency bypass surgery in 3-5%, and death in approximately 0.1%. The main drawback is the risk of restenosis (luminal diameter of less than 50%) due to intimal hyperplasia, which occurs in 30% of patients (higher in more difficult lesions), usually within the first 3 months. Repeat PTCA is often necessary, and successful, in these cases. PTCA is more expensive than medical therapy as a treatment for chronic stable angina (Parisi et al. 1992), although measures of quality of life are superior in patients undergoing PTCA (Strauss et al. 1995).

Coronary stenting, performed in conjunction with PTCA, has become widespread practise over the past 5 years. The placing of an intra-luminal prosthesis (stent) to "scaffold" the treated vessel results in a larger absolute calibre of the coronary lumen in comparison to angioplasty alone, which accounts for the reported lower rates of restenosis and need for repeated revascularisation (Fischman, et al., 1994; Serruys, et al., 1994). However, treatment options are severely limited in those 10-17% of patients that do develop restenosis within a stented lesion.

The other intravascular revascularisation procedure in widespread use is directional coronary atherectomy. However, despite a high primary success rates, randomised studies have shown no long term advantage over routine PTCA with respect to

restenosis or need for further revascularisation procedures (Baim, et al., 1998; Elliot, et al., 1995), with reports of a higher incidence of acute complications and myocardial infarcts within 6 months of the procedure in the largest published study (Elliot, et al., 1995). For these reasons, atherectomy is generally reserved for difficult lesions, such as ostial or in-stent stenoses.

1.4.3 Coronary artery bypass surgery

Coronary artery bypass surgery (CABG) has improved markedly since its introduction in the 1960s (Favaloro, 1969). While vein grafts have a 50-70% incidence of significant atheromatous narrowing at 3-5 years, internal mammary grafts show greater than 90% patency after 10 years (Loop et al. 1986). Although survival outcomes between medical therapy, PTCA, and CABG in patients with stable angina are comparable, surgery improves symptoms to a greater extent than medical therapy regardless of the severity of coronary disease, and PTCA in patients with multivessel disease (CASS Study Group, 1983b; Hamm, et al., 1994; King et al. 1994). In addition, surgery holds a significant survival advantage over medical therapy in the treatment of patients with poor left ventricular function and multi-vessel coronary disease, or in those with left main coronary artery disease, (CASS Study Group 1983a; Cohen et al. 1975).

The in-hospital mortality rates for CABG are now around 1% for single-vessel disease and 2%-3% for patients with multivessel disease and impaired left ventricular function. However, the rates in the elderly are considerably higher, with an in-

hospital mortality rate of 5.6% in octogenarians quoted in a recent review of the literature (Alexander & Peterson, 1997). Nonfatal complications are also high in the elderly, ranging between 30 and 70% in published series (Alexander & Peterson, 1997).

1.4.4 Transmyocardial laser revascularisation

Transmyocardial laser revascularisation (TMLR) has recently been evaluated as an alternative treatment strategy in patients with angina which is not controlled by conventional medical therapy and who have disease which is not suitable for conventional revascularisation techniques. Typically such patients have diffuse atherosclerotic disease throughout their coronary arterial tree, with no "target" lesions for angioplasty nor suitable distal vessels for attachment of bypass conduits.

TMLR involves the placement, via left thoracotomy, of a carbon dioxide laser probe on the surface of the ischaemic part of the left ventricle and the creation of transmural channels into the left ventricular cavity. In randomised trials, symptomatic improvement has been reported in patients treated with TMLR compared to controls, although no significant differences in survival or exercise capacity were seen at 12 months (March, 1999; Schofield, et al., 1999). Operative mortality rates for the procedure range between 3% and 10% (Burns, et al., 1999; March, 1999; Schofield, et al., 1999).

1.4.3 Limitations of current strategies

All three classes of conventional anti-anginal drugs have pronounced haemodynamic effects, often leading to adverse reactions such as low blood pressure, peripheral oedema, headaches, or worsening of heart failure. In addition, a large number of patients with angina are unable to tolerate optimal doses of β-adrenoceptor antagonists due to concomitant airways disease, peripheral vascular disease, diabetes, or conduction disturbances. All calcium antagonists are contraindicated in patients with systolic heart failure, while dihydropyridines (nifedipine and derivatives) may worsen angina due to reflex tachycardia and/or coronary "steal" (Boden et al. 1985). Chronic nitrate therapy requires a daily nitrate-free period to limit tolerance, leaving the patient susceptible to nocturnal (and possibly rebound) angina (Thadani et al. 1988).

Despite the large gains made in the medical and surgical treatment of angina pectoris, there remains a large group of patients whose angina remains refractory to maximally tolerated conventional therapy. These are often the patients who are not suitable for a first or, more commonly, repeat coronary revascularisation, due to diffuse multivessel coronary artery disease, poor left ventricular function and/or serious comorbidities. These relative contra-indications to coronary revascularisation are more common with increasing age, and despite the high incidence of CAD in the elderly, rates of coronary revascularisation are lower in this age group (Edmunds et al. 1988).

Conventional therapies may also be ineffective in myocardial ischaemia not related to CAD. One example is aortic stenosis, a condition in which there is global myocardial ischaemia as a consequence of both reduced coronary perfusion pressure and left ventricular hypertrophy. All of the conventional anti-anginal drugs are relatively contra-indicated in patients with severe aortic stenosis due to their adverse haemodynamic effects, and no medical therapy (except perhexiline (Unger et al. 1997)) has been reported to have any favourable effects on morbidity or mortality in this condition. The only proven treatment to improve the dire prognosis in symptomatic aortic stenosis is aortic valve replacement. However, as aortic stenosis is predominantly a disease of the elderly, valve replacement in practise is only offered to a small proportion of these patients without serious comorbidities (Abdul-Hamid & Mulley, 1999; Bouma, et al., 1999).

For patients with refractory symptoms who are unable to tolerate one or more of the conventional classes of drugs, or are unsuitable for revascularisation, therapeutic options are currently limited. Hence there is a need for new pharmacologic approaches in the management of chronic myocardial ischaemia; there has been little change in the therapeutic armament since the development of the calcium antagonists over 30 years ago. "Metabolic agents", which act at the cellular level and do not influence heart rate or blood pressure, potentially have a useful and unique role to play in addition to the conventional therapies for chronic myocardial ischaemia.

1.5 METABOLIC APPROACHES TO MYOCARDIAL ISCHAEMIA

As alterations in energy metabolism are integral to the pathogenesis of myocardial ischaemia, it is increasingly being recognised that optimising energy metabolism is a possible therapeutic intervention for this disorder. Conventional therapies in the medical treatment of chronic stable angina, such as calcium channel antagonists, nitrates, or β-adrenergic receptor antagonists, are aimed at restoring the balance between myocardial oxygen supply and demand, via increasing coronary blood flow and/or reducing arterial blood pressure and the afterload on the left ventricle. The advantage of metabolic therapies is the potential increase in mechanical efficiency of the left ventricle without an adverse effect on haemodynamics. This would be particularly useful in patients already on maximal haemodynamically oriented therapy.

The concept of "metabolic protection" of the ischaemic myocardium is based on the fact that glucose can be metabolised anaerobically providing glycolytic ATP in the cytosolic compartment, thereby protecting cellular membranes. In addition, increasing flux through PDH and carbohydrate (rather than fatty acid) oxidation during cardiac ischaemia would theoretically have several therapeutic benefits: a greater ATP yield for a given rate of oxygen consumption, as well as preventing the lactate and H^+ accumulation which may occur when glycolysis is uncoupled from pyruvate oxidation (Stanley et al. 1997).

The hypothesis that improving glucose supply would lead to preservation of the ischaemic myocardium has received strong experimental support (the "glucose hypothesis"), and was the rationale for the development of GIK therapy in the 1960s (see below). Because of the tight coupling between the control of both the fatty acid and glucose pathways, inhibition of one will stimulate the activity of the alternate pathway. Possible metabolic approaches to the treatment of myocardial ischaemia include enhancing glucose supply to the myocardium, decreasing FFA supply, stimulating myocardial glucose oxidation, or inhibiting fatty acid oxidation (See Figure 1.4).

The severity of the ischaemia may determine the response to a metabolic intervention. Enthusiasm for metabolic interventions such as GIK (see below) has in the past been tempered by concerns about the toxic effects resulting from increased lactate accumulation and tissue acidosis under very low or no-flow conditions (Rovetto et al. 1973). Glycolytic stimulation has been beneficial in models of moderate low-flow ischaemia (10-20% of normal coronary flow) where the glycolytic end-product of lactate is readily removed (Eberli et al. 1991). In isolated blood-perfused rabbit hearts, increasing buffer levels of glucose (to 19.5 mmol/l compared to 5.5 mmol/l in control hearts) and insulin (to 250 mU/l compared to 15 mU/l) resulted in greater developed pressure, preserved diastolic function, lower coronary resistance, increased glycolytic flux and preserved glycogen stores and high energy phosphate levels, and less loss of myocyte enzymes during a prolonged period of underperfusion, with increased recovery during reperfusion (Eberli et al. 1991).

During severe low-flow ischaemia, tissue lactate washout is ineffective, and reports have varied as to the beneficial effects of glycolytic therapies under such conditions. Several early studies in infarct models of dogs (Maroko et al. 1972) or isolated rat hearts (Hearse et al. 1976) showed a significant reduction in infarct size using a high glucose + insulin substrate. More recently, Tamm showed that increasing glucose supply to isolated rat hearts resulted in reduced left ventricular diastolic pressure and creatine kinase release and increased left ventricular pressure development during reperfusion after no-flow ischaemia (Tamm, 1994). On the other hand, some studies have reported a lack of protective effect in infarct models, although there have been no reports of increased injury compared to control glucose levels (Apstein et al. 1983; King et al. 1995).

Treatment of myocardial ischaemia with metabolic therapies should work best under conditions where there is sufficient oxygen delivery to the myocardium to support pyruvate oxidation, such as demand-induced ischaemia (e.g. exercise-induced angina) or during post-ischaemic reperfusion. Although there may be some doubt regarding the effectiveness of metabolic therapy in severely underperfused or completely anoxic tissue (e.g. during acute myocardial infarction (AMI)) (Stanley et al. 1997), it must be recognised that the acute infarct region in patients with AMI is one of moderate low-flow rather than no-flow ischemia, and the level of tissue perfusion in a significant fraction of the infarct zone appears to be above the level required for successful metabolic manipulation (Chareonthaitawee et al. 1997).

Finally, it has been postulated that patients with diabetes mellitus, a condition resulting in elevated plasma FFA levels, increased cardiac β-oxidation, and impaired glucose oxidation, should benefit in particular from metabolic therapies (Lopaschuk, 1996).

1.5.1 Increasing glucose supply to the heart

1.5.1.1 Glucose-insulin-potassium (GIK)

In animal models of ischaemia/reperfusion injury, glucose and insulin infusions decrease infarct size and prevent the associated fall in creatine phosphate, ATP, and pH. High circulating glucose ± insulin concentrations have been shown to increase resistance to ischaemia and improve mechanical recovery during reperfusion in isolated working heart models (Eberli et al. 1991; Tamm, 1994; Owen et al. 1990).

Clinical studies from the early part of this century reported an improvement in symptoms of angina with glucose ingestion or infusion, reviewed by Stanley (Stanley et al. 1997). Sodi-Pollares refined the treatment in the 1960s by adding insulin and potassium (GIK) to the infusion, and provided some evidence in a small non-randomised trial that treatment with GIK improved some of the ECG abnormalities associated with AMI, reduced ventricular arrhythmias in the post-infarct period, and improved early survival (Sodi-Pollares, et al., 1962). Subsequent studies using GIK in patients with myocardial infarction used low concentrations, or initiated therapy later (up to 48 hours after onset of chest pain) and showed mixed results (Mittra, 1965; Council, 1968), discouraging the use of such strategies. Only later did

extensive controlled studies define the infusion rates of GIK required to reduce plasma FFA and to maintain plasma K^+ concentrations (which tend to decrease in acute myocardial infarction) (Rackley, et al., 1981).

A meta-analysis overview of nine randomised placebo-controlled trials done in the pre-thrombolytic era (Faith-Ordoubadi & Beatt, 1997) concluded that GIK given within 48 hours of chest pain onset reduced in-hospital mortality by 28% (P=0.004). In the four studies in which GIK was administered intravenously at high doses, the in-hospital mortality was reduced by 48%.

Two large randomised trials of GIK in the treatment of AMI in conjunction with thrombolytic therapy have recently been reported. The ECLA (Estudios Cardiologicos Latinio-America) study (Diaz et al. 1998) study reported a remarkable 66% reduction (from 15.2% to 5.2%) in the in-hospital mortality risk when GIK was added to reperfusion with either thrombolysis or primary PTCA relative to reperfusion alone. The ECLA study also compared high-dose GIK (the Rackley regimen) to a lower dose, and found that only the high-dose GIK group had a significant survival advantage relative to the control group at one year of follow-up. In contrast, a Polish study (Ceremuzynski et al. 1999) showed no benefit from GIK therapy: however the majority of patients in this study had anticipated small infarcts, and the dose of GIK was very low, with glucose infusion rates of only about 15% of the rate used in the ECLA study.

Theoretically, GIK therapy would be of greatest benefit in patients with potentially large infarcts, and indeed improved clinical outcomes have been reported with the use of GIK in patients with AMI complicated by cardiogenic shock (Opie, 1995). Taegtmeyer and colleagues have also demonstrated the beneficial effects of GIK on cardiac function in post-cardiac surgery patients with profound left ventricular dysfunction, another form of cardiogenic shock (Coleman et al. 1989).

As mentioned above, metabolic strategies should be most effective in patients with diabetes mellitus. The recent DIGAMI study (Malmberg et al. 1995) randomised diabetic patients presenting with an acute myocardial infarct (whose early mortality rate is almost twice as high as non-diabetic patients) to either continuous insulin/glucose infusion followed by intensive subcutaneous insulin therapy for three months or conventional care. All fatal complications were significantly reduced in the active treatment group, and mortality at 12 months was reduced from 26% to 19%, a reduction of 29% ($P<0.03$). Interestingly, the most dramatic benefit of the G+I therapy was seen in the patients with only borderline or mild diabetes, suggesting the results may be applicable to non-diabetic patients. The mechanism of this benefit remains speculative. Although the intensive therapy caused only a modest effect on plasma glucose levels, it may have markedly improved cardiac utilisation of glucose and a reduction of FFAs.

1.5.2 Decreasing the fatty acid supply to the heart

As high levels of FFA dramatically inhibit glucose oxidation, another potential metabolic approach to treating ischaemic heart disease is to decrease circulating FFA levels. One possible benefit of GIK may be related to a decrease in FFA levels, as insulin inhibits the mobilisation of FFA from adipocytes, although it has yet to be determined clinically what effect GIK has on circulating FFA levels.

1.5.2.1 Nicotinic acid

Antilypolytic agents, such as nicotinic acid and its derivatives, act both by decreasing FFA release from adipocytes, and by inhibiting hepatic very low density lipoprotein (VLDL) secretion (Lopaschuk, 1999). Nicotinic acid has been shown both clinically and in experimental models of coronary occlusion to protect against myocardial ischaemic injury and reduce ST-segment elevation (Russel & Oliver, 1978; Kjekshus, 1981). However its clinical utility has been limited by significant adverse effects such as skin flushing, nausea, gastric burning, and hypotension (Vik-Mo & Mjøs, 1981). In addition, nicotinic acid and related drugs reduce peripheral arterial resistance (often causing hypotension) and increase heart rate and cardiac work, making them unsuitable for use in acute myocardial ischaemia.

1.5.2.2 β-adreneceptor antagonists

With respect to a more "conventional" therapy in acute myocardial infarction, it has been postulated that the impressive reduction in sudden cardiac deaths associated with the early administration of beta-blockers may be partially explained by their effects

on blocking the catecholamine-induced mobilisation of fatty acids from adipose tissue (Oliver & Opie, 1994), thus potentially improving the balance between glucose and fatty acid metabolism in the ischaemic zones. Although these effects are likely to be most important in patients with large infarcts, clinicians are often reluctant to give beta-blockers in these patients due to the risk of precipitating heart failure.

1.5.3 Direct stimulation of glucose oxidation

Strategies aimed at direct stimulation of glucose metabolism have also been shown to be of benefit in experimental models of ischaemia/reperfusion. By improving the coupling of glycolysis to glucose oxidation, proton production is decreased, resulting in a decrease in tissue acidosis and an improvement in cardiac efficiency. Pharmacologically, glucose oxidation can be increased either by directly stimulating pyruvate dehydrogenase, or indirectly by decreasing intra-myocardial acetyl CoA/CoA ratios.

1.5.3.1 Dichloroacetate

Several working rat heart studies have shown that direct stimulation of PDH with dichloroacetate (Lopaschuk et al. 1993; Liu, Z et al. 1996; Barak, et al., 1998), thereby increasing glucose oxidation, has resulted in enhanced recovery of post-ischaemic myocardial function. In clinical studies, dichloroacetate has been shown to increase left ventricular stroke volume in patients with coronary artery disease (Wargovich, et al., 1988). Unfortunately, the clinical use of dichloroacetate is limited by its low potency and short half-life (Stanley et al. 1997).

1.5.3.2 L-carnitine

Somewhat paradoxically given its role as a co-factor in the transport of FFA into the mitochondria, exogenous L-carnitine seems to play an important metabolic role by enhancing carbohydrate metabolism and reducing FFA toxicity. L-carnitine has been shown to increase pyruvate metabolism, reduce lactate production and acidosis, and to act as a scavenger of the toxic catabolic products of FFA which accumulate in the heart during ischaemia (Rizzon et al. 1989). The ability to increase glucose oxidation occurs secondary to an increase in PDH activity, due to a lowering of the intramitochondrial acetyl CoA/CoA ratio (Stanley et al. 1997).

Clinically both L-carnitine and its analogue propionyl L-carnitine have been shown to have anti-ischaemic properties. The available short-term controlled studies in patients with stable angina have shown that administration of high doses of L-carnitine improves exercise tolerance and increases the ischaemic threshold (Canale, et al. 1988). In a double-blind, placebo-controlled study in 31 men with angina, administration of propionyl L-carnitine intravenously prior to atrial pacing attenuated ST depression and prevented ischaemia-induced ventricular dysfunction without any effect on oxygen supply or demand (Bartels et al. 1994). Cardioprotective effects of these agents, including attenuation of left ventricular dilatation and improved exercise capacity, have also been observed with long-term therapy following acute myocardial infarction (Ileceto, et al., 1995).

1.5.4 Inhibition of fatty acid oxidation

1.5.4.1 CPT-1 inhibitors

Direct CPT-1 inhibitors have been widely investigated as both potential hypoglycaemic and anti-ischaemic agents, although this approach has received little clinical attention to date. Inhibition at this step in fatty acid metabolism has two theoretical benefits in myocardial ischaemia: a reduction in the build-up of toxic fatty acid intermediates (LCACs), and a secondary switch to glucose oxidation during reperfusion, necessary for functional recovery of the myocardium. A number of CPT-1 inhibitors have been tested for effects on cardiac metabolism, including sodium 2(5-(4-chlorophenyl)-pentyl)-oxirane-2-carboxylate (POCA), ethyl 2-(6-(4-chlorophenoxy(hexyl)oxirane-2-carboxylate (etomoxir), 2-tetraglycidic acid (TDGA) and oxfenicine (S-4-hydroxyphenylglycine) (see Figure 1.5).

POCA and its more potent analogue, etomoxir, were initially developed as potential hypoglycaemic drugs, and are converted *in vivo* to active CoA derivatives. In the normoxic anaesthetised dog heart, POCA has been shown to almost completely inhibit FFA uptake while increasing lactate utilisation, accompanied by a fall in myocardial oxygen consumption by 15-20% (Seitelberger et al. 1984). Studies in the isolated working rat heart have shown that both POCA (Paulson, et al., 1986) and etomoxir (Lopaschuk & Saddick, 1992; Lopaschuk et al. 1990; Lopaschuk et al. 1988) have demonstrated significant protection of hearts from fatty acid-induced ischaemic injury, associated with increased myocardial efficiency. In working hearts taken from diabetic rats, etomoxir, either when given added to the perfusate in

vitro(Wall & Lopaschuk, 1989) or as pre-treatment intraperitoneally (Reinauer et al. 1990), significantly improves cardiac function even under normoxic conditions. Using isolated blood-perfused rabbit papillary muscles, Yamada et al. showed that both POCA and oxfenicine completely prevented an increase in LCACs during ischaemia, and this was associated with a marked delay in the onset and progression of cellular uncoupling and ischaemic contracture (Yamada, et al., 1994).

Oxfenicine, also a prodrug, is converted *in vivo* to the active metabolite HPG (4-hydroxy phenyl glyoxylate). Studies in isolated rat hearts (Higgins, 1980) under normoxic conditions showed that oxfenicine increased carbohydrate oxidation and cardiac performance without increasing oxygen consumption, confirming improved myocardial efficiency due to CPT-1 inhibition. Following a period of global ischaemia, oxfenicine-treated hearts recovered more completely. These results were confirmed in later studies using perfused rat (Liedke et al. 1984) and swine hearts (Bielefeld et al. 1985; Molaparast-Saless et al. 1987), which also showed a reduction in the accumulation of LCACs in both normal flow and ischaemic conditions. It was postulated that the beneficial effects of CPT-1 inhibition were due to both the switch from fatty acid to glucose utilisation, and the reduced accumulation of the toxic fatty acid metabolites.

However, as mentioned above, the contribution of LCAC accumulation in the post-ischaemic recovery of fatty-acid-perfused hearts remains controversial. POCA, despite complete inhibition of LCAC accumulation, failed to attenuate the incidence

of reperfusion-induced ventricular fibrillation, or reduce infarct size, in whole animal models using both rats (Heathers et al. 1993) or dogs (Vogel et al., 1994). Lopaschuk et al. have shown that while etomoxir significantly improved the functional recovery of fatty-acid-perfused isolated rat hearts, this was unrelated to changes in levels of LCACs, but was more likely due to increased glucose use by the reperfused heart resulting in the observed fall in oxygen consumption per unit work (Lopaschuk et al. 1988). In addition, the LCAC accumulation during normoxic conditions induced by perfusion of isolated rat hearts with the specific CPT-2 inhibitor aminocarnitine has no detrimental effect on cardiac function (Hülsmann et al. 1991).

Oxfenicine is the only direct CPT-1 inhibitor to be studied in patients with angina. An acute intravenous dose was shown to both increase the pacing time to angina and decrease myocardial oxygen consumption during atrial pacing, while reducing myocardial fatty acid extraction (Bergman et al. 1980).

However, plans for the long-term oral administration of oxfenicine (and other direct CPT-1 inhibitors) in clinical trials were aborted following reports that chronic dosing with oxfenicine in rats and dogs led to cardiac hypertrophy (also reported in rats with the long-term use of TDGA) (Bachmann et al. 1983), adverse effects on myocyte membrane functions and uncoupling of oxidative phosphorylation (Higgins et al. 1985; Bachmann & Weber, 1988). With respect to histological changes, Greaves et al. reported that the increase in heart weight in both rats and dogs undergoing long-term oxfenicine treatment was due to uniform myocardial fibre hypertrophy, with

only a minor increase in intracellular lipid content (Greaves et al. 1984). Bachman and Weber described a transient accumulation (first month only) of phospholipids and triglycerides in hearts, livers, and kidneys of rats during chronic treatment with oxfenicine (Bachmann & Weber, 1988). Chronic dosing of rats with etomoxir has also been associated with changes in cardiac phenotype, as evidenced by alterations in the expression of myosin isoenzymes and sarcoplasmic calcium pumps, which result in cardiac growth (Rupp & Jocob, 1992).

Interestingly, chronic treatment with etomoxir does not affect ventricular mass in rats following aortic banding, and actually prevents the impairment in contractile function seen in this model of chronic ventricular overload (Rupp et al. 1995). Recent reports have also shown beneficial effects of chronic treatment with etomoxir on attenuation of post-infarct remodelling in rats (Theres et al. 1998), and improved symptoms and haemodynamic parameters over three months in an open pilot study of etomoxir in patients with chronic heart failure (Schmidt-Schweda & Holubarsch, 1998).

1.5.4.2 Thiazolidinediones

Thiazolidinediones, such as rosiglitazone and troglitazone, are a relatively new class of antidiabetic agents which enhance sensitivity to insulin in the liver, adipose tissue and muscle, resulting in improved insulin-mediated glucose disposal. These agents modulate several processes to increase sensitivity to insulin, including effects on insulin receptor kinase activity, insulin receptor phosphorylation, numbers of insulin receptors, and hepatic glucose metabolism (Grossman & Lessem, 1997). Another key

action of thiazolidinediones is to activate the nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ), a key regulatory enzyme in adipogenesis in mammalian adipose tissue (Bishop-Bailey, 2000).

In addition to their effects on the liver and adipose tissue, thiazolidinediones may have direct metabolic protective effects in the heart. Troglitazone has been reported to increase cardiac glucose uptake via increased GLUT-1 and GLUT-4 protein expression in cultured adult rat cardiomyocytes (Bahr et al. 1996). Treatment of streptozotocin-induced diabetic rats with troglitazone for 6 weeks partially restored the *ex vivo* basal heart rate and cardiac work towards control values and improved the post-ischaemic functional deficits in a working heart model (Shimabakuro et al., 1996).

1.5.4.3. Piperazine derivatives

Trimetazidine (see Figure 5 for chemical structure), in use in Europe for over a decade and now used in over 80 countries worldwide (Kantor et al. 2000), was the first widely used antianginal agent that has a mechanism of action that can be attributed to an optimisation of energy metabolism. It exerts beneficial anti-ischaemic effects, both *in vitro* (Libersa et al. 1990) and *in vivo* (Dalla-Volta et al. 1990; Detry, et al. 1994) without displaying systemic haemodynamic activity or negative inotropic effects. Clinically trimetazidine improves exercise tolerance in comparison to β-blockers (Detry et al. 1994) and nitrates (Michaelides et al. 1997), and is comparable in effect in relief of angina to calcium channel blockers (Dalla-Volta et al. 1990). In a

recent randomised clinical trial of 94 patients, trimetazidine infusion resulted in less ST segment elevation both during and immediately after percutaneous transluminal coronary angioplasty compared to placebo (Steg et al. 1999).

The novel mechanism of action appears to involve direct myocardial cytoprotection, as evidenced by its *in vitro* ability to maintain cellular homeostasis, preserve electrical and contractile function activity, limit cytolysis, inhibit neutrophil accumulation, and afford direct membrane protection against the effects of oxygen free radicals (Cheirchia & Fragasso, 1993; Cargoni et al. 1999).

It has recently become evident that trimetazidine's cardioprotective effects are primarily due to a shift in cardiac metabolism from fatty acid to glucose oxidation. Fantini et al reported that trimetazidine inhibits mitochondrial palmitoyl carnitine oxidation, while only slightly altering pyruvate oxidation and preserving mitochondrial oxidative function (Fantini et al. 1994). More recent studies have reported an increase in glucose utilisation both in open-chest dog (Mody et al., 1998) and isolated working rat heart (Lopaschuk, 1998) experimental models of ischaemia, in the absence of any haemodynamic effects. Boucher et al reported an *in vitro* cardioprotective effect on post-ischaemic recovery of isolated perfused rat hearts with low concentrations of trimetazidine, in the absence of any change in glycolysis (rates of glucose oxidation were not measured). However no such cardio-protective effects were seen in *ex vivo* experiments of pre-treated animals (Boucher et al. 1994).

In isolated cardiac mitochondria, Demaison et al. reported that respiration was reduced when the substrate was palmitoylcarnitine but was not affected when other substrates were used (Demaison et al. 1995), suggesting that trimetazidine may act as an inhibitor of β-oxidation. More recently we have shown that trimetazidine is a weak CPT-1 inhibitor in rat heart mitochondria (Kennedy, 1998), while Lopaschuk's group went on to demonstrate a significant inhibition of the last enzyme involved in long chain fatty acid β-oxidation, 3-ketoacyl coenzyme A thiolase, associated with a shift from fatty acid oxidation to glucose oxidation in working rat hearts during both normal and low-flow conditions (Kantor et al. 2000). Although the drug has been in use for years in humans, there have been no reports of cardiac growth or lipidosis.

A similar mechanism of action has been proposed for a newer piperazine derivative, ranolazine (Figure 5), which has also been shown to shift myocardial substrate usage toward carbohydrates rather than fatty acids (possibly in part due to preservation of PDH activity during ischaemia) (Clarke et al. 1993) in the absence of any haemodynamic effects. In isolated rat hearts, ranolazine increases glucose oxidation under a variety of normoxic conditions, and also during both low-flow ischaemia and during reperfusion following severe ischaemia (McCormack et al. 1996). In patients with exertional angina, ranolazine monotherapy has been shown to improve exercise tolerance (Pepine & Wolff, 1999; Rousseau et al. 1992) and reduce the severity of post-ischaemic diastolic dysfunction without affecting the determinants of myocardial oxygen demand (Rousseau et al. 1992). However, a larger clinical study (using lower

dosages) failed to demonstrate any clear-cut anti-anginal efficacy over placebo (Thadani et al. 1994), and the drug has not yet been approved for routine clinical use.

A summary of the published studies of monotherapy with trimetazidine or ranolazine is shown in Table 1.1. The numbers are surprisingly scant, given the widespread use of trimetazidine in Europe. The observed effects on exercise duration or anginal frequency are also generally small when compared with the results of similar studies with perhexiline (see below, Table 1.2). In contrast to perhexiline, long-term hepato- or neuro-toxic effects have not been reported with the piperazine derivatives.

1.5.5 Other pharmacologic approaches

1.5.5.1 Coenzyme Q_{10}

The vitamin-like nutrient Coenzyme Q_{10} (CoQ_{10}), discovered in 1957, has a crucial role in cellular ATP production as the electron and proton carrying coenzyme for the inner mitochondrial enzyme complexes I, II, and III of oxidative phosphorylation (Langsjoen & Langsjoen, 1998). In addition it is a potent antioxidant, residing at the primary site of free radical production within the lipid environment of the mitochondrial membranes (Langsjoen & Langsjoen, 1998).

CoQ_{10} has been used in many small clinical trails of heart failure and angina since the late 1960s. The rationale behind the use of CoQ10 in heart failure is primarily the correction of a measurable deficiency of CoQ_{10} in both blood and myocardial tissue, the degree of CoQ_{10} deficiency correlating directly with the degree of impairment in

left ventricular function (Mortensen et al. 1990). In a meta-analysis of 14 controlled trials using CoQ_{10} in heart failure between 1986 and 1995, Soja and Mortensen reported significant improvements in stroke volume, cardiac index, cardiac output, and end-diastolic volume with the use of 60 to 200 mg daily doses of CoQ_{10} as a supplement to conventional treatment (Soja & Mortensen, 1997). Several controlled clinical trials have also documented benefits in chronic stable angina, with reports of less angina, improved exercise tolerance, and less ST depression during exercise (Kamikawa et al. 1985; Langsjoen & Langsjoen, 1998).

1.6 PERHEXILINE

Perhexiline [2-(2,2-dicyclohexlethyl)piperidine] maleate was developed in the mid-1960s as a chemical analogue to the investigational vasodilator hexadiline, which caused significant gastrointestinal irritation in man (Rowe et al. 1963) (see Figure 1.5 for chemical structure). Perhexiline was initially investigated as a possible anti-anginal agent on the basis of its coronary and peripheral vasodilator effects *in vitro* and in intact animal preparations (Cho et al. 1970; Hudak et al. 1970). However, later clinical and laboratory studies have suggested that the major mechanism of action of perhexiline involves an improvement in myocardial efficiency via an alteration in cardiac metabolism (see below).

1.6.1 Haemodynamic effects

1.6.1.1 Animal studies

In several early studies in dogs, perhexiline was reported to be a weak coronary vasodilator (Cho et al. 1970; Hudak et al. 1970), with selectivity for the coronary circulation such that coronary flow was increased simultaneously with a fall in systemic arterial pressure (Hudak et al. 1970). However this was not confirmed in further animal experiments (Rowe et al. 1970; Michelin et al. 1980), nor in human subjects (see below) (Pepine et al. 1974; Rowe et al. 1970).

Fleckenstein-Grün (Fleckenstein-Grün, Fleckenstein et al. 1978) classified perhexiline as a weak L-type calcium antagonist due to its inhibition of excitation-contraction coupling in guinea pig papillary muscle, which was antagonised by an excess of calcium in the medium. Further *in vitro* studies confirmed negative inotropic effects and peripheral vasodilatation (Barry et al. 1985; Perez et al. 1982). However, these effects were only seen with relatively high concentrations of perhexiline ($2\mu M$ or higher), and therapeutic perhexiline concentrations in patients on long-term therapy are unlikely to be sufficient to induce clinically significant calcium channel blockade (Barry et al. 1985). In addition, some investigators have described a mild positive inotropic action with perhexiline (Jeffrey et al. 1995; Silver & Monteforte, 1988).

Several other studies have reported the effects of intravenous perhexiline in anaesthetised open-chest dogs. In doses on 0.03-0.3 mg/kg, perhexiline injected

intravenously over two minutes caused dose-dependent increases in pulmonary artery flow, venous return, and right atrial pressure, followed by decreases in all three parameters at higher doses (Ono & Hashimoto, 1981). Coronary blood flow was increased while myocardial oxygen consumption decreased in a dose-dependent fashion. Perhexiline decreased heart rate, systemic blood pressure and left ventricular work dose-dependently, except for lower doses of perhexiline which slightly increased cardiac output and ventricular work. Overall mechanical efficiency of the left ventricle was increased with perhexiline, except at high doses (Ono et al. 1982). The effects of perhexiline at the higher doses again would be consistent with calcium channel antagonism.

Klassen et al. found that intravenous perhexiline in an ischaemic canine model resulted in a redistribution of coronary blood flow toward the vulnerable subendocardial regions, associated with a reduction in myocardial oxygen consumption and an increase in myocardial lactate uptake (Klassen et al. 1976). This preservation of subendocardial flow represents another possible anti-ischaemic effect of perhexiline, and was confirmed in a later study of dogs pre-treated with oral perhexiline by the same group (Klassen et al. 1980).

Daniell et al. reported that both oral pre-treatment with perhexiline for 1-2 weeks, and acute intravenous perhexiline (3 mg/kg), reduced the infarct size in dogs following ligation of the left anterior descending coronary artery (Daniell et al. 1977). This was associated with lower peak creatine phosphokinase activity, reduced heart rates, and a

reduced incidence of ventricular ectopic beats. In noninfarcted open chest dogs intravenous perhexiline resulted in increases in coronary blood flow, narrowing of the coronary arterio-venous oxygen content difference, and a 14% reduction in myocardial oxygen consumption. The protective effects of perhexiline were postulated to result from a combination of reduced myocardial oxygen demand, reduced heart rate, as well as an anti-arrhythmic effect.

1.6.1.2 Human studies

The haemodynamic effects of perhexiline have been studied only to a very limited extent in human subjects, largely because of the lack of a commercially available intravenous preparation and the slow onset of effect of orally administered perhexiline. In the only published report of parenteral perhexiline administration in man, a single bolus dose of perhexiline gluconate (40-60 mg) was injected directly into the right atrium or right ventricle in six patients with angina (Rowe et al. 1970). The haemodynamic effects were strikingly different to the dog studies, with a decrease in heart rate, cardiac output, left ventricular work and stroke work indices, while total peripheral and pulmonary resistances increased. In addition, there was evidence of reduced coronary blood flow, with an increase in coronary arterio-venous oxygen content difference. However, continuous flow measurements were not made in these experiments and a transient vasodilatory effect may have been missed as the single coronary flow determination was made 10-20 minutes after administration of the drug.

Pepine et al. (Pepine et al. 1974) reported that, in a group of 19 patients with angina, two weeks of perhexiline therapy had no significant effect on the resting heart rate or left ventricular filling pressure, but increased both the cardiac index and the stroke work index. With exercise, the heart rate increased significantly less (than during the control period) and the stroke work index increased at lower left ventricular filling pressures, while both lactate and oxygen extraction improved. With pacing-induced tachycardia, perhexiline's favourable effects on ischaemia persisted, as evidenced by improved myocardial lactate extraction and increased anginal threshold. Thus, perhexiline appears to lessen exercise-induced ischaemia and help preserve left ventricular function. The precise basis for limitation of exercise-induced tachycardia was not determined, but was not the complete answer given the comparable protective effects when heart rates were controlled during atrial pacing.

The only study of effects on coronary flow in man was reported in 13 patients with angina and evidence of subnormal coronary flow (as measured by de Vernejoul's radiocardiographic technique) at baseline (Vacheron, 1978). Compared with 7 patients administered a placebo, 6 subjects treated with perhexiline maleate 400 mg daily for 8 weeks in a double-blind manner exhibited no significant difference in the coronary fraction of cardiac flow, suggesting a lack of clinical coronary vasodilatation (although anti-anginal effects were not reported in this study).

Although some clinical studies have also reported a modest affect of perhexiline on limiting tachycardia during treadmill testing (Grupp et al. 1970; Morgans & Rees,

1973; Morledge, 1973), this effect was not seen in other studies (Horgan et al., 1981; Lyon et al. 1971; Mir & Kafetzakis, 1978), and is insufficient to explain the marked anti-anginal efficacy of the drug. The greater efficacy of perhexiline, both in comparison to, and incremental to, maximal beta adrenoceptor blockade also suggests an alternative mechanism of therapeutic action (see below).

1.6.2 Clinical efficacy

In a large number of short-term (2-12 weeks) double-blind placebo-controlled crossover trials during the 1970s, perhexiline maleate monotherapy (usually given empirically at 300-400 mg daily) was shown to be effective in over 70% of patients with angina. Perhexiline therapy produced a significant decrease in frequency of anginal attacks, consumption of sublingual nitro-glycerine tablets, and an increase in exercise duration on treadmill or bicycle exercise tests compared to placebo (see Table 1.2) (Bleifer et al. 1972; Brown et al. 1976; Burns-Cox et al. 1971; Hoekanga et al. 1973; Horgan et al. 1981; Lyon et al. 1971; Mir & Kafetzakis, 1978; Morgans & Rees, 1973; Morledge, 1973). Many of these studies involved patients with angina refractory to β-adrenoceptor antagonists. Furthermore, in all of these studies, there were only minor adverse effects (especially nausea and dizziness) during the period of perhexiline therapy.

In another early study, 6 weeks of perhexiline monotherapy was directly compared to β-adrenoceptor antagonist monotherapy in a double blind crossover study of 45 patients with frequent stable angina. Despite a significant improvement in both

anginal frequency and glyceryl trinitrate consumption with the use of β-adrenoceptor antagonists compared to placebo, there was a further highly significant ($P < 0.001$) advantage of perhexiline over the β-adrenoceptor antagonists in both measures of anti-anginal efficiency. (Armstrong, et al., 1974).

Two studies have confirmed the incremental antianginal effects of perhexiline in patients refractory to 'conventional' antianginals (Cole, et al., 1990; White & Lowe, 1983), as well as improved exercise performance (Cole, et al., 1990; White & Lowe, 1983) and quality of life (Cole, et al., 1990). In a double-blind placebo-controlled crossover trial in 20 patients with severe angina despite maximal β-adrenoceptor antagonist therapy, significant incremental effects on anginal frequency, nitroglycerin consumption, and mean exercise duration were observed with the addition of perhexiline (White & Lowe, 1983). Cole et al., in the most recent published study, used a randomised double-blinded placebo-controlled crossover design in 17 patients with angina refractory to maximal 'conventional' antianginal therapy (maximally tolerated doses of nitrates, β-adrenoceptor antagonists, and calcium channel antagonists), and found that 63% had a significant objective response to perhexiline on exercise testing (c.f. 18% with placebo), while 65% noted a significant subjective improvement in their symptoms while on perhexiline (c.f. none on placebo) (Cole et al., 1990).

In addition, short-term perhexiline therapy for patients on the waiting list for elective coronary artery bypass surgery was also shown to be highly effective in control of

anginal symptoms (Armstrong, 1976). Short-term therapy appeared safe, but following the reports of serious toxicity during long-term (> 3 months) therapy in the late 1970's (see below), perhexiline was no longer utilised as a first line antianginal agent, but rather as an alternative when other agents were ineffective or contra-indicated, or alternatively as a short-term agent prior to revascularization.

1.6.3 Toxicity

Despite its proven antianginal efficacy, enthusiasm for the use of perhexiline waned in the late 1970s due to increasing awareness of side effects associated with the drug. These may be divided into short-term toxicity, long-term toxicity, the specific interaction with oral hypoglycaemic agents, and the potential for torsade de pointes..

1.6.3.1 Short-term toxicity

The early studies, using doses of 300-400 mg/day, reported a high incidence (up to 50%) of minor side effects such as dizziness, nausea, and tremor, which were all reversible on withdrawal of the drug (Burns-Cox et al. 1971; Lyon et al. 1971). In a more recent study, a loading regimen of 400 mg/day for three days only, followed by 200 mg/day, was associated with the development of transient nausea and/or dizziness in approximately 10% of patients (Stewart et al. 1996).

1.6.3.2 Interaction with oral hypoglycaemics

An increased incidence of hypoglycaemic attacks has been reported in diabetics on oral hypoglycaemic agents during therapy with perhexiline (Button et al. 1993). Perhexiline appears to induce or aggravate hyperinsulinism as reflected by delayed

and increased insulin secretion following an oral glucose load (Luccioni & Trigano, 1973). Many diabetics may require a decrease in dosage of oral hypoglycaemics when commenced on perhexiline.

1.6.3.3 Long-term toxicity

In prolonged treatment (>12 weeks), weight loss and mild elevations in liver transaminases have been noted, but are reversible on withdrawal of the drug. Of most concern was the infrequent but unpredictable occurrence of serious hepatic (Lewis et al. 1979; Pessayre, et al., 1979) and neurological (Lhermitte et al. 1976) toxicity amongst patients receiving chronic perhexiline therapy. On the other hand, treatment with perhexiline for less than three months is rarely associated with side-effects requiring cessation of treatment, regardless of the treatment regimen used (Bleifer, et al. 1972; Hoekanga et al. 1973; Horowitz et al. 1986; White & Lowe, 1983).

Serious neuro- or hepatotoxicity were usually seen in patients with high plasma concentrations and slow plasma clearance of perhexiline compared to patients without these complications on equivalent doses of the drug, indicating that toxicity was associated with impaired metabolism or clearance of perhexiline (Singlas et al. 1978) (see below). These toxic effects were also reversible on withdrawal of perhexiline, except when cirrhosis developed from chronic liver disease (Pessayre et al., 1979). Interestingly, the early studies in the 1970s involved empiric perhexiline doses of 300-400 mg daily, a much higher dose than is currently recommended in most patients. With the advent of straightforward reliable methods of quantifying blood

levels of perhexiline, individualization of dosing has led to a pronounced decrease in the incidence of side effects (Cole, et al., 1990; et al. 1986).

The cellular mechanism of perhexiline toxicity is moderately well understood. Histological investigations indicate the progressive development of drug-induced phospholipidosis, with enlarged lysosomes containing myeloid figures in hepatocytes, Schwann cells, and other tissues (Albert & Lullmann-Rauch, 1983; Lhermitte, et al. 1976; Pessayre & Larrey, 1988). The lysosomal phospholipidosis has been ascribed to the cationic amphophilic structure of perhexiline, resulting in trapping of the protonated form of the drug in the acidic intralysosomal milieu and the formation of reversible complexes with phospholipids, which inhibit the action of intralysosomal phospholipases, resulting in impaired digestion of phospholipids which accumulate within the enlarging lysosomes (Kodavanti & Mehendale, 1990; Lullmann, et al. 1978; Pessayre & Larrey, 1988). These same ultrastructural changes have been documented with amiodarone, another cationic amphophilic drug (Lullmann, et al. 1978; Simon et al. 1984). This phospholipidosis appears to have little clinical significance (Pessayre & Larrey, 1988). However, in a few patients, it is associated with pseudoalcoholic liver lesions, including the presence of microvesicular and macrovacuolar steatosis and Mallory bodies, which may progress to overt liver disease and even cirrhosis (Guigui, et al., 1988; Lewis et al. 1979; Pessayre, et al., 1979; Pessayre & Larrey, 1988).

1.6.3.4 Torsade de pointes

A single case of torsade de pointes, associated with QT prolongation, has been reported (Kerr & Ingham, 1989). The underlying mechanism is probably blockade by perhexiline of the rapidly activating delayed rectifier K+ channel, which has been reported in both human atrial myocytes and in cells cloned from human heart (Rampe, et al., 1995). Although reports of ventricular arrhythmias with perhexiline have been rare, the combination of perhexiline therapy with other drugs acting in a similar fashion on potassium channels (e.g. amiodarone (Sato, et al., 1994)) may potentially lead to an increased risk of torsade de pointes.

1.6.4 Pharmacokinetics

Perhexiline is rapidly absorbed from the gastrointestinal tract after oral administration and distributes to the tissues as a mixture of unchanged perhexiline and more water-soluble mono- and dihydroxylated metabolites (Pexid Product Summary for Physicians, 1982). Perhexiline is a very lipophilic compound, and appears to have a large volume of distribution, with mean tissue to plasma ratios of radioactivity 2 hours after oral dosing with perhexiline-^{14}C maleate in rats of 47.4 in lung, 52.3 in liver, 20.5 in kidney, and 6.8 in heart (Leeson, et al. 1969).

Perhexiline undergoes classic "phase I" oxidative metabolism in the liver by a specific isoform of the cytochrome P-450 family of related enzymes (P-450_{dbl}), the major products being the stereoisomers cis- and trans-monohydroxyperhexiline (M_1 and M_3, respectively) and, to a lesser extent, dihydroxyperhexiline (Cooper et al.

1987). There is very little information available as to the possible antianginal activity of the various hydroxylated perhexiline metabolites, although these are unlikely to contribute significantly to toxicity (Singlas et al. 1978). Importantly, hepatic metabolism has been shown to be saturable at "therapeutic" doses of the drug (Horowitz et al. 1981). Hence the pharmacokinetics of perhexiline should best be regarded as Michaelis-Menten, with K_m and V_{max} values of approximately 500ng/ml and 600mg/day respectively in most patients (Horowitz, et al. 1981).

The enzyme responsible for oxidation of perhexiline (P-450dbl/CYP2D6) exhibits genetic polymorphism, and is under single gene control, recently localised to chromosome 22 (Bertilsson, 1995). Oxidation is regulated by two alleles: D^H for rapid and extensive metabolism, and D^L for slow, impaired metabolism (Sloan et al. 1978). 5-10% of Caucasians are homozygous for D^L ('slow hydroxylators'), and have an impaired ability to oxidise several drugs, including perhexiline, debrisoquine, and several β-adrenoceptor antagonists and Class 1C antiarrhythmics (Cooper et al. 1984). Substrate stereoselectivity is apparent, as slow hydroxylators demonstrate markedly reduced production and excretion of the major metabolite, M_1, compared to fast hydroxylators (Cooper et al. 1984).

In slow hydroxylators, saturation of hepatic metabolism and a switch from first-order to zero-order elimination kinetics occurs at relatively low doses of the drug, resulting in dramatically lengthened perhexiline elimination times (Horowitz et al. 1981; Pilcher et al., 1985). These patients have been shown to have a markedly increased

risk of peripheral neuropathy and hepatotoxicity if plasma perhexiline levels are not closely monitored (Morgan et al., 1984; Shah et al. 1982).

1.6.5 Circumvention of toxicity

It was first reported in 1978 that long-term adverse effects of perhexiline only occurred in the presence of high plasma perhexiline. Singlas et al. (et al. 1978) reported higher mean plasma perhexiline levels in 13 patients, each receiving 100-400 mg daily, with documented neuropathy compared to patients without neuropathy. In thc "non-toxic" group, mean plasma perhexiline concnetrations were 1.07 ± 0.19 (SD) mg/L, with M_1 metabolite concentrations of 3.83 ± 0.47 mg/L. In the "toxic" group, perhexiline and M1 concentrations were 3.78 ± 0.43 and 1.62 ± 0.23 mg/L respectively. Hence the long-term adverse effects of perhexiline were strongly associated with elevation of steady-state plasma drug concentrations and impaired perhexiline metabolism. All patients with toxicity had plasma levels of >2 mg/L, although due to its retrospective nature this study was unable to determine the lowest levels at which early toxicity may occur.

While subsequent studies confirmed 'slow hydroxylators' had increased risk of serious toxicity (Morgan, et al., 1984; Shah et al. 1982), peripheral neuropathy and hepatotoxicity have also been documented in the presence of 'normal hydroxylator' status (Jallon et al. 1978; Morgan et al., 1984). Two prospective studies involving patients on chronic perhexiline therapy, with dosages adjusted according to clinical response, showed that adverse effects only occurred with plasma perhexiline levels of

>700ng/mL (Horowitz et al. 1986; Pilcher et al., 1985). Further long-term studies using plasma drug monitoring to guide dosages, aiming for a 'therapeutic' range of 0.15-0.60 mg/L, confirmed complete absence of toxicity without loss of therapeutic efficacy (Cole et al., 1990; Horowitz et al. 1986). Even with plasma levels in the range 0.6-2.0 mg/L, reports of severe toxicity are rare, and it is possible that in some cases incremental therapeutic response may be achieved associated with such higher plasma perhexiline concentrations.

1.6.6 Mechanism of therapeutic action

The mechanism of the antianginal action of perhexiline remains incompletely understood, the early theories based on its possible haemodynamic effects having been discounted in clinical practice. On an extensive review of the literature up to 1980, Vaughan Williams (Vaughan Williams, 1980) summarised fragmentary evidence for unique effects of perhexiline on myocardial metabolism, consisting largely of a shift from fatty acid to carbohydrate utilisation by ischaemic myocardium. This would result in sparing myocardial oxygen utilisation, due to an approximate 6-13% increase in ATP produced per O_2 consumed when glucose rather than fatty acid is used as substrate (see below) (Liedtke, 1981; Vik-Mo & Mjos, 1981), thus providing a critical margin of increased efficiency when oxygen supplied to the myocardium is limited by obstructive coronary artery disease. This change in substrate utilisation, associated with an improvement in myocardial efficiency, has recently been confirmed in an *in vitro* animal model (Jeffrey et al. 1995). In an isolated working rat heart model, the metabolism of palmitate was reduced while

lactate utilisation was increased in the presence of "therapeutic" concentrations in the perfusate (2 μM, i.e. 0.56 mg/L). These changes corresponded to an increase in cardiac output in the absence of any significant change in oxygen consumption, indicating enhanced cardiac efficiency (Jeffrey et al. 1995). Furthermore, perhexiline has recently reported to inhibit CPT-1 in isolated rat myocardial and hepatic mitochondria (Kennedy et al. 1996).

The metabolic anti-ischaemic actions of perhexiline could also explain the reported anti-arrhythmic benefits of the drug (Vaughan Williams, 1980). Furthermore, this biochemical shift would accord with the efficacy of perhexiline in patients with classical, rather than vasospastic, angina pectoris (Theroux et al., 1979); its lack of negative inotropic effects in normal therapeutic doses (indeed even a weak positive inotropic effect may occur) (Pepine et al. 1974); and even its potential for eventual development for toxicity, leading to both hypoglycaemia and the formation of intracellular fatty inclusion bodies in the liver and Schwann cells via possible tissue deposition of non-metabolised lipids (Vaughan Williams, 1980). Indeed, Deschamps et al, examining the biochemical basis for toxic effects of perhexiline in cultured rat hepatocytes, reported that moderately high concentrations of perhexiline (5 μ*M*) inhibited mitochondrial β-oxidation of fatty acids (Deschamps et al., 1994).

Another potentially favourable action of perhexiline in myocardial ischaemia is inhibition of platelet aggregation, although its mechanism of action remains uncertain (Ono & Kimura, 1981; Willoughby et al., 1998). Inhibition of aggregation induced

by ADP, adrenaline and collagen in human platelet-rich plasma by perhexiline did not correlate in potency with its calcium channel blocking effect, when compared with the anti-aggregatory agents such as verapamil (Ono & Kimura, 1981). Perhexiline has also been reported to inhibit platelet aggregation in response to multiple agonists (adrenaline, ADP, serotonin, and thrombin) in whole blood, although no such anti-aggregatory effects were seen with the CPT-1 inhibitors malonyl-CoA, etomoxir, or hydroxyphenylglyoxylate (Willoughby et al., 1998).

1.6.7 The role of perhexiline in the 21st century

The treatment of stable angina aims to relieve symptoms and in at-risk cases prolong life. "Conventional" (haemodynamically active) medical therapy, revascularisation techniques, and "metabolic" therapies (including perhexiline) should be seen as complimentary, not competitive, strategies. Utilisation of all three treatment modalities either alone or in combination may be necessary to provide optimal anti-ischaemic benefits in patients with angina pectoris. At present, the major clinical role of perhexiline is the treatment of severe angina refractory to all other "conventional" (medical or surgical) therapies.

With the ageing of the population in Western countries, the elderly are making up an ever greater proportion of patients suffering from myocardial ischaemia. In this age group, the two major causes of myocardial ischaemia are atherosclerotic coronary disease and calcific aortic stenosis. In these patients in particular, surgical revascularisation approaches are less likely to be utilised (Edmunds et al. 1988), and

valve replacement for aortic stenosis is often not considered (Abdul-Hamid & Mulley, 1999; Bouma, et al., 1999). The reasons for not operating in the elderly include increased peri-operative mortality and risk of serious complications such as stroke, increased prevalence of significant comorbidities, and increased cost to the public health system due to more prolonged stays in intensive care facilities.

In these patients, particularly those with aortic stenosis in whom haemodynamically active therapies are contra-indicated, metabolic treatments may have much to offer. Perhexiline, which in clinical trials seems to be the most effective non-haemodynamically active agent in the treatment of chronic angina pectoris, seems ideally suited in these patients. In a recent study of 15 elderly patients with severe symptomatic aortic stenosis, perhexiline therapy (dosage titrated according to steady state plasma perhexiline concentrations) resulted in a marked improvement in clinical status over a period of three months (Unger et al. 1997).

In addition, metabolic strategies such as GIK have shown great promise when added to conventional treatment in the setting of acute myocardial infarction (Diaz, et al. 1998; Faith-Ordoubadi & Beatt, 1997). The development of a parenteral formulation of perhexiline may potentially benefit those patients presenting to the coronary care unit with acute coronary syndromes. In the only study in such patients to date (Stewart et al. 1996), perhexiline was added using an oral loading dose of 400 mg daily to maximal conventional therapy (including heparin, intravenous nitrates and verapamil). Although the numbers were small, a correlation between early resolution

of angina and achievement of therapeutic plasma perhexiline levels was suggested (P = 0.06). There is clearly a need for further investigations into the rate of myocardial uptake of perhexiline, to determine whether parenteral formulations of perhexiline would potentially provide further anti-ischaemic benefits in acute coronary syndromes.

1.7 EXPERIMENTAL TECNIQUES USED TO MEASURE CARDIAC ENERGY METABOLISM

The major aim of the proposed research is to confirm the metabolic shift induced by perhexiline, under both *in vitro* and *in vivo* conditions. It would be appropriate at this stage to briefly review the various experimental techniques available in the assessment of myocardial metabolism. They include biochemical measurement of metabolites and enzymes of intermediary metabolism, measurement of high energy phosphates with ^{31}P nuclear magnetic resonance, measurement of tricarboxylic cycle activity with ^{13}C nuclear magnetic resonance, measurement of the rate of flux through the major pathways of intermediary metabolism with ^{14}C- and ^{3}H-labelled carbon substrates, measurement of glucose uptake and oxidative metabolism with positron emission tomography, and measurement of the rate of fatty acid utilisation using radio-iodinated fatty acids.

1.7.1. Coronary sinus catheterisation

The most direct approach to measuring energy metabolism in man involves catheterisation of the coronary sinus. This allows the simultaneous measurement of

carbohydrate and fatty acid levels in arterial blood and the coronary sinus. Measurement of the coronary flow rate allows calculation of net rates of carbohydrate and lipid utilisation. This approach was first used by Bing in the 1950s (Bing, 1955), who went on to characterise energy substrate preference by the heart under a variety of physiologic and pathologic conditions (Bing, 1964).

This approach was refined by Wisneski et al who utilised ^{14}C glucose and ^{13}C lactate, allowing direct comparison of glucose uptake in relation to oxidation and simultaneous measurement of lactate uptake and release (Wisneski et al. 1990). These investigators showed that the previous methods gave erroneous estimates of flux through the various pathways of intermediary metabolism. Using ^{14}C glucose, they determined that only a portion of the glucose taken up by the heart is immediately oxidised, particularly if the subject has hyperglycaemia or is fasting. As a result, previous estimates of the contribution of glucose to ATP production under these conditions were found to be overestimates.

Although coronary sinus catheterisation and the use of radiolabelled substrates is a sophisticated approach to measuring energy metabolism directly in vivo, it is also a highly invasive procedure. Availability of research subjects, cost, and ethical considerations have limited this approach to the study of energy metabolism (Lopaschuk, 1997).

1.7.2 Biochemical tissue analysis

Biochemical tissue analysis allows measurement of most intermediates of energy metabolism. Examples include the levels of high energy phosphates, lactate and triglyceride levels in frozen or lypophilised tissue samples, and the isolation and measurement of the activity and kinetics of individual enzymes involved in intermediary metabolism in either intact tissue or subcellular particles such as mitochondria. The need for tissue samples limits this approach to in vitro experiments, or to invasive procedures such as myocardial biopsy during cardiac bypass surgery or explanted hearts from cardiac transplant recipients.

The comparison of the ratios of high energy phosphates such as ATP and creatine phosphate to ADP and AMP makes it possible to estimate the energetic status of the heart at the time the tissue sample is frozen (Lopaschuk, 1997). However, this technique does not provide information about the rates of production or utilisation of ATP, nor about the carbon source used for the high energy phosphate production. This requires the use of ^{13}C nuclear magnetic resonance spectroscopy or radiolabelled substrates (see below).

Analysis of mitochondrial preparations requires large amounts of tissue but allows measurement of many of the enzymes involved in fatty acid transport and β-oxidation, pyruvate oxidation, tricarboxylic acid cycle activity, and the electron transport chain. However, while biochemical measurement of enzyme activities and tissue expression can provide useful information about the control sites of cellular and

mitochondrial metabolism, the rates of flux through individual enzymes in an intact working heart cannot be determined with this approach (Lopaschuk, 1997).

1.7.3 Nuclear magnetic resonance (NMR) spectroscopy

The use of nuclear magnetic resonance spectroscopy allows assessment of molecular structure and concentration. When a magnetic field is imposed on a tissue sample, the nuclei align with and against the field, producing a "magnetic moment" (a magnetic field generated by spinning nuclei) parallel to the applied field. A radiofrequency pulse is used to perturb the orientation of the net magnetisation, and on discontinuation of the pulse a radiofrequency signal is released. If nuclei of the same species have the same resonant frequency, a single narrow peak in the NMR spectrum is observed.

A number of biologically important nuclei can be measured with this approach, including ^{31}P and ^{13}C. ^{31}P NMR enables the direct measurement of intracellular pH and provides information on the levels of high energy phosphates in the intact heart (Lopaschuk, 1997). Madden et al used this technique in isolated Langendorff-perfused rat hearts and reported less severe acidosis during 20 minutes of global ischaemia, and increased ATP content during reperfusion (associated with improved post-ischaemic recovery of function), in hearts perfused with both palmitate and hexanoate (a medium-chain fatty acid not dependent on CPT-1 to enter mitochondria) compared to hearts perfused with palmitate alone (Madden et al. 1995). The addition of the CPT-1 inhibitor oxfenicine to those hearts perfused with both palmitate and

hexanoate resulted in a small further benefit in pH during ischaemia and post-ischaemic ATP content and recovery of function, although glucose was not present in the perfusate. However, Burkhoff et al, using the same technique, reported that myocardial efficiency and high energy phosphate levels during low coronary flow rates were lower when hexanoate was the sole substrate, compared to glucose alone (Burkhoff, et al., 1991). As with biochemical techniques, the major limitations of ^{31}P NMR spectroscopy are the inability to measure the rates of ATP production or utilisation, and to elucidate which pathways are responsible for the ATP production (Lopaschuk, 1997).

^{13}C NMR spectroscopy, utilising ^{13}C-enriched substrates such as glucose, long chain fatty acids, lactate, and acetoacetate, allows direct measurement of flux through various metabolic pathways (Jeffrey et al. 1995). For example the use of ^{13}C glucose allows one to follow the metabolic fate of glucose, including incorporation of ^{13}C into the intracellular glycogen pool.

Much of the experimental cardiac work using this approach comes from Malloy's group, who have reported that carbohydrate flux through the PDH complex is increased more than eightfold in non-ischaemic working rat hearts in the presence of dichloroacetate. However, dichloroacetate had no significant effect on PDH flux during reperfusion following 30 minutes of ischaemia despite improving mechanical recovery, a finding they were unable to explain (Barak, 1998). This group was also the first to report a "metabolic shift" induced by perhexiline (Jeffrey et al. 1995). In

this landmark paper, they reported that perhexiline (2μM) improved myocardial efficiency, accompanied by a reduction in FFA and endogenous substrate utilisation and increased lactate utilisation, in non-ischaemic working rat hearts. However, the authors were unable to explain the lack of glucose utilisation (0%) in either control or perhexiline preparations. This may be due to flawed methodology or to non-physiological conditions, as no insulin was present in the perfusate.

One major limitation to the widespread use of ^{13}C NMR spectroscopy is the high cost of the NMR system and stable ^{13}C isotopes, particularly ^{13}C fatty acids. Another limitation is the complicated kinetic modelling and requirement of non-steady state conditions (Lopaschuk, 1997), as once the intracellular metabolite becomes equilibrated with the label, flux through the pathways can no longer be measured. Although this technique is potentially amenable to in vivo studies, most work to date, including that reported by Malloy's group, has been performed on tissue samples from isolated heart preparations.

1.7.4 ^{14}C- and ^{3}H- labelled substrates

The use of beta-emitting radiolabelled energy substrates, such as ^{14}C or ^{3}H labelled carbohydrates and fatty acids, allows direct analysis of the source of cardiac ATP production, as well as the rates of flux through the various pathways involved in energy metabolism. This includes direct measurement of glycolysis, glucose oxidation, lactate oxidation, and fatty acid oxidation. Use of ^{3}H- or ^{14}C-isotopes is a

relatively inexpensive way to measure energy metabolism, and no highly specialised equipment is needed to detect these isotopes.

Although this method has been used *in vivo* in conjunction with coronary sinus catheterisation (see above) (Wisneski et al. 1990), the use of this approach has been limited by cost and technical difficulties involved in the quantitative collection of $^{14}CO_2$ It is more ideally suited to isolated heart models, such as the working rat heart (see Chapter 4). With the use of radioisotopes labelled at specific hydrogen or carbon sites on the various energy substrates, it is possible to quantitate the rate of flux via individual metabolic pathways via measurement of the production of $^{3}H_2O$ or $^{14}CO_2$ produced by the heart. For example, the addition of [5-^{3}H]glucose to the perfusate results in the production of $^{3}H_2O$ at the enolase step of glycolysis while the use of ^{14}C-labelled glucose results in $^{14}CO_2$ production during glucose oxidation. Palmitate oxidation can be measured with the use of either ^{3}H- or ^{14}C- labelled palmitate. The catabolism of ^{3}H-palmitate and ^{14}C-glucose in the heart are shown in Figures 1.6 and 1.7 respectively.

This experimental approach does not require complex kinetic modelling, and allows direct steady-state measurement of flux through the various metabolic pathways. However, theoretical concerns with the use of these isotopes relate to the oversimplification of cardiac metabolic pathways as shown in Figures 5 and 6. $^{14}CO_2$ production from ^{14}C glucose not only can originate from glucose oxidation (passage through the pyruvate dehydrogenase complex and the tricarboxylic acid cycle) but

also can pass through the pentose phosphate shunt. In practise however the cardiac pentose phosphate pathway is orders of magnitude slower than flux through glucose oxidation (Lopaschuk, 1997). In the liver, it has been reported that ^{14}C-labelled fatty acids may be incompletely oxidised (Veerkamp et al. 1986); however this does not appear to be the case in the working heart model, with identical fatty acid β-oxidation rates with the use of either [U-^{14}C]palmitate or [9,10-^{3}H]palmitate under identical conditions (Lopaschuk et al. 1986; Saddick & Lopaschuk, 1992).

1.7.5 Positron emission tomography

Positron emission tomography (PET) allows noninvasive evaluation and quantitation of regional metabolic rates in the heart in vivo. This approach involves the use of high spatial and temporal resolution PET cameras and energy substrates which are labelled with positron-emitting isotopes such as ^{15}O, ^{11}C, ^{13}N, and ^{18}F. Positrons emitted from these nuclei will annihilate with an electron and produce two 511 keV γ-rays travelling in opposite directions. The detection of such a "coincidence event" by scintillation detectors placed on either side of the radiation source (heart) localises the positron annihilation to a point lying somewhere on the line that joins the two detectors. A typical PET scanner consists of hundreds of such detectors forming a ring that surrounds the subject, providing information about the quantity and spatial localisation of positron emitters within the patient. The short half-life of the positron-emitting compounds means that radiation exposure to research subjects is limited.

The use of physiologic substrates such as ^{11}C palmitate, while theoretically the most appropriate, is limited in may instances because metabolism of the tracer gives rise to numerous labelled intermediary compounds that cannot be independently distinguished on PET imaging. The early use of ^{11}C palmitate in PET metabolic studies was based on the assumption that following cardiac uptake of ^{11}C palmitate, rapid clearance of ^{11}C from the myocardium represents the production and release of $^{11}CO_2$ from the tricarboxylic acid cycle, and therefore that the rate of this clearance correlates directly with β-oxidation rates. However, back-diffusion of unmetabolised ^{11}C palmitate is a significant problem, and the kinetics of this tracer are markedly affected by circulating fatty acid concentrations, which are difficult to control *in vivo* (Bergmann, 1989). Cycling of fatty acids through endogenous triacylglycerol pools can also complicate interpretation of ^{11}C clearance kinetics in hearts from patients infused with ^{11}C palmitate, especially in the post-ischaemic period (Bergmann, 1989). Due to the complex nature by which ^{11}C palmitate is handled by the myocardium, some early investigators concluded incorrectly that fatty acid oxidation rates were impaired in postischaemic hearts (Bergmann, 1989; Schelbert, 1989). However, direct measurements of fatty acid oxidation with ^{14}C palmitate showed that fatty acid oxidation recovers rapidly and often exceeds preischaemic rates (Lopaschuk et al. 1990; Saddick & Lopaschuk, 1992; Benzi & Lerch, 1992).

The use of ^{11}C acetate overcomes many of these problems, as it has a much simpler fate in the heart, and has become an established research tool to assess oxidative metabolism and overall rates of acetyl-CoA entry into the tricarboxylic acid cycle

(Bergmann, 1989; Brown et al. 1988). However it cannot delineate the source of the acetyl CoA (PDH or fatty acid β-oxidation), and therefore cannot be used to detect shifts in energy substrate preference (Lopaschuk, 1997).

The most widely utilised cardiac PET tracer is [^{18}F]-2-deoxyglucose (FDG). While ^{11}C glucose is metabolised to numerous labelled intermediates, making analysis of the PET images difficult, FDG is taken up by the myocardium via glucose transporters and phosphorylated by hexokinase, in a similar manner to glucose. FDG-6-phosphate is however not available for further metabolism either to glycogen or pyruvate, and remains trapped intracellularly (Lopaschuk, 1997). Although its accumulation is a measure of total myocardial glucose uptake, or exogenous glucose utilisation, this cannot be extrapolated to overall glucose metabolism, not does this technique give any information regarding rates of glycolysis or glucose oxidation (Lopaschuk, 1997). The primary clinical use for FDG is its ability to identify jeopardised but viable "hibernating" myocardium, which shows evidence of a flow/metabolism "mismatch" on PET imaging when FDG is combined with a flow tracer such as ^{13}N ammonia.

The greatest limitation to PET research is the high cost, and as a result, limited availability of PET facilities. The short half-life of the positron-emitting compounds, while limiting the radiation exposure to research subjects, also requires that an expensive cyclotron be located within the vicinity of the PET imaging equipment.

1.7.6 Nuclear imaging with radio-iodinated fatty acids

The use of radio-iodinated fatty acid analogues allows in vivo imaging with standard gamma cameras available in any nuclear medicine department (see Figure 1.8 for structures of ^{123}I-labelled fatty acids used both clinically and in animal models). Myocardial imaging using radiolabelled fatty acids was first performed by Evans et al using ^{131}I-labelled oleic acid (Evans et al. 1965). However this substance never became clinically useful because of its low specific activity, the poor imaging properties of ^{131}I, and limitations in administered activity. Moreover, the addition of radioiodine to the double bond of the long chain fatty acid resulted in altered extraction and elimination compared to its natural analogue.

The 159 keV γ photon energy and 13 hour half-life of ^{123}I makes this an ideal tracer for imaging in nuclear medicine, although its availability is somewhat limited by the need for a cyclotron for its production. In 1978 Machulla et al. experimentally used various radiolabelled FFA and showed that terminally labelled [17-^{123}I]-heptadecanoic acid (HDA) had a myocardial uptake and elimination almost the same as that of ^{11}C-palmitate (Machulla et al., 1978). In vitro work by Luthy et al. showed that the deiodination of ^{123}I-HDA was reduced in isolated rat hearts by either the addition of the CPT-1 inhibitor POCA to the perfusate, or by pre-treatment of the rats with POCA (Luthy et al. 1988). Further clinical studies however were hampered somewhat by rapidly increasing background radioactivity due to release of free radioiodide into the circulation as the radioactive by-product of ^{123}I-HDA oxidation in the heart and liver.

Machulla was also the first to propose the use of radioiodinated phenyl fatty acids (Machulla et al. 1980) such as 15-(*p*-[^{123}I]iodophenyl)pentadecanoic acid (IPPA). The advantages of these compounds over the alkyl fatty acids include more stable carbon-iodine bonds, and less radioactive background in clinical models. The radioactive end-product resulting from β-oxidation of these compounds is iodobenzoic acid, which is rapidly eliminated from the circulation via hepatic conjugation with glycine to form iodo-hippuric acid which is renally excreted (see figure 1.9). Hence no correction for catabolites is required, in contrast to ^{123}I-HDA.

Reske et al compared IPPA with ^{14}C-palmitic acid in a series of experiments in the mid-1980s. Analysis of radiolabelled metabolites in the aqueous, solid and lipophilic subfractions in hearts, lungs, livers, and kidneys of rats following intravenous injection of IPPA and ^{14}C-palmitic acid showed significant correlation (Reske, 1985; Reske et al. 1984). When IPPA and ^{14}C-palmitic acid were administered simultaneously to perfused Langendorff rat hearts, both catabolic markers of oxidative metabolism (^{123}I-benzoic acid and $^{14}CO_2$ respectively) were released concurrently and in the same order of magnitude (Reske et al. 1984). This relationship remained unchanged during metabolic interventions designed to increase (isoproterenol) or decrease (lactate) fatty acid oxidation (Reske, et al., 1986). They concluded that IPPA can be considered an ideal metabolic analogue of palmitic acid for the investigation of cardiac metabolism.

Although ideal for functional studies of myocardial fatty acid turnover rates, IPPA is not well suited to clinical investigation of coronary artery disease, as the rapid washout precludes the use of single photon emission computed tomography (SPECT), the optimal method for localisation of perfusion or metabolic abnormalities. The time required by SPECT for camera rotation (approximately 20 minutes) requires minimal tracer distribution during the acquisition period. Later modifications included the incorporation of one or more methyl groups at various positions along the carbon chain of the phenyl fatty acids. While these agents, such as 15-(*p*-[^{123}I]iodophenyl)-3-R,S-methylpenta-decanoic acid (BMIPP), are extracted by the heart in a similar fashion to native fatty acids (such as palmitic acid), they are not catabolised and remain trapped in the myocardium for prolonged periods of time (c.f. FDG in PET imaging), allowing 3 dimensional SPECT imaging (Schelbert, 1999).

Experimental studies have revealed similar pharmacokinetics in the heart for both ^{11}C-palmitic acid and for radioiodinated fatty acids such as HDA or IPPA (Machulla et al., 1978; Poe et al. 1976; Reske et al. 1984). The kinetics exhibit a fast uptake, representing cardiac extraction from the blood, which has been shown to be a reflection of perfusion in studies comparing $^{13}NH_3$ and ^{11}C-palmitic acid (Schelbert, et al. 1981). This is followed by two elimination phases, a fast and a slow one. The fast elimination phase is considered to represent β-oxidation and is clinically the most relevant phase. The third phase can be attributed to the slow release of fatty acids which have been stored initially as triglycerides and phospholipids (Reske et al. 1985; DeGrado et al. 1989). Hence, in an *in vivo* model, a biexponential time activity curve

can be obtained using planar imaging, and half-clearance times calculated for each phase.

DeGrado et al. were the first to use IPPA to analyse the response to a CPT-1 inhibitor (DeGrado et al. 1989). Following bolus injection of ^{125}I-labelled IPPA, radioactivity in the isolated perfused working heart was monitored using external scintillation probes. The early clearance of activity was significantly delayed in hearts from rats pre-treated with POCA. In another study, rats were injected with IPPA two hours following intraperitoneal injection of POCA, and then sacrificed at various time points (Kaiser et al., 1990). Compared to control rats, the POCA treated animals showed progressive myocardial retention of IPPA, reaching a plateau at 10 to 20 minutes. Dormehl et al. (Dormehl et al. 1993) used larger animals for in vivo imaging of the effect of CPT-1 inhibition. Six baboons underwent dynamic planar scintigraphy following intravenous IPPA, with a mean half clearance time of the first phase of 24.0 $\pm$ 2.6 minutes. A repeat study performed 2 hours post intravenous etomoxir showed significant prolongation of this measure to 55.0 $\pm$ 13.0 minutes. These studies confirm that IPPA is a sensitive tool for analysing the effect of CPT-1 inhibition on cardiac metabolism.

1.8 SCOPE OF THE CURRENT STUDY

Despite being in clinical usage as an anti-anginal agent for nearly 30 years, the precise nature of perhexiline's anti-ischaemic effect has not been fully elucidated. Fragmentary evidence points to a primary metabolic effect, including the lack of

clinically significant haemodynamic effects and toxic effects including hypoglycaemia and long-term lipid accumulation in various tissues in animals and man. This may point to a role for perhexiline as one of the most widely used and clinically efficacious agents in a new class of anti-anginals, the "metabolically active agents", which also includes among its members GIK therapy, L-carnitine, CoQ10, trimetazidine, ranolazine, and potentially the thiazolodinediones. Although the role of perhexiline as a prophylactic anginal agent has been well documented, its role in acute ischaemic syndromes remains unclear.

CPT-1 inhibitors, although widely proven in laboratory models of cardiac ischaemia, have not survived the transition to clinical usage due to fears of long-term toxicity. Few studies to date have linked CPT-1 inhibition with clinically proven anti-anginal efficacy. Recent work in our own laboratory however has shown that perhexiline, and to a lesser extent trimetazidine and amiodarone, inhibit CPT-1 in isolated rat heart and liver mitochondria (Kennedy, 1998; Kennedy et al. 1996). Inhibition of fatty acid β-oxidation by perhexiline in rat hepatocytes had also been reported (Deschamps, et al., 1994), and a reduction in palmitate use, combined with increased lactate utilisation, was recently reported in isolated rat hearts treated with perhexiline by Jeffrey et al. (Jeffrey et al. 1995).

The aims of the current study were directed at further elucidating the link between perhexiline and alterations in cardiac substrate utilisation. The major aims were to (i) assess the haemodynamic effects of perhexiline in an *in vivo* model; (ii) to assess the

metabolic effects of perhexiline for the first time in an *in vivo* model; and (iii) quantitate the changes in substrate utilisation induced by perhexiline in an *in vitro* model, and correlate these changes to effects on haemodynamics and myocardial efficiency.

Chapter Two describes the set-up of a chronically instrumented sheep model, which is used to assess the pharmacokinetics and haemodynamic effects of intravenous perhexiline in conscious animals.

In Chapter Three, an *in vivo* model, using nuclear medicine techniques with ^{123}I labelled long chain fatty acids in sheep, is used to assess cardiac fatty acid utilisation. Effects of both acute and longer term (24 hours) intravenous perhexiline administration are presented, and correlated with plasma perhexiline concentrations.

Chapter 4 describes development of the isolated working rat heart model, and its use in the measurement of myocardial metabolism using ^{3}H and ^{14}C labelled substrates. Results of experiments using acute perhexiline exposure are presented, and metabolic and haemodynamic effects are compared to those of known CPT-1 inhibitors, etomoxir and oxfenicine. Further *ex vivo* experiments in perhexiline pre-treated rats are also described.

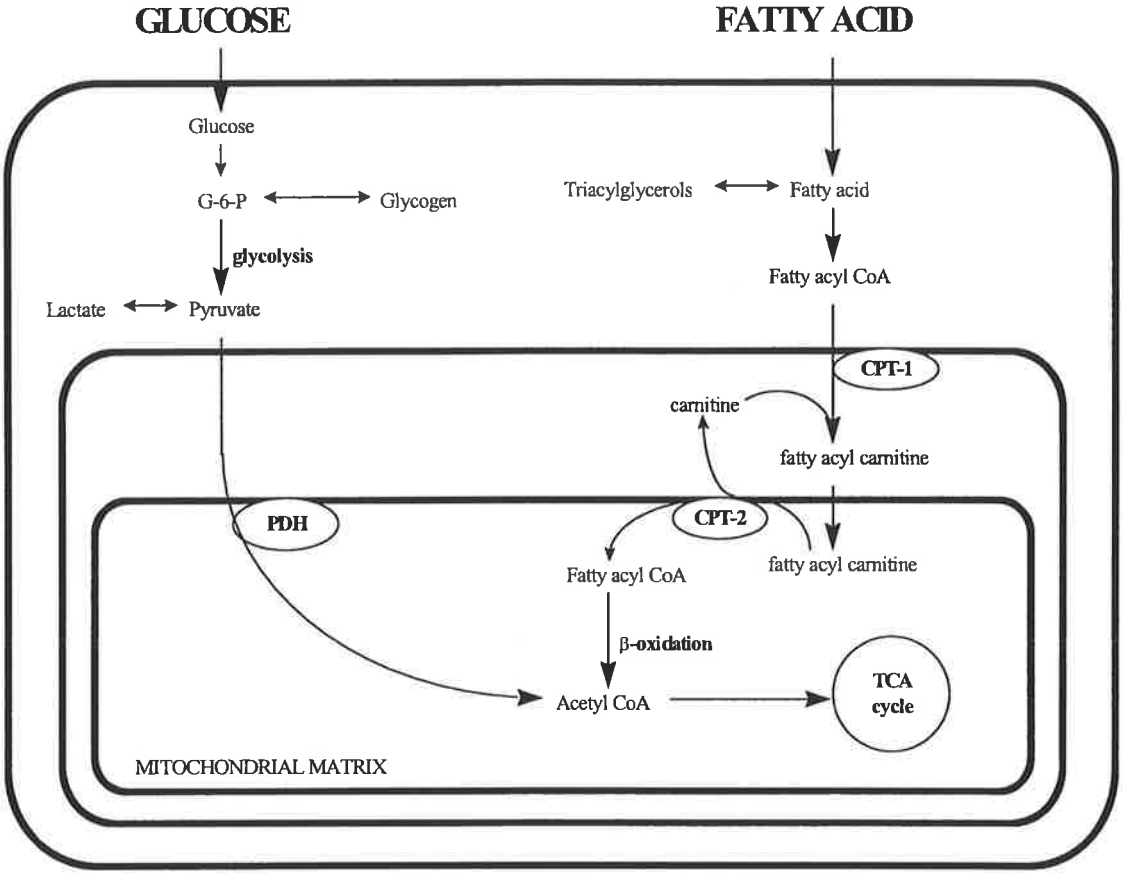


Figure 1.1 Overview of glucose and fatty acid metabolism in the heart. G-6-P = glucose-6-phosphate; PDH = pyruvate dehydrogenase; CPT-1 = carnitine palmitoyl-transferase-1; CPT-2 = carnitine palmitoyl-transferase-2; TCA = tricarboxylic acid.

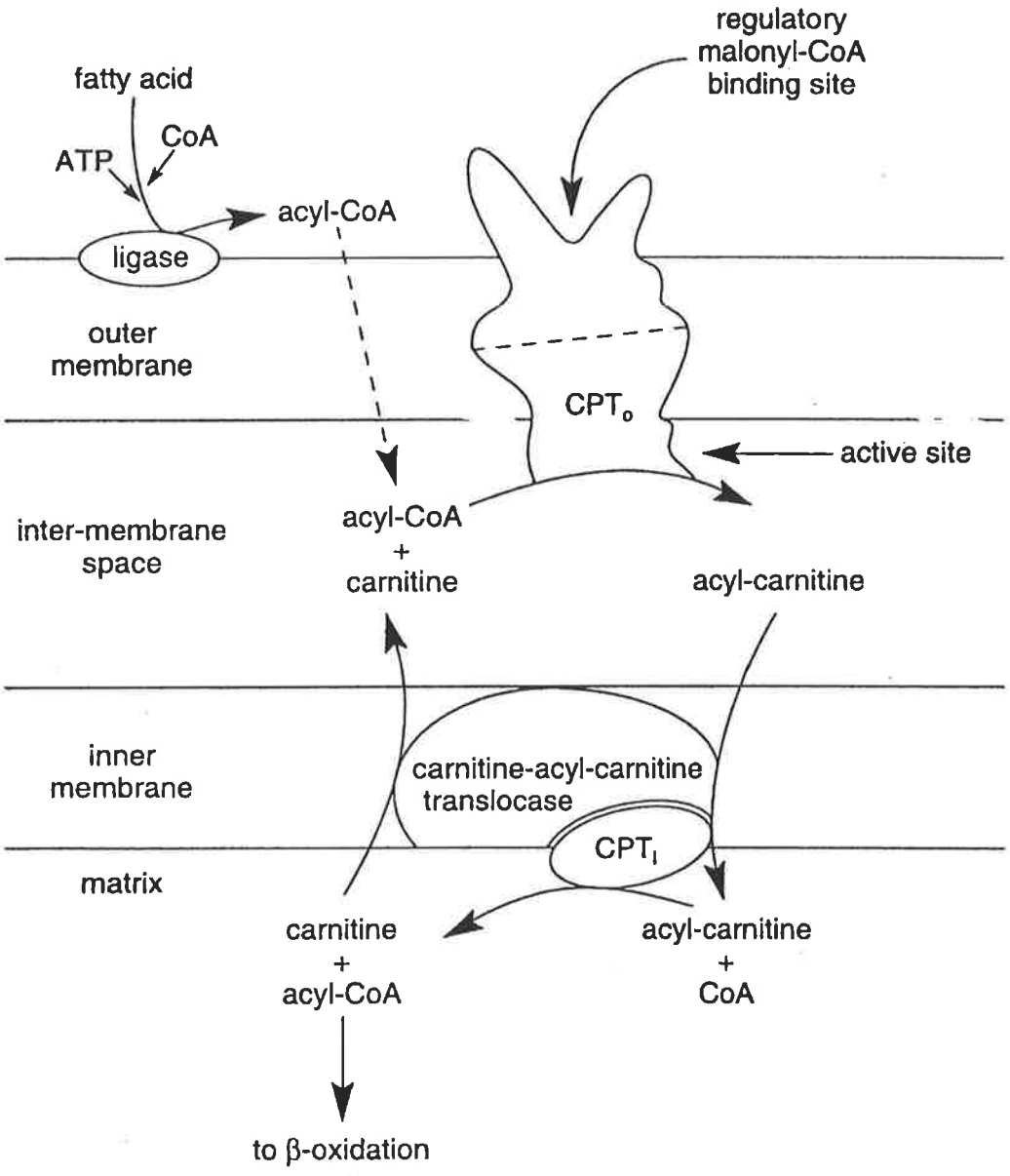


Figure 1.2: Schematic representation of the carnitine-dependent transport of fatty acyl groups into the mitochondria. Carnitine palmitoyltransferase 1 (CPT_o) is situated on the outer mitochondrial membrane, while carnitine palmitoyltransferase 2 (CPT_i) is on the inner mitochondrial membrane, adjacent to the matrix space. (Reproduced from Fritz and Arrigoni-Martelli, 1993).

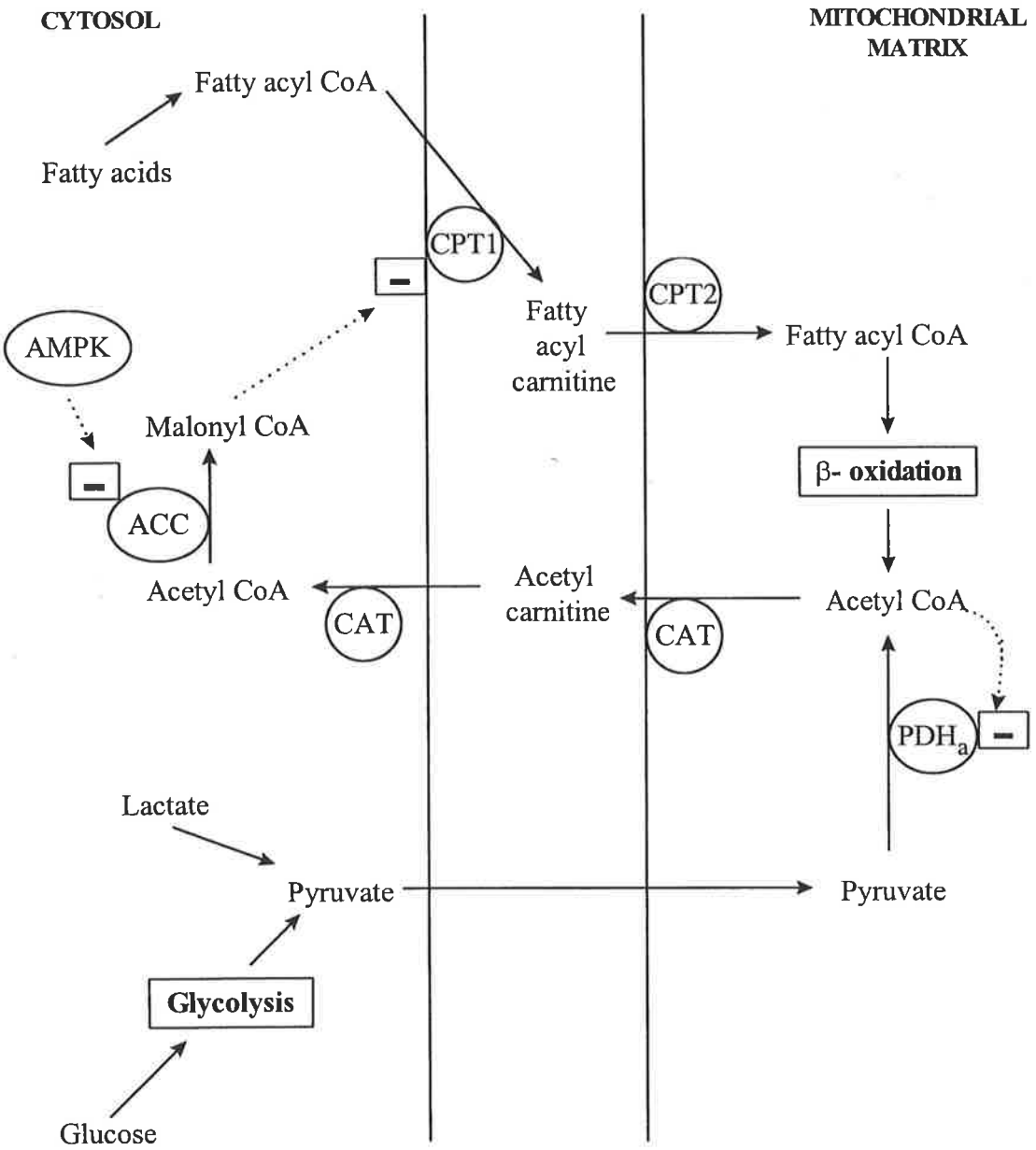


Figure 1.3: Inter-regulation of fatty acid and pyruvate oxidation. The dotted lines denote inhibition of enzymatic activity. Abbreviations: ACC, acetyl CoA carboxylase; AMPK, 5"AMP-activated protein kinase; CPT-I, carnitine palmitoyltransferase 1; CPT-II, carnitine palmitoyltransferase 2; CAT, carnitine acetyltransferase; PDHa, active dephosphorylated pyruvate dehydrogenase.

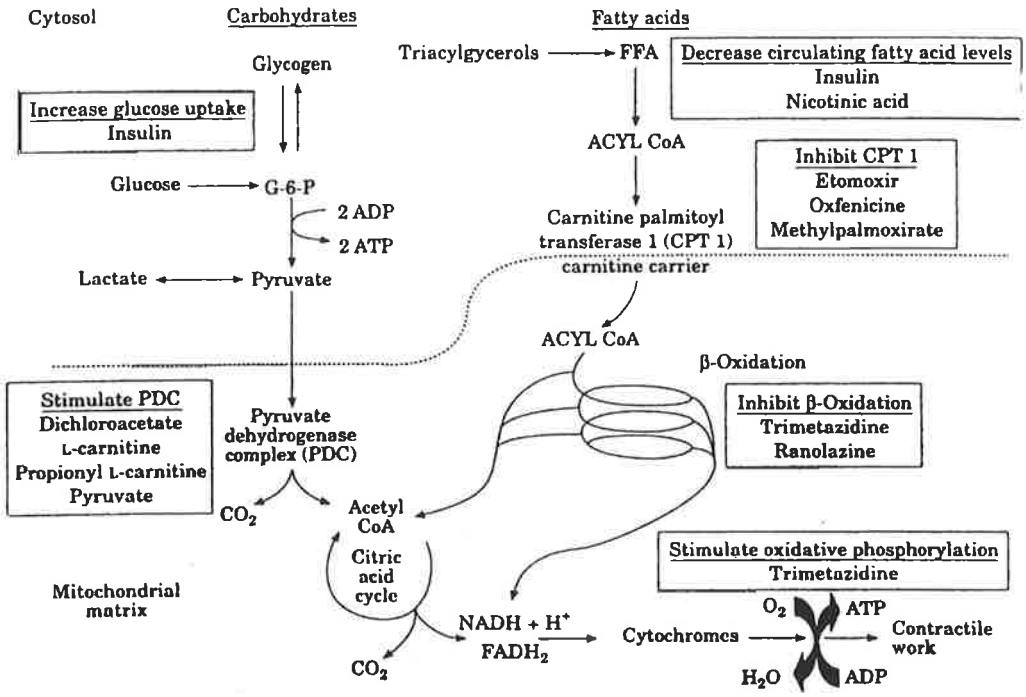


Figure 1.4: Potential sites at which metabolic interventions can protect the ischaemic heart. Simplified catabolic pathways for both glucose and fatty acids in the cardiomyocyte are shown. Potential sites of pharmacological action are enclosed in boxes, as are some of the agents known to affect these sites. (Reproduced from Lopaschuk, 1999).

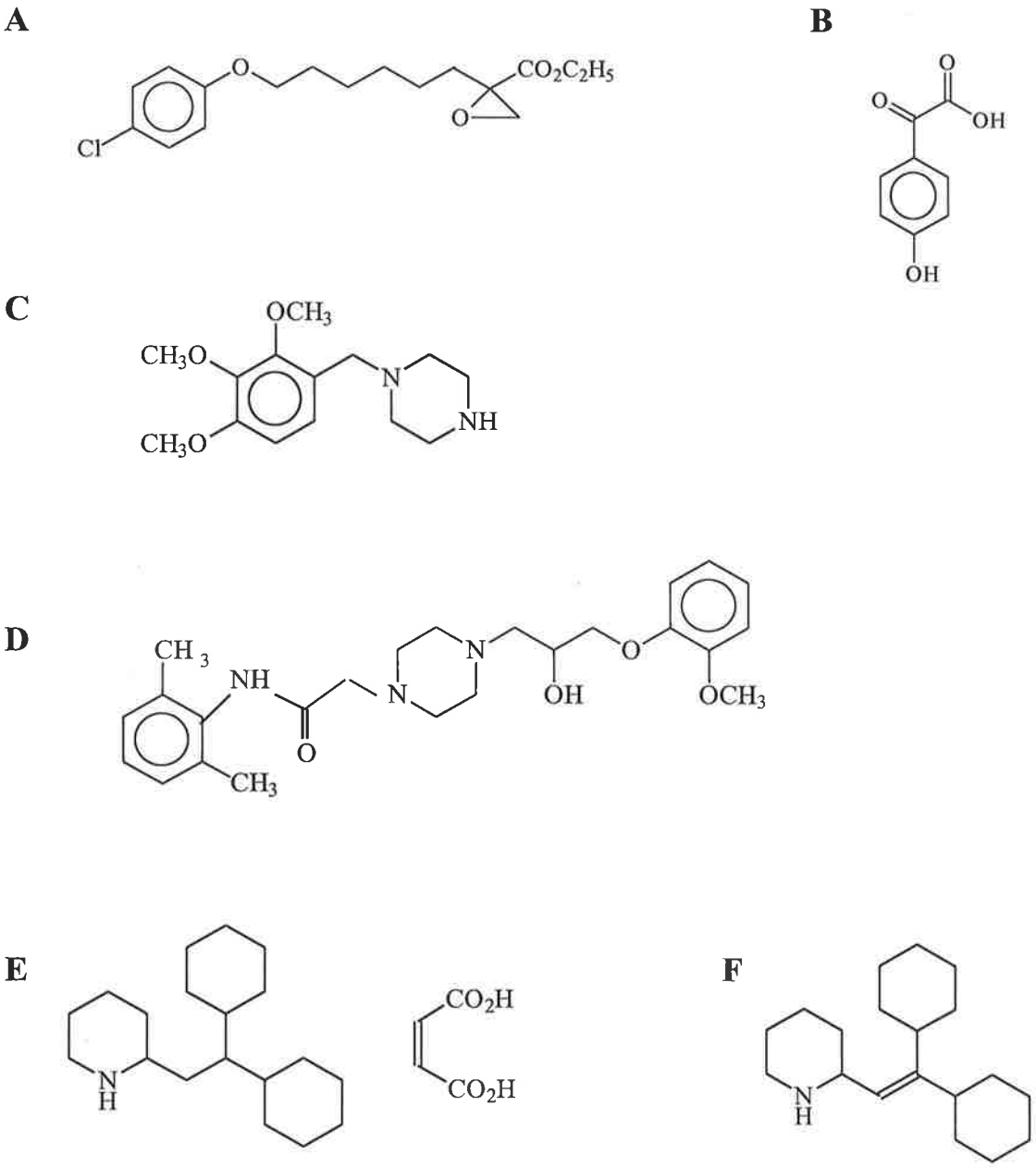


Figure 1.5: Chemical structures of (A) etomoxir, (B) 4-hydroxyphenylglyoxylic acid (the active metabolite of oxfenicine); (C) trimetazidine; (D) ranolazine; (E) perhexiline maleate; and (F) hexadiline.

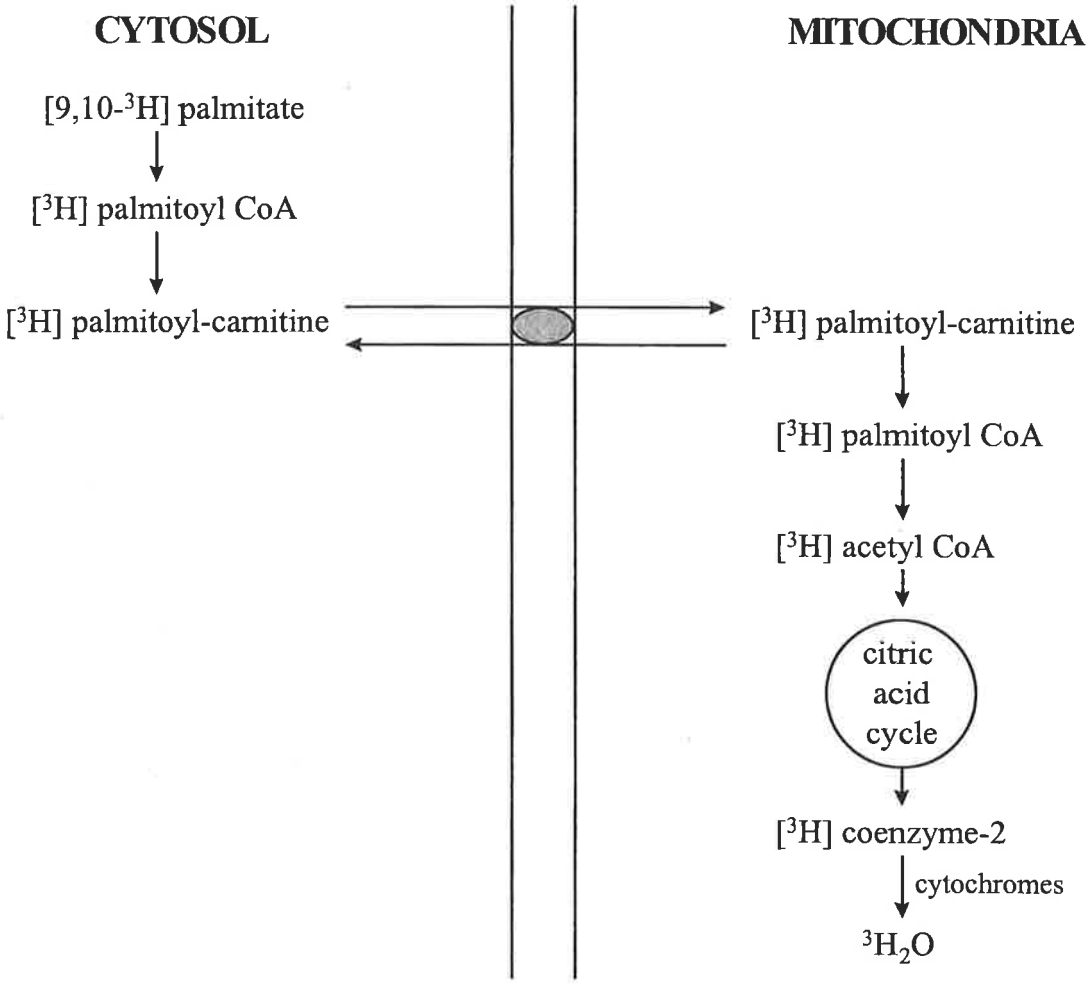


Figure 1.6: The catabolism of ^{3}H-labelled palmitate in the heart, resulting in the production of $^{3}H_2O$.

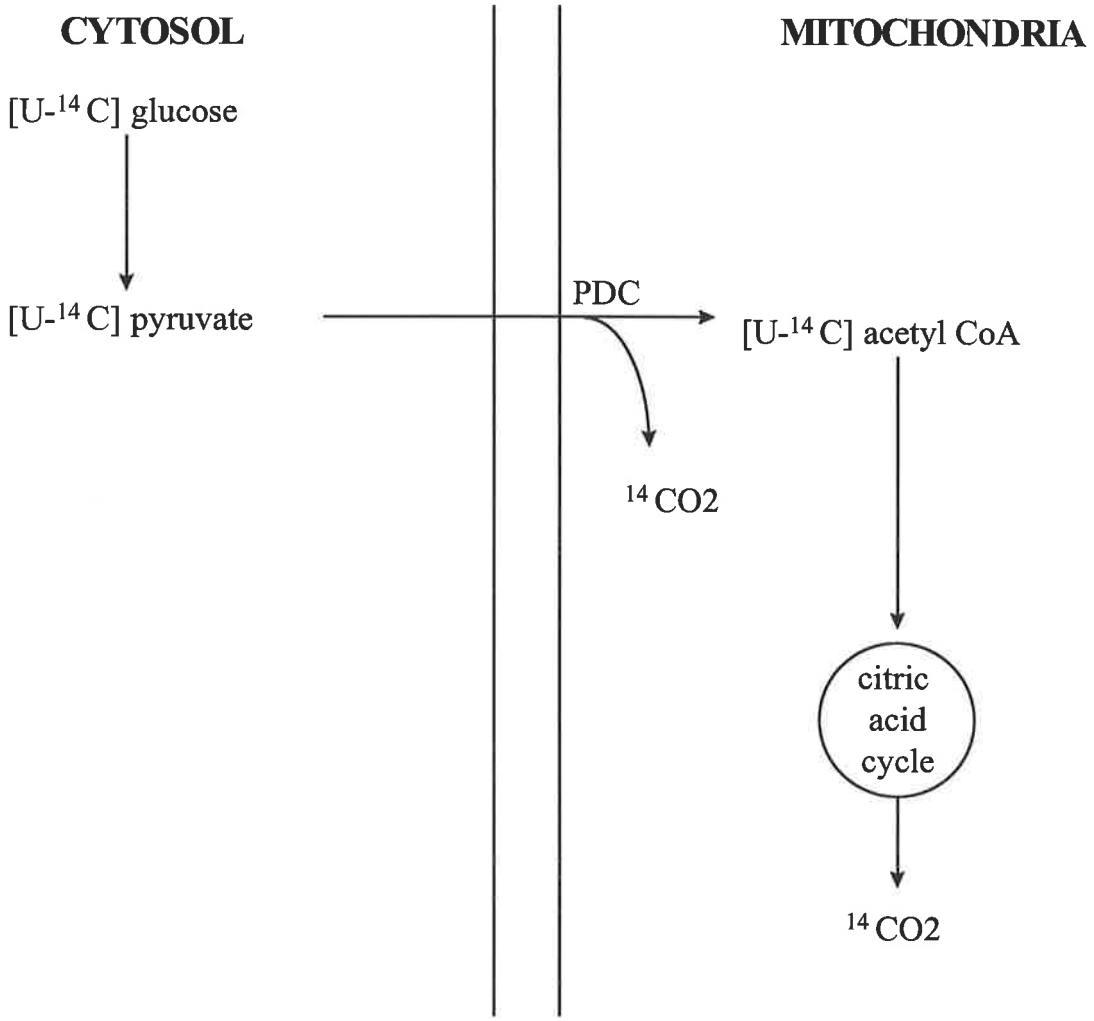


Figure 1.7: The catabolism of ^{14}C-labelled glucose in the heart, resulting in the production of $^{14}CO_2$. PDC = pyruvate dehydrogenase complex .

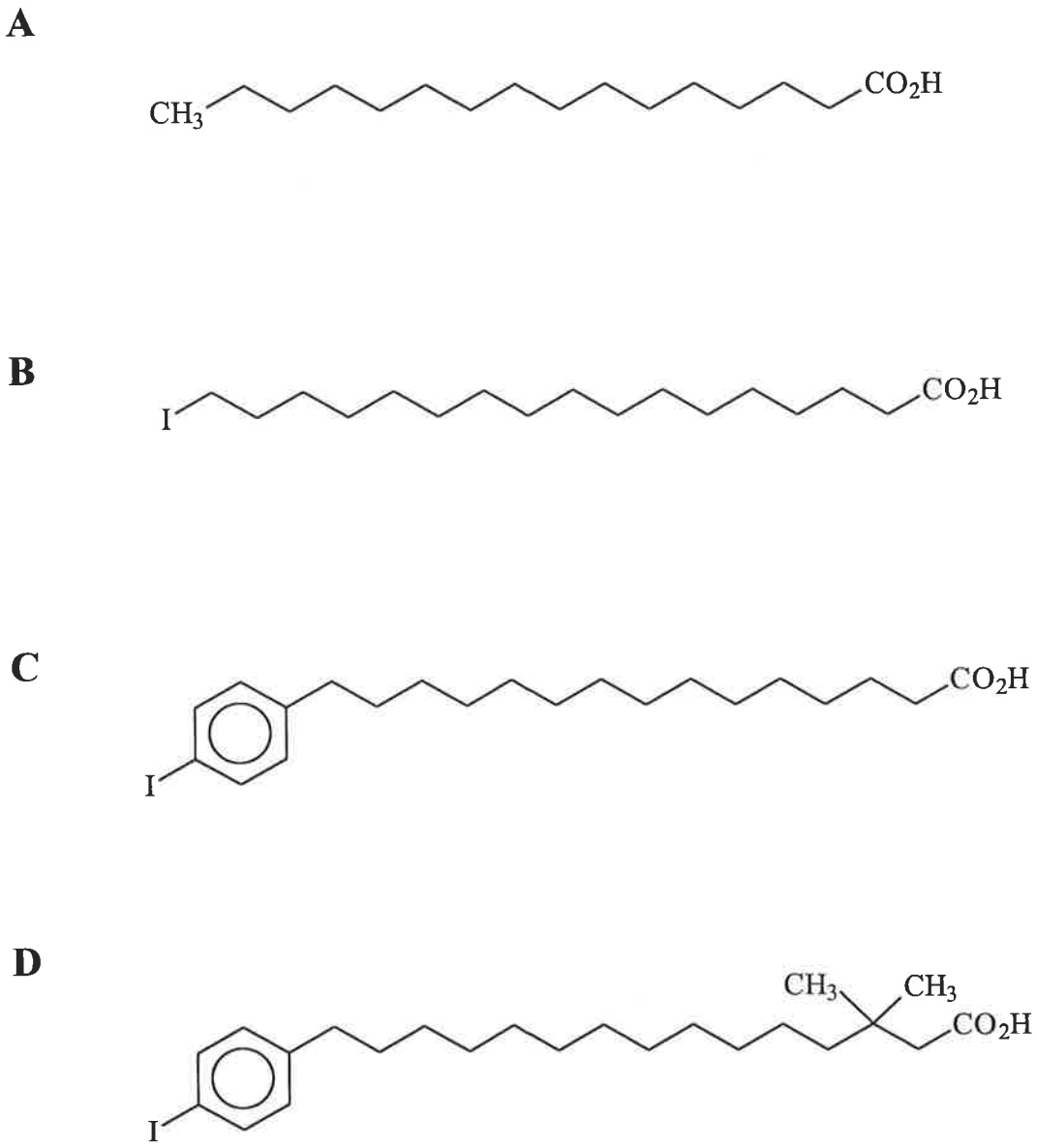


Figure 1.8. Chemical structures of: (A) palmitic acid; (B) [17-^{123}I]-heptadecanoic acid (HDA); (C) 15-(*p*-[^{123}I]iodophenyl)pentadecanoic acid (IPPA); and (D) 15-(*p*-[^{123}I]iodophenyl)-3-R,S-methylpenta-decanoic acid (BMIPP).

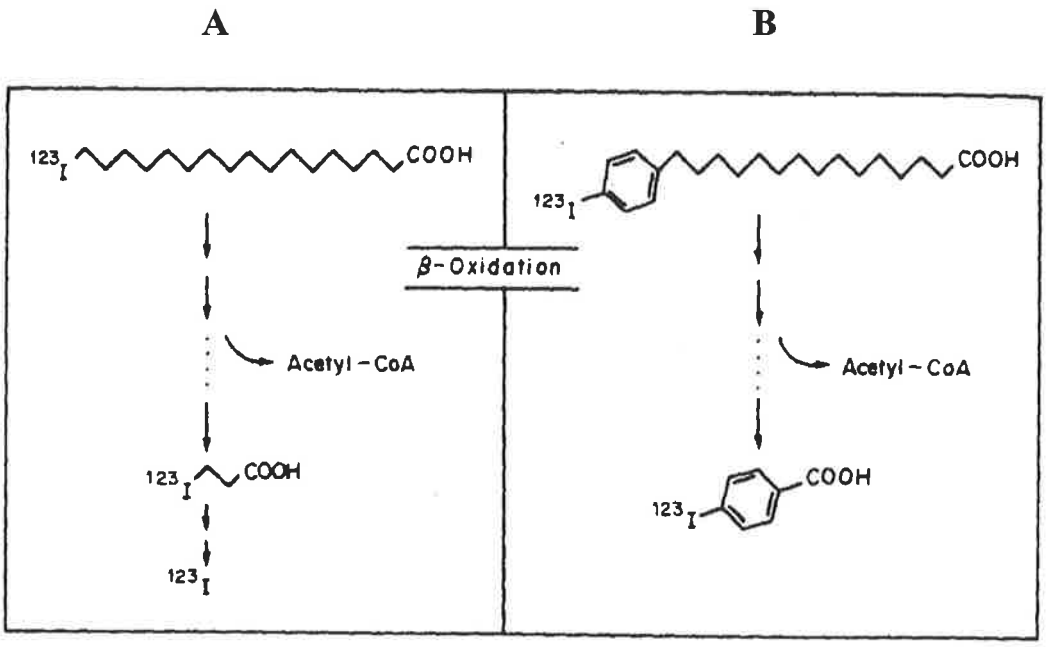


Figure 1.9. Catabolic pathways of (A) ^{123}I-HDA and (B) ^{123}IPPA in the heart. The radio-labelled end product of ^{123}I-HDA is free iodide, which contributes to high background blood pool activity, complicating analysis of myocardial time-activity curves. The radio-labelled end product of ^{123}IPPA is iodo-benzoic acid, which is rapidly cleared by the kidneys and contributes very little to background blood pool activity. (Reproduced from Machulla et al, 1986).

Reference	n	Drug	Daily dose (mg)	Duration (weeks)	E.D.	A.F.
Gallet 1986	32	Trimetazidine	60	4	+ 17%*	+ 10%
Passeron 1986	54	Trimetazidine	60	2	+ 37%*	- 30%*
Thadani 1994	318	Ranolazine	30	4	- 11%	+ 7%
			60		- 5%	- 5%
			120		- 9%	+ 12%
Pepine 1999	312	Ranolazine	800-1200	1	+ 5%*	na

Table 1.1: Summary of the randomised placebo-controlled trials assessing the efficacy of trimetazidine or ranolazine monotherapy on exercise duration (E.D.) ± anginal frequency (A.F.). Values shown are the incremental percentage changes in parameters compared to placebo group. n = number of patients in trial; * = statistically significant from placebo; na = not assessed.

Reference	n	Daily dose (mg)	Duration (wks)	End-points A.F.	E.D.
Burns-Cox, 1971	45	400	8	-84%*	na
Lyon, 1971	12	400	8	-31%*	+21%*
Bleifer, 1972	19	300-400	8	-57%*	na
Morgans, 1973	11	400	2	na	+77%*
Morledge, 1973	30	300	12	-51%*	+30%*
Hoekanga, 1973	85	400	8	-48%*	na
Brown, 1976	7	200-400	8	-47%*	na
Mir, 1978	28	200	6	-54%*	+41%*
Horgan, 1981	15	200	2	na	+39%*

Table 1.2: Summary of the randomised placebo-controlled trials assessing the efficacy of perhexiline monotherapy on exercise duration (E.D.) ± anginal frequency (A.F.). Values shown are the percentage changes in parameters compared to placebo group. n = number of patients in trial; * = statistically significant from placebo; na = not assessed.

CHAPTER 2:

THE HAEMODYNAMIC EFFECTS OF PERHEXILINE IN CONSCIOUS SHEEP

2.1 INTRODUCTION

The available information on the cardiovascular actions of perhexiline in animals or man (in the presence or absence of ischaemia) is fragmentary and often contradictory. In particular, the early emphasis on coronary vasodilatation in animal experiments does not seem relevant to studies of parenteral perhexiline in man, nor to its therapeutic effects. Most of the published literature involves anaesthetised dogs, with reports of dose-related bradycardia and coronary and systemic vasodilatation in some studies (Hudak et al. 1970; Ono & Hashimoto, 1981) but not in others (Rowe et al. 1970) with a consistent finding of increased myocardial efficiency (Cho et al. 1970; Daniell et al. 1977; Klassen et al. 1976; Ono et al. 1982). There has been only one study of the effects of parenteral perhexiline in man (Rowe et al. 1970) which gave qualitatively different results suggestive of systemic and coronary vasoconstriction, although a transient vasodilatory effect could not be excluded as continuous measurements of coronary flow were not performed in this study.

As no parenteral perhexiline formulation is commercially available, previous haemodynamic studies in dogs have utilised various forms of perhexiline, including perhexiline hydrochloride (Daniell et al. 1977; Hudak et al. 1970), perhexiline maleate (Leeson et al. 1969) in ethanol, and perhexiline gluconate (Rowe et al. 1970). The only commercially available perhexiline salt, perhexiline maleate (Sigma Chemical Company, St Louis, MO, USA) is extremely insoluble in aqueous solutions, and therefore unsuitable for administration *in vivo* in suitable doses in large animals. A perhexiline lactate salt was therefore formulated by the Victorian College

of Pharmacy which was soluble in water at concentrations of up to 7 mg per litre, but remained insoluble in saline solutions. Hence all infusions used in sheep consisted of perhexiline lactate dissolved in 5% dextrose solution.

As there are no published studies on the parenteral use of perhexiline lactate, nor on the haemodynamic effects of perhexiline in sheep, it was important to assess any potential cardiovascular effects before proceeding to metabolic studies in this species (see Chapter 3). The experiments described in this chapter are aimed at quantitation of both haemodynamic effects and pharmacokinetics associated with the intravenous administration of perhexiline in sheep, and comparing these with previously published work in dogs and man.

2.2 METHODOLOGY

2.2.1 Animals and materials

Sheep were used as a prelude to imaging studies (see Chapter 3), and because they have proven tolerant of the anaesthesia and surgery necessary for the catheterisation of multiple blood vessels (Huang et al. 1992; Runciman et al. 1984). These catheters can be maintained over several weeks. Because of their size, repeated blood sample collections can be performed during pharmacokinetic studies without significant decreases in their haemoglobin status or alterations in their haemodynamic status.

Adult Merino sheep between one and two years old, weighing between 40-50 kg, were supplied from a constant breeding stock by the research farm of the Institute of

Medical and Veterinary Science, Adelaide. They were housed in metabolic crates in air-conditioned rooms maintained at 22°C with a 12 hour light-12 hour dark cycle. All studies were approved by the Animal Ethics Committee of the University of Adelaide.

Perhexiline lactate was manufactured by the Victorian College of Pharmacy from the perhexiline maleate salt (Sigma Chemical Company, St. Louis, MO, U.S.A.).

Stock solutions used in the measurement of plasma perhexiline concentrations were: (i) internal standard: hexalidine HCl (Merrell Dow Pharmaceuticals, Australia) diluted in 0.1 M HCl to give concentrations of 14 and 1.4 mg/l; (ii) derivatising agent: dansyl chloride (Sigma Chemical Company, USA) 0.005 M prepared in acetone on the day of use; (iii) sodium bicarbonate solution 0.1M, adjusted to pH = 10 with NaOH; (iv) trizma base (tris-hydroxymethyl-aminomethane, Sigma Chemical Company, USA) buffer 2M, adjusted to pH = 8.75 with concentrated HCl solution; and (v) mobile phase solution: 86:14 (v:v) mixture of methanol and glass distilled water, vacuum filtered through a 0.2 mm Millipore filter prior to use in the HPLC columns.

2.2.2 Preliminary experiments using bolus doses of perhexiline

Preliminary experiments, described in 2.3.1, were performed to assess the haemodynamics and pharmacokinetics of bolus doses of perhexiline lactate administered intravenously. Cannulation of the sheep used for these studies was

performed under general anaesthesia with pentobarbitone sodium (20 mg/kg injected slowly into the jugular vein), and tracheal intubation. Using the Seldinger technique, 7-French gauge intravascular catheters (7-French grade; Multi-purpose B1 catheter, Cordis Corporation, Miami, Fl, U.S.A.) were placed in both the external jugular vein and the carotid artery. The latter was attached to pressure transducer (Transpac IV, Abbott Critical Care Systems, USA), allowing monitoring of intra-arterial pressures, and enabling arterial blood samples to be drawn. Arterial pressure traces were printed on a chart recorder (Hewlett Packard) at minutely intervals.

The preliminary experiments were performed under two conditions: (i) under general anaesthesia, maintained with 2% halothane and 100% oxygen, allowing external ECG monitoring via clips attached to each leg for assessment of any electrophysiologic effects, and (ii) in conscious animals to exclude a potential cardiodepressor effect of the anaesthesia (as has been reported previously with barbiturate anaesthetic agents in sheep (Huang, et al., 1997)), with monitoring of intra-arterial blood pressure but not ECG.

2.2.3 Set-up of chronically instrumented sheep model

The remainder of the experiments described in this chapter were performed in conscious, chronically instrumented sheep, using perhexiline lactate infusions. Preparation of the sheep for these more extensive haemodynamic studies was based on the methods described by Huang et al. (Huang et al. 1992)), and was performed in two stages. Approximately two weeks before experimentation, sheep were

anaesthetised with 30 mg/kg thiopentone sodium, and the trachea intubated with a cuffed endotracheal tube. Anaesthesia was maintained with 2% halothane and 100% oxygen. Under aseptic conditions, the right femoral artery and vein were exposed via a groin excision. Using the Seldinger technique, one 7-French and one 9-French gauge catheter (Multi-purpose A1 catheter, Cook Australia) were placed in the abdominal aorta for (i) arterial blood sampling and (ii) monitoring of mean arterial pressures via an external pressure transducer (Model 4-327-I, Bell and Howell Inc., Salt Lake City, UT, U.S.A.). Through the femoral vein, a 8.5 French introducer catheter (Biosensors International Pty Ltd, Singapore) was placed in the inferior vena cava. Through the introducer catheter, a 7.5 French multi-lumen thermodilution catheter (Biosensors International Pty Ltd, Singapore) was placed in the pulmonary artery , and its position was confirmed by monitoring of the pressure wave pattern.

Two days later, the sheep were anaesthetised as described above for probe placement and catheterisation. A left thoracotomy at the 4th intercostal space and a pericardiotomy were performed, and modified silastic cuff-type pulsatile Doppler flow probes (Titronics Medical Instruments, Iowa, U.S.A.) were placed on the left main coronary artery (for measurement of an index of left coronary blood flow) and the trunk of the pulmonary artery (for measurement of cardiac output). The probes were secured around the arteries using cotton tape, which acted as a "cuff" around the left main coronary artery and ensured a constant vessel calibre at the point of Doppler sampling. The apex of the left ventricle was stitched with 02 silk suture, and a microtransducer (Codma MicroSensor, Johnson & Johnson Professional, Inc.,

Raynham, MA, U.S.A.) catheter was inserted 3 cm into the left ventricle through a 5-gauge needle and fixed securely with the suture. After ensuring that the left coronary and pulmonary arteries were not constricted, the leads of both Doppler probes and the micro transducer were exteriorised through the chest incision and a subdermal tunnel, and the incisions were closed. In one sheep, a 7-French gauge (Cordis, Cordis Corporation, Miami, FL, U.S.A.) was also placed into the coronary sinus via the right jugular vein. In this sheep, the hemiazygous vein was ligated during the thoracotomy, so that all of the blood draining into the coronary sinus was from the coronary circulation. The position of all catheters was confirmed under fluoroscopy.

Haemodynamic studies were not performed until at least 10 days after the placement of the catheters, allowing sheep to recover from the surgery and the Doppler flow probes to become firmly embedded in scar tissue. This minimises changes in the angle between the ultrasonic beam and the direction of blood flow (α), and ensures good acoustic coupling. The scar tissue also minimises changes in vessel diameter due to changes in perfusion pressure or drug effects The catheters were flushed on a daily basis with heparinised (50 iU/ml) 0.9% saline, and the sheep were housed in metabolic crates with free access to food and water.

2.2.4 Haemodynamic measurements

During the experiments the Doppler frequency shifts from the coronary and pulmonary artery flow probes were recorded at a sampling rate of 1 Hz using a four-channel pulsed Doppler flowmeter (Bioengineering, The University of Iowa, Iowa

City, IO, U.S.A.) and an analog-to-digital card (Metrabyte DAS 16-G2) in a personal computer (Microbits 486-based IBM compatible). In all experiments, the Doppler sampling points were chosen as the points with the highest flow velocities in the coronary and pulmonary arteries. Blood flow velocity was calculated from the Doppler frequency shifts using the Doppler principle:

$$\text{Blood flow velocity} = \frac{F \times C}{2F_t \times \cos\alpha} \qquad (2.1)$$

where F is the recorded Doppler frequency shift, C is the speed of sound in blood, F_t is the incident sound frequency from the Doppler flow probe, and α is the angle between the sound beam and the direction of blood flow.

The pulmonary artery flow probe was calibrated *in vivo* by correlating the pulmonary artery Doppler shifts with the cardiac outputs determined by the standard thermodilution method using the thermodilution catheter placed in the pulmonary artery and a cardiac output computer (Model 9520A, Edwards Laboratories, Inc.. Irvine, CA, U.S.A.). Good linear correlations between cardiac outputs measured by the thermodilution method and a Doppler flowmeter have previously been reported in experimental rabbits (White et al. 1974). Although the range of cardiac outputs was too small during the current experiments to accurately plot linear regressions, previous work from this laboratory has shown excellent correlation between the two techniques in sheep, with coefficients of correlation (r) between 0.97 and 0.99 (Huang et al. 1992). In the current study, correlation between the two methods was also good in the sheep with the widest fluctuations in cardiac output ($r = 0.95$). The diameter of

the coronary artery could not be measured *in vivo*. Changes in coronary blood flow velocity were analysed as a marker of alterations in coronary blood flow (assuming minimal change in coronary artery diameter during the experiments due to the cotton tape cuff and scar tissue).

Left ventricular pressure and mean arterial pressure were recorded using the same data acquisition system. The positive peak value of left ventricular dP/dt represents the maximum rate of left ventricular pressure increase (LV dP/dt_{max}). Heart rate was also calculated from the left ventricular pressure wave. Stroke work, the external work performance of the left ventricle, was defined (in the absence of PCWP data) as the product of stroke volume and mean arterial pressure, and converted from ml.mmHg to g.m using the conversion factor of 0.0136 (Grossman, 1980).

$$\text{Stroke work (g.m)} = \text{MAP} \times \text{SV} \times 0.0136 \qquad (2.2)$$

(SV = CO/HR)

where MAP = mean arterial pressure (mmHg), SV = stroke volume (ml), CO = cardiac output (ml/min), and HR = heart rate (beats/min)

The determination of the resistance in a vascular bed requires measurement of the mean pressure of the proximal and distal ends of the vascular bed as well as the total flow through it. Systemic vascular resistance (SVR) was calculated from the mean arterial pressure, right atrial pressure and cardiac output, using a correction factor of 80 to convert the units from mmHg/l/min to absolute resistance units dynes.sec.cm^{-5} (Grossman, 1980).

$$SVR\ (dynes.sec.cm^{-5}) = \frac{(MAP - RAP) \times 80}{CO} \qquad (2.3)$$

where RAP = mean right atrial pressure (taken at baseline and assumed not to vary significantly during the experiments).

2.2.5 Perhexiline assay and estimation of myocardial drug content

For determination of plasma perhexiline concentrations, each sheep blood sample was centrifuged, and the plasma was frozen for later analysis via high performance liquid chromatography (HPLC) as per Morris et al (Morris, et al., 1992). Each 0.5 ml plasma sample was spiked with 200µl of the internal standard hexadiline (1.4 mg/l), 50 µl of Trizma buffer, and 4 ml of n-hexane in 15 ml screw cap disposable glass tubes. This mixture was mixed on a horizontal shaker for 15 minutes, followed by centrifugation at 2500 rpm for 10 minutes. The organic layer was pipetted into 5 ml disposable glass tubes and evaporated to dryness using an evacuated centrifuge. When dry, 100 µl of sodium bicarbonate solution and 100 µl of derivatising agent (dansyl chloride) were added to the residue and vortex mixed. Tubes were immediately capped and incubated in a 37°C water bath for 20 minutes. 1.5 ml of n-hexane was then added and the tubes were vortex mixed prior to centrifugation for 3 minutes at 2500 rpm. The phases were separated by snap freezing the aqueous layer in a dry ice/ethanol bath, and the organic layer was decanted into a second 5 ml tube. The samples were again evaporated in the evacuated centrifuge, and the residue was dissolved in 100 µl of the mobile phase solution, and 50 µl was injected into an autosampler (Millipore/Watres WISP, model 710B) for chromatographic separation.

Compounds separated by HPLC were quantified by fluorescence detection (Perkin Elmer, model LS-1) at excitation and emission wavelengths of 366 and 470 nm, respectively. The output to a dual-pen chart recorder was plotted and perhexiline concentrations were derived by comparison to the known content of the internal standard (hexadiline) in each sample. In later experiments, 2M NaOH was substituted for the Trizma buffer, allowing analysis also of the major perhexiline metabolite, mono-hydroxyperhexiline.

In order to estimate myocardial drug content, the following assumptions were made: (i) the coronary flow velocity data from the Doppler flow probe on the left coronary artery can be taken as an index of myocardial blood flow, and (ii) the plasma drug concentrations in the abdominal aorta and at the entrance to the coronary arteries are equivalent, due to the mixing of blood that occurs in the chambers of the heart. Although there is not a large body of work to confirm the latter assumption, one study reported no differences in the time-courses of lignocaine concentrations in the aorta and femoral artery following intravenous bolus administration(Horowitz, et al., 1986).

The theoretical myocardial drug content was then calculated using the following method (Horowitz, et al., 1986):

$$\text{Myocardial drug uptake (MDU)} = \delta C \times F$$

where δC is the transcoronary drug concentration gradient, taken as the difference between aortic and coronary sinus perhexline concentrations, and F is a measure of coronary flow, taken in this case as the readings from the Doppler flow probe in the

left coronary artery (hence the units are arbitrary). From this data, the myocardial drug content (MDC) was estimated:

$$MDC = MDC_1 + \frac{(MDU_1 + MDU_2)}{2} \times t$$

where MDC_1 = myocardial drug content at the end of the previous sampling period, and MDC_2 = instantaneous myocardial drug uptake at beginning and end of current sampling periods, and t = duration of current sampling period.

2.2.6 Experimental protocol

During the experiments, the sheep were supported in a comfortable sling inside their metabolic crates in order to minimise movement that would influence the haemodynamic measurements. Baseline haemodynamic measurements were obtained after the sheep were allowed to settle in the sling for at least 15 minutes. Perhexiline lactate was dissolved in a 30 ml volume of 5% dextrose. Allowing for dead-space, 25 ml was infused into the inferior vena cava over 5 minutes using a Terumo syringe pump. Two doses of perhexiline were used for the infusions in the chronically instrumented sheep, 37.5 mg (7.5 mg/min over 5 minutes) and 75 mg (15 mg/min over 5 minutes). These doses were equivalent (in terms of mg per kg) to those used previously in dogs, which resulted in improvements in indices of myocardial efficiency (Cho et al. 1970; Daniell et al. 1977; Klassen et al. 1976; Ono et al. 1982).

Haemodynamic variables were recorded continuously for 30 minutes from the start of the infusion, and arterial samples were taken at 0, 5, 10, 15, and 30 minutes for blood

gas analysis (Radiometer ABL System 625, Medical A/S, Copenhagen, Denmark) and determination of plasma perhexiline concentration. In one experiment, blood samples were obtained simultaneously from the coronary sinus and abdominal aorta at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, and 30 minutes, allowing pharmacokinetic modeling and estimation of myocardial uptake of perhexiline.

2.2.7 Statistical analysis

All the recorded haemodynamic parameters were averaged for 10 second periods consisting of 5 seconds before and 5 seconds after each of the following time points: minutely for five minutes prior to drug infusion (baseline), half-minutely during the infusion, and minutely for 25 minutes post-infusion. These averaged parameters were entered into a Microsoft Excel spreadsheet program. Cardiac output, LV dP/dt_{max}, heart rate, stroke work, and systemic vascular resistance were calculated for each time point as described above

One way analysis of variance with repeated measures was used for statistical analysis of haemodynamic parameter changes between the 5 minute baseline control periods, the drug infusion periods, and the "washout" period taken as between 10 and 15 minutes after the end of the infusion. If this analysis showed a significant effect, Dunnett's procedure was performed to compare the infusion and washout periods to the control period. Two way analysis of variance was used to compare the haemodynamic effects during different time periods between the two perhexiline doses utilised. $P < 0.05$ was considered to be statistically significant.

2.3 RESULTS

2.3.1 Bolus perhexiline administration (preliminary experiments)

Baseline haemodynamic monitoring showed a relative tachycardia in anaesthetised sheep compared to conscious animals, which has been previously reported with barbiturate anaesthesia in sheep (Huang, et al., 1997). Intravenous bolus injections of more than 5 mg of perhexiline were associated with acute haemodynamic effects, particularly bradycardia and consequent hypotension, which were seen both in conscious and anaesthetised animals. Figure 2.1A shows the haemodynamic effects of intravenous boluses of 5 mg and 20 mg of perhexiline (in 5 ml 5% dextrose) in the same sheep. The dose of 5 mg resulted in a small fall in systemic blood pressure and heart rate, these values returning to baseline within 6 minutes. The bolus of 20 mg perhexiline was associated with marked systemic hypotension (fall in mean arterial pressure from 108 mmHg to 74 mmHg at 2 minutes post injection), followed by moderate bradycardia. Despite the haemodynamic effects, there were no changes in electrocardiographic parameters including PR interval, QRS duration, and QT interval, with either dose.

Arterial blood samples were taken over 4 hours following the bolus injections, and the samples were assayed for plasma perhexiline concentration. At the time of these preliminary experiments, the perhexiline assay was not set up to measure levels of perhexiline metabolites. The pharmacokinetic profiles are shown in Figure 2.1B. These curves suggested at least 2 clearance phases: an early rapid phase for the first 15 minutes, followed by a second slow phase. Venous samples taken from the

contralateral jugular vein at 15 and 60 minutes showed that the arterio-venous perhexiline concentration gradient at 15 minutes (plasma perhexiline 0.85 mg/l arterial, 0.62 mg/l venous) was no longer apparent at 60 minutes (0.40 mg/l arterial; 0.42 mg/l venous), suggesting complete tissue distribution within 60 minutes.

2.3.2 Perhexiline infusions

2.3.2.1 Perhexiline phamacokinetics

Figure 2.2 shows the arterial plasma perhexiline concentrations following 5 minute infusions of either 37.5 mg (n = 3) or 75 mg (n = 3) of perhexiline via the inferior vena cava. Non-linear regression analysis of both curves revealed bi-exponential elimination kinetics (r = .95 for 75 mg, r = .93 for 37.5 mg), with an early rapid clearance phase of approximately 15 minutes (similar to the post-bolus pharmaco-kinetics described above). Plasma levels of the major metabolite, mono-hydroxyperhexiline, were unrecordable during the 30 minute sampling period.

In one sheep, sampling was also performed via the coronary sinus, allowing analysis of the myocardial uptake of the drug. The simultaneous coronary sinus and arterial plasma perhexiline concentrations following a 5 minute infusion of 75 mg of perhexiline are shown in Figure 2.3A. The transcoronary drug gradient reaches a maximum at the end of the infusion period, but net myocardial uptake continues for a further 8 minutes. The point of reversal of the arterio-venous concentration gradient corresponds to the time of maximal myocardial drug content (Horowitz, et al., 1986). The theoretical myocardial content of perhexiline was calculated as described in

Section 2.2.5. These data, expressed as a percentage of maximal myocardial drug content, are plotted in Figure 2.3B.

2.3.2.2 Haemodynamic effects

Haemodynamic effects of the perhexiline infusion were divided into two phases: (i) early changes observed during the infusion, occurring well before peak myocardial drug content; and (ii) changes occurring at least 10 minutes after the end of the infusion, more consistent with interaction with myocardial function. A control infusion of lactate in 5% dextrose was administered to two sheep, with no changes in any haemodynamic parameter (data not shown).

The time courses of the haemodynamic effects during and after both low dose (37.5 mg, n = 4) and high dose (75 mg, n = 4) perhexiline infusions are shown in Figures 2.4, 2.5, and 2.6. Mean values for each parameter for three 5 minute time periods (pre-infusion, infusion, and 10 minutes post-infusion) are shown in Table 2.1, together with the F and P values resulting from a comparison between each time period via one-way analysis of variance with repeated measures.

(i) Heart rate and cardiac output (see Figure 2.4)

During the lower dose infusion of perhexiline, there was no early change in heart rate, but there was a significant fall in heart rate immediately following the infusion ($P < 0.05$) which persisted for at least 25 minutes. In contrast, the higher dose infusion produced a marked early rise in heart rate, from a baseline value of 79 ± 9 beats/min

to a peak at 30 seconds into the infusion of 123 ± 27 ($P < 0.01$). The heart rate remained elevated during the infusion, but fell post-infusion, although this fall did not achieve statistical significance ($P = 0.14$).

There was no significant change in cardiac output during either the low- or high-dose perhexiline infusions. Following the infusion, there was a non-significant trend with both doses towards a fall in cardiac output.

(ii) LV dP/dt_{max} and stroke work (see Figure 2.5)

No significant changes in LV dP/dt_{max} were observed during the low-dose infusion, but there was a significant fall ($P < 0.05$), in parallel with the decrease in heart rate, following the infusion. During the higher dose infusion, LV dP/dt_{max} rose significantly to approximately 115% of baseline during the infusion ($P < 0.01$ compared to baseline; $P < 0.005$ compared to low-dose), and returned to baseline values post-infusion.

Stroke work fell briefly in the first minute of the high-dose infusion, but then returned to baseline during the remainder of the infusion. There were no significant changes during the low-dose infusion. Following both the low- and high-dose infusions, there was a trend towards a persistent increase in stroke work, although this did not become statistically significant.

(iii) Mean arterial pressure and systemic vascular resistance (see Figure 2.6)

Mean arterial pressure rose markedly during the high-dose infusion ($P < 0.01$), and remained elevated post-infusion ($P < 0.01$). There was a non-significant trend towards a rise in mean arterial pressure during and after the low-dose infusion ($P = 0.06$). There was a trend towards a sustained increase in systemic vascular resistance during and after all infusions, becoming significant ($P < 0.05$) in the period following the high dose infusion.

(iv) Coronary blood flow (see Figure 2.7)

Coronary Doppler data were only available in 7 of the sheep. An unexpected qualitative difference was noted in coronary blood flow velocity (shown as percentage change from baseline), with trends toward a decrease associated during the low-dose infusion ($P = 0.08$), but increased coronary flow ($P = 0.08$) during the higher dose of perhexiline. The effects of the two doses on coronary blood flow velocity during infusion of the drug were statistically different to each other ($P = 0.03$). The changes returned to baseline values post-infusion.

2.3.2.3 Other effects

No significant changes were seen in any of the arterial blood gas parameters (pH, pO_2, pCO_2) (data not shown) with either dose of perhexiline. Arterial plasma catecholamine levels were measured at baseline, mid-infusion, and 10 minutes post-infusion in one sheep receiving the 75 mg dose of perhexiline. There was a slight rise in noradrenaline from 1.5 nmol/l at baseline to 2.6 nmol/l mid-infusion, falling to 2.1

nmol/l post-infusion (reference range in man 0.5 - 4.1). None of the sheep showed any outward signs of agitation during any of the perhexiline infusions.

2.4 DISCUSSION

The pharmacokinetic studies show a rapid plasma clearance following both infusion and bolus doses of intravenous perhexiline. This clearance occurred in at least two phases: an early rapid clearance phase, lasting approximately 15 minutes, most likely related to rapid tissue distribution; and a slower phase which may reflect hepatic metabolism of the drug. The initial phase corresponded to the period of net myocardial drug uptake in the sheep in which transcoronary drug sampling was performed

The haemodynamic effects of intravenous perhexiline in the conscious sheep differed depending on the speed of administration (bolus vs. infusion) and the dose administered. Bolus doses of 5 mg and 20 mg resulted in a transient marked bradycardia with consequent systemic hypotension. In contrast, following infusions of perhexiline at 7.5-15 mg per minute, bradycardia was preceded by elevation of the mean arterial pressure accompanied by an increase in systemic vascular resistance and a trend towards increased stroke work. Despite the evidence of systemic vasoconstriction, there were no major changes in coronary blood flow velocity in the post-infusion period.

The underlying basis for the bradycardic effects remain uncertain, given the different time-course of effects between bolus administration and infusion of perhexiline. Despite the presumed calcium antagonist action of the drug in past reports (Barry et al. 1985; Fleckenstein-Grün et al. 1978; Perez et al. 1982), an electrophysiologic study in open-chest dogs reported no significant changes in sinus rate, PR interval or intra-atrial, atrioventricular, and His-Purkinje conduction times with intravenous doses of 3 mg/kg of perhexiline (Vera et al., 1975). The immediate bradycardia observed in this study following bolus administration suggests an interaction with central autonomic outflow, as myocardial content at this stage would be low (based on the pharmacokinetic data from one sheep). The more prolonged negative inotropic effect observed following the infusions may also reflect a central autonomic reflex mechanism related to the early increase in systemic vascular resistance.

Both LV dP/dt_{max} and stroke work are relatively afterload-independent indices of left ventricular contractility under physiological conditions. Both are significantly affected by alterations in left ventricular end-diastolic pressure, but are accurate if large changes in loading conditions do not occur. In man and several other mammals, LV dP/dt_{max} may also be affected by simultaneous changes in heart rate (the positive rate staircase or Treppe phenomenon) (Koch-Weser & Blinks, 1963; Mason, 1969), although this phenomenon has not been observed in sheep. A previous study in conscious sheep reported no significant change in LV dP/dt_{max} during intra-cardiac pacing to heart rates of up to 140 beats per minute (Huang et al. 1998). In the present study, there was a trend towards an increase in stroke work following the higher dose

of perhexiline, although this did not reach statistical significance.. This suggests a lack of significant negative inotropic effects due to calcium channel antagonism in this model.

There have been no previous reports of the pharmacokinetic or haemodynamic effects of perhexiline in sheep. Most of the previous animal work has been in dog models, and the results of the more extensive studies are summarised in Table 2.2. Unfortunately the exact timing of these observations with respect to the perhexiline infusions is not made clear in these reports; only "peak" effects are presented. Vascular effects in dogs have been consistent, with evidence of both coronary (Cho, et al. 1970; Hudak et al. 1970; O'Hara et al. 1981; Ono & Hashimoto, 1981; Rowe, et al. 1970) and systemic (Hudak et al. 1970; O'Hara et al. 1981) vasodilatation following brief infusions of 0.3-3 mg/kg of perhexiline intravenously.

Studies in dogs have reported negative inotropic effects at doses of > 1 mg/kg (Cho, et al. 1970; Ono & Hashimoto, 1981). However lower doses have been associated with increased stroke work (Ono et al. 1982; Rowe et al. 1970). A consistent finding has been a reduction in myocardial oxygen consumption (Cho et al. 1970; Ono & Hashimoto, 1981; Rowe et al. 1970), regardless of effect on the major determinants of myocardial oxygen demand including heart rate, arterial pressure, and myocardial contractility (Braunwald, 1969). This has been associated with improved cardiac efficiency, defined as the amount of cardiac work performed per unit of oxygen consumption. As discussed in Chapter 1, these results suggest that the improvement

consistently observed in myocardial efficiency are due to a direct effect on cardiac intermediary metabolism, with enhanced ATP production per unit oxygen consumption.

There had only been one published study of parenteral perhexiline administration performed in man (Rowe et al. 1970). In this uncontrolled study, 40 mg of perhexiline maleate was infused over 3-5 minutes via the right atrium in 3 patients, and 60 mg was infused via the right ventricle in another 3 patients, all with angiographic evidence of severe coronary atherosclerosis. Haemodynamic data for all 6 patients were pooled. In contrast to the vasodilatory effects in dogs, increased peripheral vascular resistance and reduced coronary flow were observed compared to baseline values, although these effects were not measured until several minutes after the dose. In addition there was a small but significant fall in heart rate, (from 70 ± 11 to 66 ± 9; $P < 0.05$), and falls in stroke index ($P < 0.05$), cardiac index ($p < 0.01$) and left ventricular work index ($P < 0.02$). There were no significant changes in left ventricular oxygen consumption or cardiac efficiency.

The current study suggests that haemodynamic responses to intravenous perhexiline in sheep are strikingly different to those in dogs, and similar in some respects to those in man. A confounding factor in the previous reports in dogs is that all haemodynamic studies were performed under barbiturate anaesthesia, and an interaction between perhexiline and the anaesthetic agent may have led to some of the observed effects. Barbiturates themselves have been associated with various

cardiovascular effects in animals, including decreases in afterload and myocardial contractility (Huang, et al., 1997; Parker & Adams, 1978). This potential interaction was excluded in the current study by performing the experiments in conscious animals.

The major findings in this study are the increased peripheral vascular resistance, and a lack of any significant negative inotropic effects following intravenous infusion of perhexiline in sheep. These results confirm that perhexiline does not act like a typical calcium antagonist on either the myocardium or the vasculature. In keeping with this, the observed negative chronotropic effects probably represent an autonomic reflex mechanism rather than any direct effect on the sino-atrial node.

The lack of observed calcium channel antagonism at these doses would fit with a primary metabolic, rather than haemodynamic, cardio-protective effect in this conscious sheep model. In comparison, studies on the effects of parenteral CPT-1 inhibitors such as oxfenicine or POCA have not demonstrated any significant changes in resting haemodynamics in dogs (Higgins et al. 1985; Seitelberger et al. 1984) or man (Bergman et al. 1980). Despite this, the mean pacing time to angina was significantly increased in 18 patients following intravenous oxfenicine (3-12 mg/kg), associated with a shift in cardiac substrate utilisation and a fall in myocardial oxygen consumption (Bergman et al. 1980).

The major weaknesses of the current study are the small numbers involved, and the inability to measure oxygen consumption, myocardial efficiency, or changes in cardiac metabolism (see Chapter 3). Further studies are needed to investigate these effects in the conscious sheep model, which from the current work appears to represent a suitable animal model. In addition to coronary sinus sampling for measurements of myocardial substrate and oxygen consumption, the use of intra-cardiac pacing in future experiments would allow the comparison of these effects under conditions of increased myocardial oxygen demand.

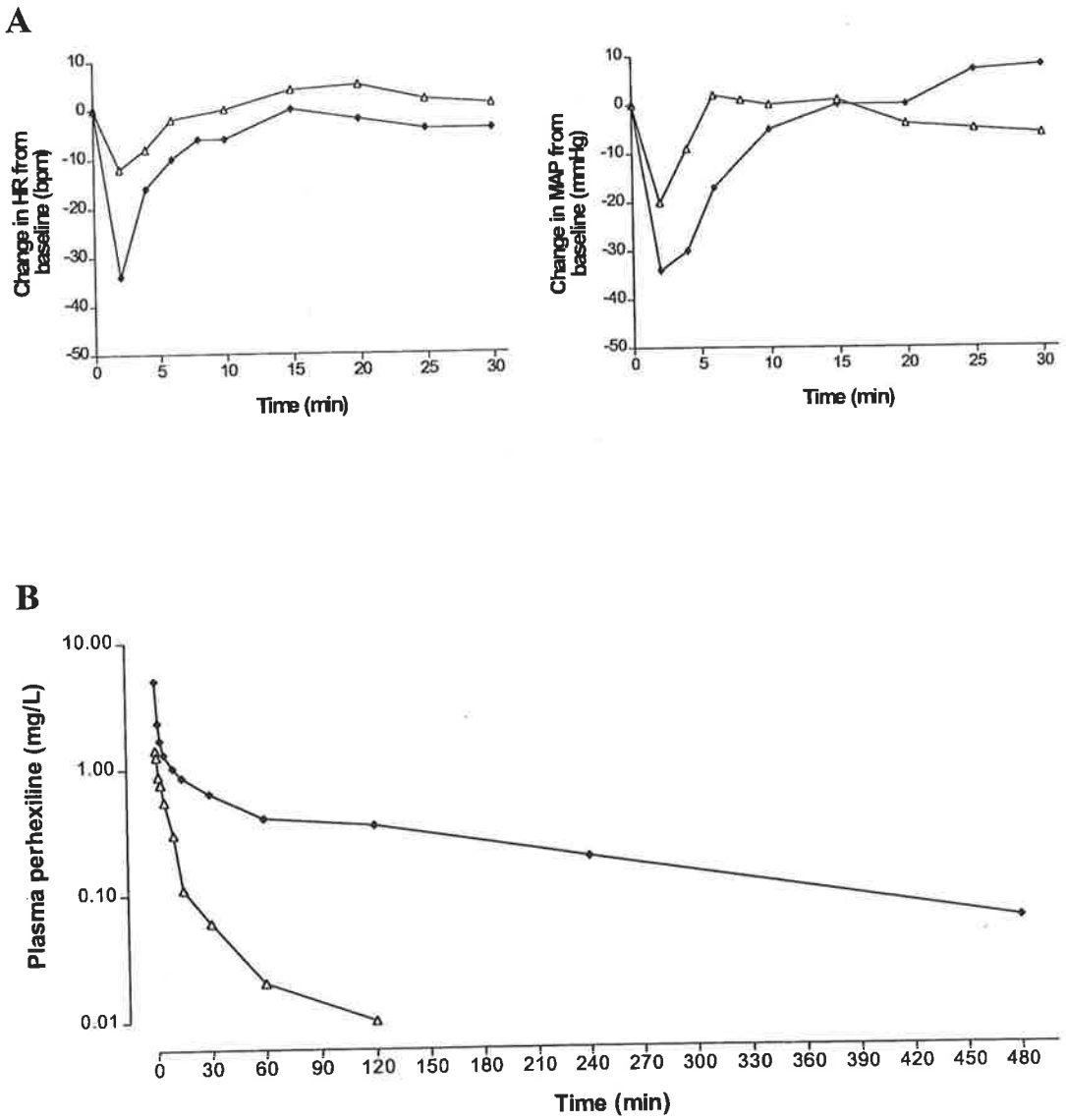


Figure 2.1: Effects on heart rate and mean arterial pressure (**A**) and arterial plasma perhexiline concentrations (**B**) in 1 sheep following bolus injections of 5 mg (Δ) and 20 mg (♦) of perhexiline.

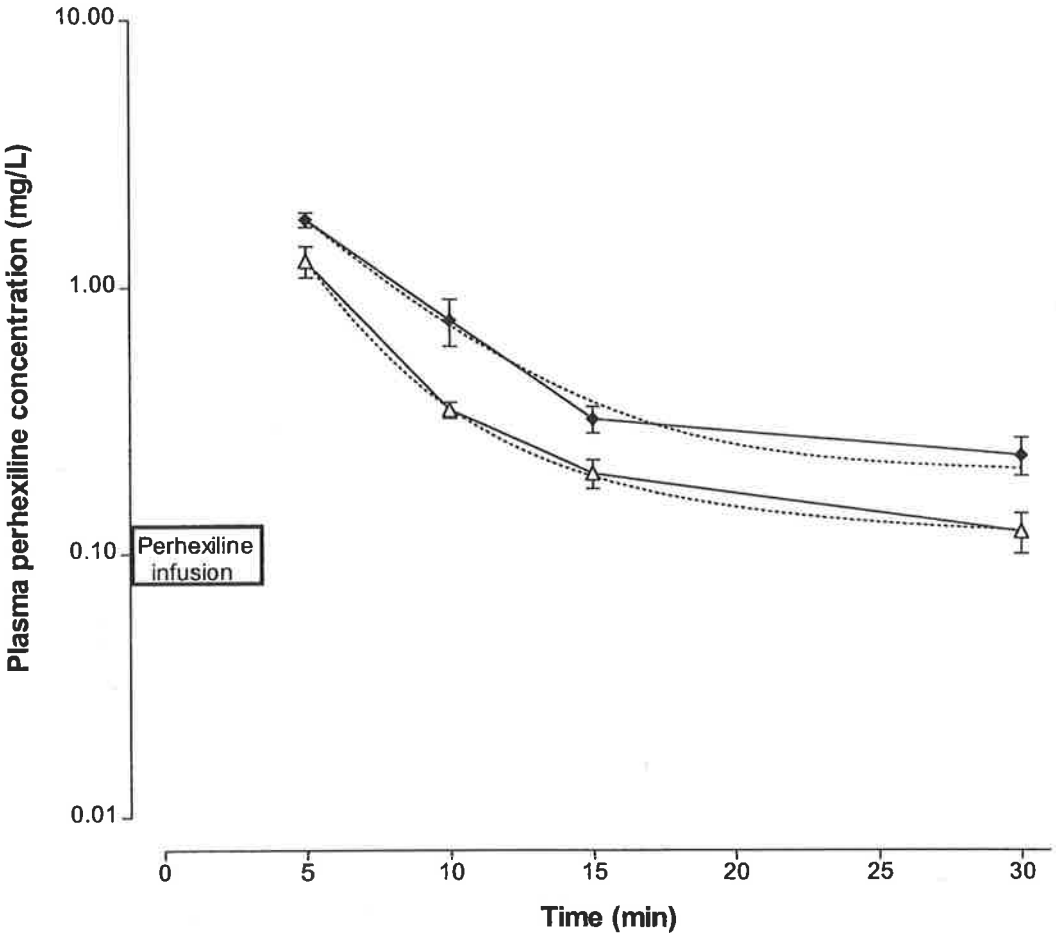


Figure 2.2: Arterial plasma perhexiline concentrations (mean ± SD) in conscious sheep following infusions of perhexiline over 5 minutes. Total doses administered were either 37.5 mg (Δ, n = 3) or 75 mg (♦, n = 3). The dotted curves represent bi-exponential curves of best fit (Δ, r = 0.93; ♦, r = 0.95).

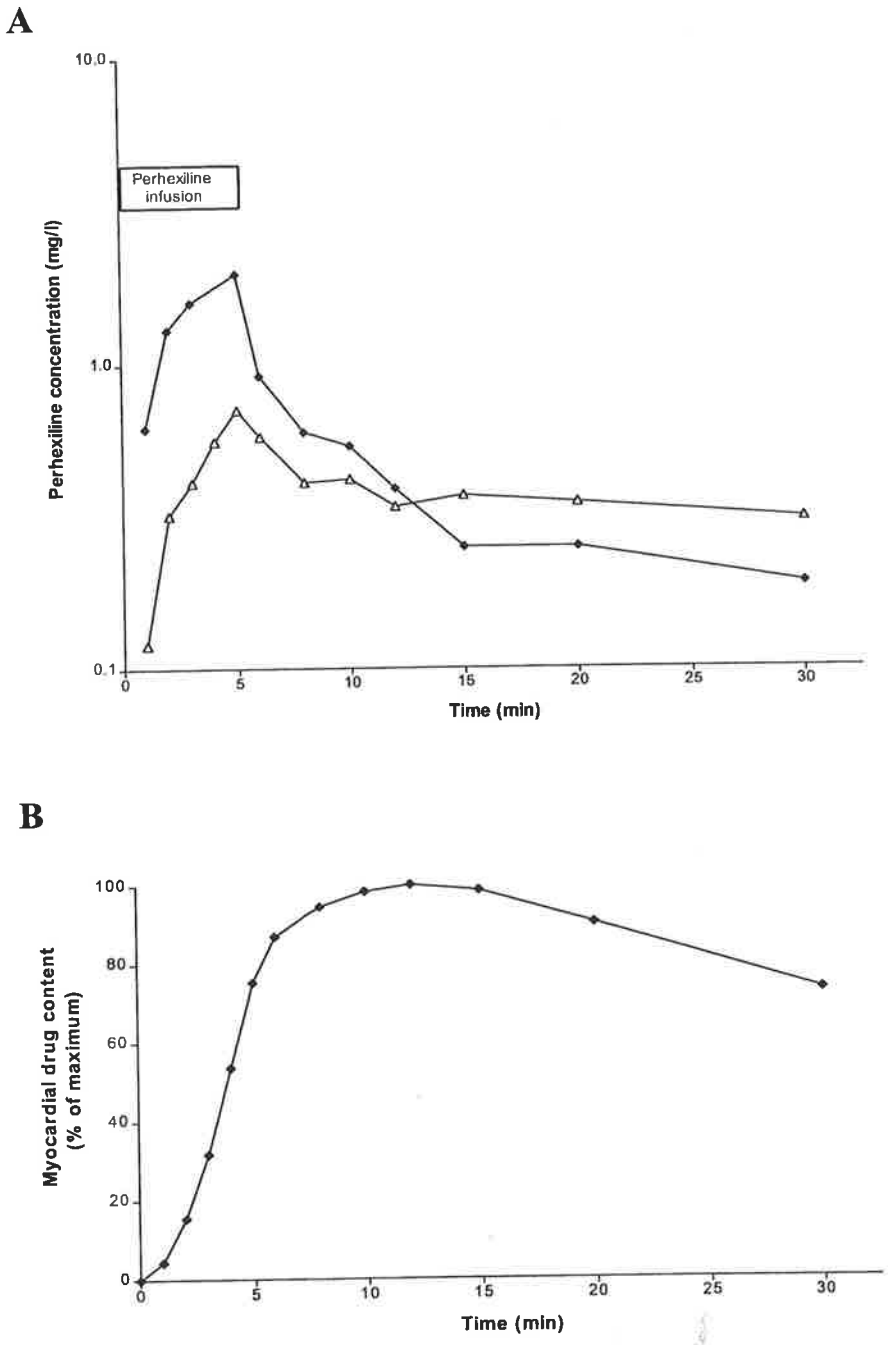


Figure 2.3: (**A**) Plasma perhexiline levels in femoral artery (♦) and coronary sinus (Δ) in one sheep following a five minute intravenous infusion of 75 mg perhexiline (15mg/min). (**B**) theoretical myocardial content of perhexiline in the same sheep, shown as a percentage of maximal myocardial drug content.

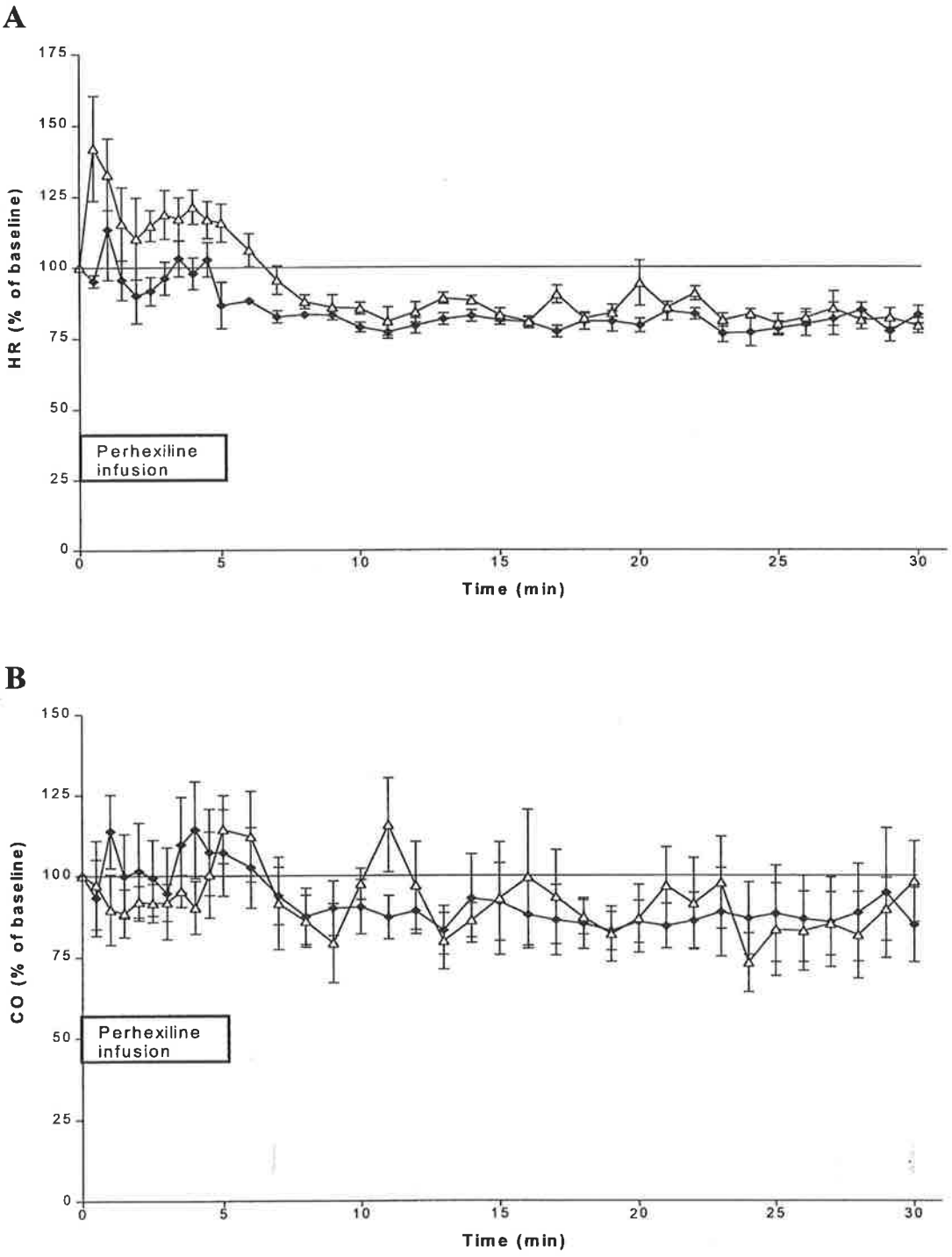


Figure 2.4: The time-courses of changes in heart rate (**A**) and cardiac output (**B**) expressed as percentages of their baseline values (mean ± SEM) following 5 minute infusions of 37.5 mg (♦; n = 4) and 75 mg (Δ; n = 4) of perhexiline intravenously.

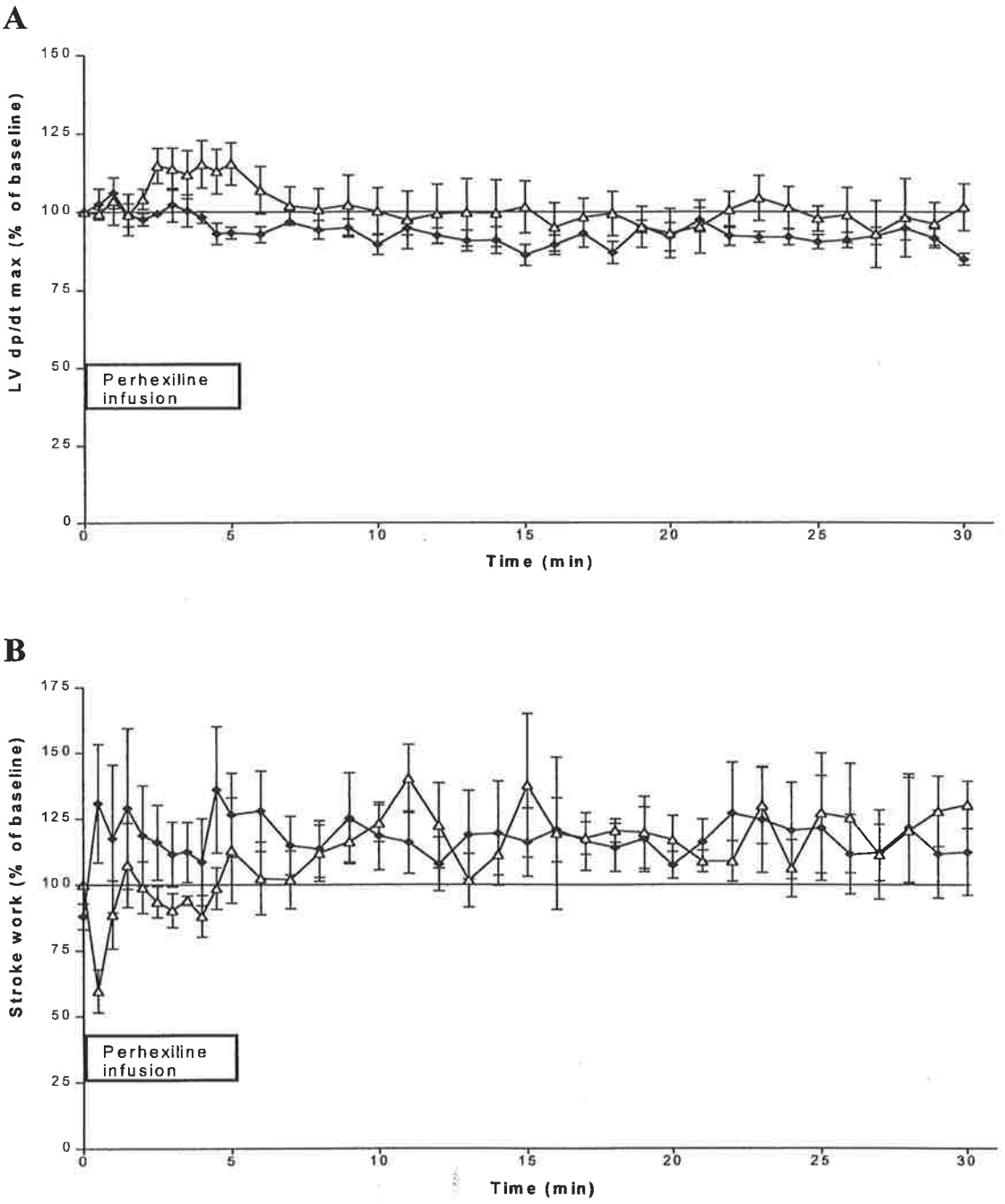


Figure 2.5: The time-courses of changes in (**A**) the maximum rate of left ventricular pressure rise (LV dP/dtmax) and (**B**) stroke work expressed as percentages of their baseline values (mean ± SEM) following 5 minute infusions of 37.5 mg (♦; n = 4) and 75 mg (Δ; n = 4) of perhexiline intravenously.

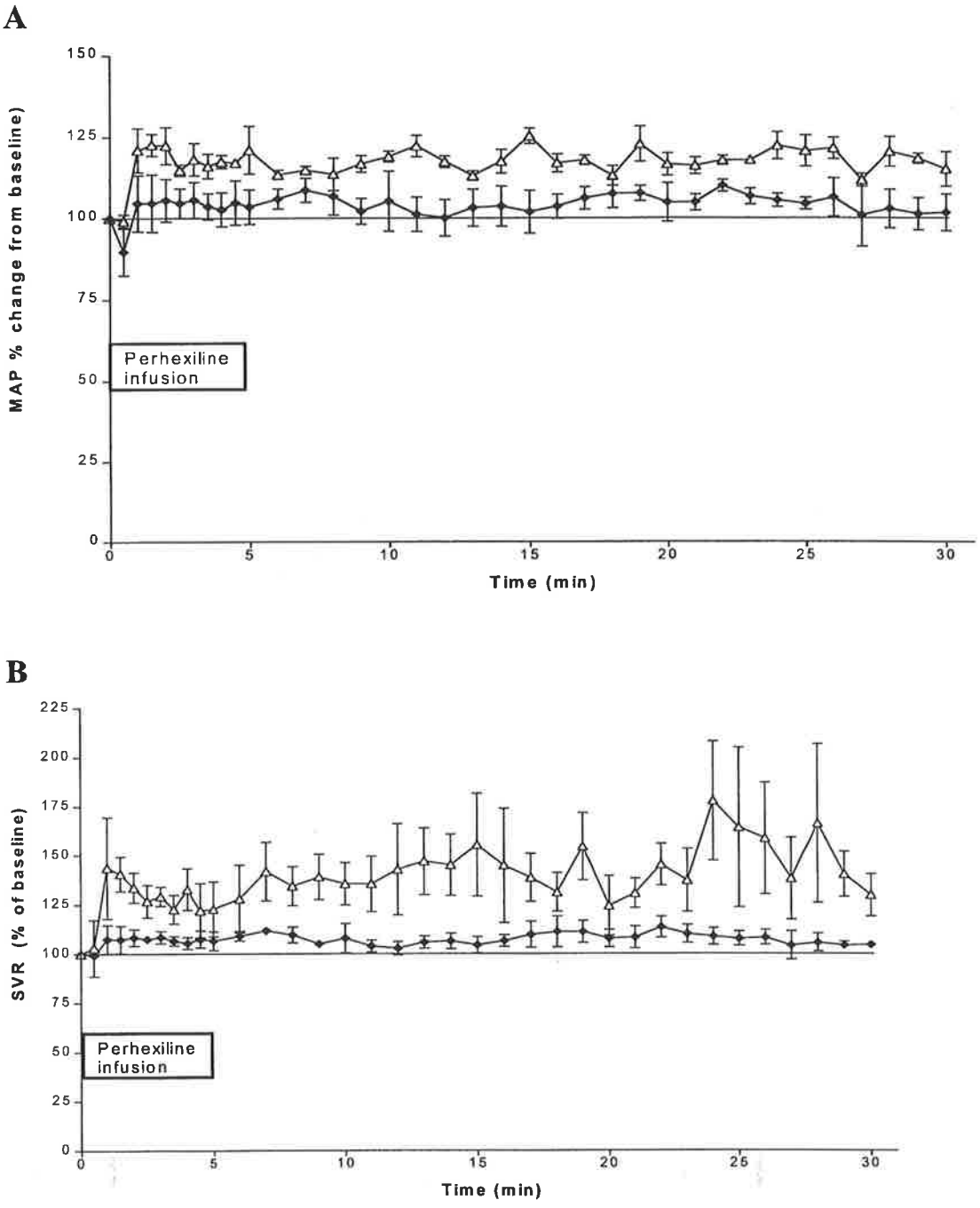


Figure 2.6: The time-courses of changes in mean arterial pressure (**A**) and systemic vascular resistance (**B**) expressed as percentages of their baseline values (mean ± SEM) following 5 minute infusions of 37.5 mg (♦; n = 4) and 75 mg (Δ; n = 4) of perhexiline intravenously.

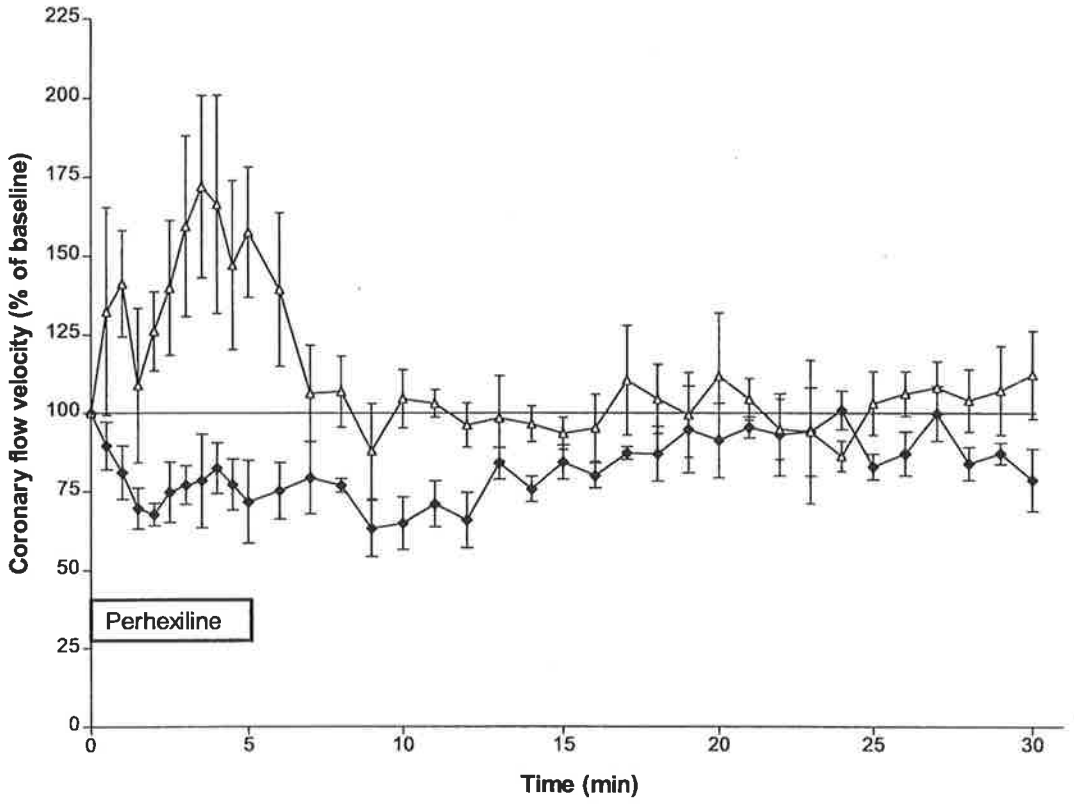


Figure 2.7: The time-courses of changes in Doppler coronary blood velocity expressed as percentages of baseline values (mean ± SEM) following 5 minute infusions of 37.5 mg (♦; n = 3) and 75 mg (Δ; n = 4) of perhexiline intravenously.

	Dose (mg)	Baseline	Infusion	Post-infusion	F	P
Heart rate (BPM)	37.5	88.7 ± 15.0	82.0 ± 10.5	72.0 ± 12.0*	13.52	0.006
	75	78.4 ± 7.8	92.1 ± 21.0*	67.0 ± 9.1	13.16	0.006
Cardiac output (L/min)	37.5	5.5 ± 0.9	5.6 ± 0.7	4.7 ± 0.5	1.31	0.34
	75	5.2 ± 0.7	4.8 ± 1.0	4.6 ± 1.1	1.66	0.34
LV dP/dt_{max} (mmHg/sec)	37.5	3935 ± 311	3846 ± 297	3527 ± 183*	8.79	0.02
	75	3375 ± 375	3739 ± 495*	3220 ± 578	12.98	0.007
Stroke work (g.m)	37.5	85.6 ± 14.1	100.8 ± 20.2	95.9 ± 18.0	1.28	0.35
	75	74.2 ± 16.8	73.1 ± 25.7	86.2 ± 25.6	4.77	0.06
MAP (mmHg)	37.5	98.4 ± 2.9	105.6 ± 7.6	103.3 ± 8.1	4.52	0.06
	75	81.4 ± 3.5	97.6 ± 5.6*	96.6 ± 6.1*	18.53	0.003
SVR (dynes.sec.cm^{-5})	37.5	1439 ± 187	1529 ± 239	1717 ± 178	1.96	0.22
	75	1289 ± 185	1697 ± 388	1917 ± 626*	5.57	0.04

Table 2.1: Mean values (± SD) for haemodynamic parameters at baseline, during the 5 minute perhexiline infusions, and between 10 and 15 minutes post-infusion of either 37.5 (n = 4) or 75 (n = 4) mg of perhexiline over 5 minutes. Statistical comparison between each time period was via one-way analysis of variance with repeated measures. Calculated F values and P values are shown. MAP = mean arterial pressure, SVR = systemic vascular resistance, * = significantly different from baseline via Dunnett's multiple comparison test.

Reference	Hudak 1970	Cho 1970	Rowe 1970	Ono 1981
Perhexiline salt	HCl	HCl	maleate	HCl
Perhexiline dose	0.3-3 mg/kg	1-2mg/kg	0.6 mg/kg	0.3-3 mg/kg
Heart rate	↓	↓	↑	↓
Cardiac output	↑	↔	↔	↔
Contractility		↓	↑	↑ ↓*
MAP	↓	↔	↔	↓
SVR	↓	↔	↔	
CBF	↑	↑	↑	↑
LV work		↔	↔	↓
MVO_2		↓	↓	↓
Cardiac efficiency		↑	↑	↑

Table 2.2: Summary of haemodynamic effects following brief (2-5 minute) intravenous infusions of perhexiline in various dog models: open chest (Hudak, 1970; Ono, 1981), closed chest (Rowe, 1970), and right heart bypass (Cho, 1970). HCl = hydrochloride, MAP = mean arterial pressure, SVR = systemic vascular resistance, CBF = coronary blood flow, LV work = left ventricular work, MVO_2 = myocardial oxygen consumption. * = bidirectional effect: ↑ at low dose, ↓ at high dose.

CHAPTER 3:

THE EFFECT OF PERHEXILINE ON FATTY ACID OXIDATION RATES IN SHEEP

3.1 INTRODUCTION

As discussed in Chapter 1, the use of radio-iodinated fatty acid analogues allows the non-invasive assessment of fatty acid metabolism in *in vivo* models using standard gamma cameras. 15-(*p*-[^{123}I]iodophenyl)pentadecanoic acid (IPPA) is an ideal agent, as its metabolism in the heart parallels that of palmitic acid (Reske, 1985; Reske, et al. 1984; Reske, et al., 1986). The rate of early clearance of radioactivity from the heart after bolus administration of IPPA is a surrogate measure of fatty acid β-oxidation, and effects of drug therapies on this rate of clearance can be quantified by analysis of the myocardial time-activity curves (DeGrado et al. 1989; Dormehl, et al. 1993; Kaiser, et al., 1990).

Sheep were used as the experimental model as they were readily available and relatively large compared to other commonly used experimental animals, necessary for the imaging procedures. Although the main natural sources of cardiac energy in ruminants such as sheep are volatile fatty acids, it was evident from a preliminary experiment that IPPA was cleared from the myocardium in an identical manner to previously published studies in other mammals, with a half-clearance time of approximately 30 minutes. However there were no data in the literature regarding the administration or metabolism of perhexiline in sheep, requiring preliminary dose-ranging studies.

The purpose of the experiments in this chapter were to investigate the effects of perhexiline on substrate utilisation under aerobic conditions in an *in vivo* sheep

model. The effects of both acute and 24 hour parenteral perhexiline administration on myocardial IPPA clearance kinetics are presented. Given the *in vitro* results showing inhibition of CPT-1 by perhexiline in isolated cardiac and hepatic mitochondria, a known CPT-1 inhibitor, etomoxir, was used as a positive control. Etomoxir was selected on the basis of a previous study by Dormehl et al. (Dormehl et al. 1993) which showed significantly delayed myocardial clearance of IPPA following administration of etomoxir in baboons.

3.2 Methods

3.2.1 Animals and materials

Female adult sheep (weight range 25-38 kg) were obtained from the University of Adelaide Waite Institute, and housed in air-conditioned rooms maintained at 22°C with a 12 hour light-12 hour dark cycle. All experimental procedures were approved by the University of Adelaide Animal Ethics Committee. Between experiments, radioactive waste was disposed of according to radiation safety guidelines.

Unlabelled phenyl-pentadecanoic acid was supplied by MDS Nordion, Canada, and raw ^{123}I by the National Medical Cyclotron (Sydney, Australia). Perhexiline lactate was manufactured by the Victorian College of Pharmacy from the perhexiline maleate salt (Sigma Pharmaceuticals, Australia), and etomoxir (sodium salt) was obtained from Dr HPO Wolf (Allensbach, Germany).

3.2.2 Preliminary experiments

Initial attempts at oral administration of commercially available perhexiline maleate tablets (Sigma Pharmaceuticals, Australia) to sheep were unsuccessful, as plasma levels of both perhexiline and its major metabolite, hydoxy-perhexiline, were unrecordable despite large oral doses of up to 600 mg twice a day for 3 days. This suggested very poor bioavailability, probably related to the ruminant physiology of the sheep.

Further dose ranging studies were performed using intravenous perhexiline lactate (prepared by the Victorian College of Pharmacy) dissolved in 5% dextrose. Given the rapid elimination kinetics following bolus doses or brief infusions of perhexiline, longer infusions were used for further dose-ranging studies. With the aim of maintaining plasma levels within the "therapeutic range" of between 0.15 and 0.60 mg/l (as quoted for patients on long-term therapy) (Cole, et al., 1990; Horowitz et al. 1986) during planned imaging with IPPA, a Terumo syringe pump was used to infuse the perhexiline at a constant rate over 60 minutes.

In preliminary studies, a total dose of 5 mg/kg perhexiline, administered in a volume 60 ml of 5% dextrose at a rate of 1 ml/min (83 μg/kg/min) was not associated with any changes in heart rate, systemic blood pressure, or ECG parameters. The plasma perhexiline concentrations during and after administering this dose to three sheep are shown in Figure 3.1. This dose and infusion rate was utilised in the imaging studies

performed to assess the effects of acute perhexiline and etomoxir exposure on myocardial IPPA metabolism (see below).

In order to assess effects of longer-term perhexiline exposure, intravenous infusions of perhexiline dissolved in dextrose were administered over several days. Initial attempts at infusing 1 gram of perhexiline per day (approximately 30 mg/kg per day) led to adverse reactions including anorexia, tachypnoea, diarrhoea, and haematuria after the first 24 hours, with one sheep dying on Day 3. Another sheep died during sodium thiopentone anaesthesia just prior to imaging on the third day, suggesting a toxic combination of chronic perhexiline levels and barbiturate anaesthesia. Plasma levels for these two sheep at 48 hours were 0.50 and 0.62 mg/l respectively. Due to the toxicity encountered with more prolonged infusions, the imaging studies descibed below were performed following 24 hour infusions only.

3.2.3 Preparation of IPPA

Raw [^{123}I]sodium iodide was supplied on a weekly basis by the National Medical Cyclotron (Sydney, Australia). Phenylpentadecanoic acid (MDS Nordion, Canada) was radioiodinated at the Queen Elizabeth Hospital radiochemistry laboratory on the day of planned imaging. This was done via an organothallium intermediate, as per Kulkarni and Parkey(Kulkarni & Parkey, 1982). 0.5 mg PPA was dissolved in 0.2 ml trifluoroacetic acid with 2 mg of thallium trifluoroacetate and incubated at room temperature for at least one hour. The in-situ formed PPA-thallium complex was treated with 185 to 300 MBq of Na^{123}I in 100μl of 0.1N NaOH along with 12 μg of

the carrier KI. The reaction mixture was heated at 100°C for 15 minutes and the labelled product purified by C18 solid phase extraction and reverse phase HPLC. After evaporation of the solvent, the product was dissolved in 100 μl ethanol and reconstituted with 6% bovine albumin solution. The final product was filtered through a 0.1 μm membrane filter to remove aggregates.

3.2.4 Experimental protocols

At least 24 hours prior to the initial imaging procedure, sheep were cannulated using the Seldinger technique via the external jugular vein while under general anaesthesia with pentobarbitone sodium 20 mg/kg. The central venous sheaths (8.5 Fr, Arrow International Inc., Reading, PA, U.S.A) were sutured in place and left in situ for serial imaging procedures in each sheep.

Before each experiment, the sheep were fasted for 24 hours. They were anaesthetised with sodium thiopentone (20 mg/kg) and intubated with a cuffed tracheal tube prior to transfer to the Nuclear Medicine Department at the Queen Elizabeth Hospital. Sheep were placed supine under the large field of view gamma camera (either the GE or Siemens Diacam) and were strapped to prevent movement during imaging (see Figure 3.2). Anaesthesia was topped up as necessary during the experiment with 2mg/kg boluses of pentobarbitone sodium.

The short term experiments were performed to observe the effect of acute exposure to perhexiline, with plasma perhexiline levels within the "therapeutic range" as quoted

for patients on long-term therapy (i.e. 0.15-0.6 mg/l) (Cole, et al., 1990; Horowitz, et al. 1986) during a short (60 minute) infusion of perhexiline. Imaging was commenced half-way through this infusion so that the early clearance phase of IPPA, corresponding to β-oxidation, occurred while plasma perhexiline levels remained in this range, and sufficient time had elapsed to allow myocardial uptake of perhexiline (see Section 2.3.2.1). Venous plasma samples were taken at 30 and 60 minutes during the perhexiline infusion for assay of plasma levels (see section 2.2.5 for methodology).

To assess the effects of short-term exposure to perhexiline, each sheep underwent serial imaging procedures: baseline, using a control infusion of 50 ml 5% dextrose; a second scan during an infusion with perhexiline lactate (total dose 5 mg perhexiline base per kg) in 50 ml of 5% dextrose; and a third experiment, using 10 mg/kg of etomoxir in 25 ml of 5% dextrose over 30 minutes, immediately followed by IPPA injection (and imaging) and then a further 25 ml of 5% dextrose. This was done to mimic the other infusions, but also to give the entire dose of etomoxir prior to analysis of β-oxidation, as was the case in the previously published work (Dormehl, et al. 1993). Etomoxir was always the final drug administered, due to its irreversible inhibition of CPT-1 (Zarain-Herzberg & Rupp, 1999). Infusions of perhexiline were administered via the jugular vein over 60 minutes, with IPPA injected at 30 minutes, at which time imaging was commenced and a plasma perhexiline level was taken (from the opposite jugular vein). Imaging experiments in each individual animal were performed at least one week apart.

Similar methodology was utilised in the assessment of longer-term perhexiline exposure. These sheep underwent two sets of imaging experiments: following an infusion of 1000 ml of 5% dextrose via the central venous catheter over 24 hours; and following a similar infusion with a total dose of 1 gram of perhexiline lactate dissolved in the 5% dextrose solution. Plasma perhexiline levels were measured at the time of IPPA injection (approximately 1 hour before the end of the 24 hour infusion).

3.2.5 Imaging and data analysis

60-80 MBq IPPA was injected as a bolus via the jugular vein catheter. Imaging was performed using a Siemens Diacam single-headed gamma camera. A low-energy all-purpose collimator was used, and a 20% energy window was set around the 159 keV photopeak of ^{123}I. Data acquisition was commenced immediately on injection of IPPA, and continued for 50 minutes (50 one minute frames). At 35-40 minutes, a bolus of 30-50 MBq $Na^{123}I$ was injected to correct for radio-iodine blood pool background.

Data processing was performed on the Diacam Icon AppleMac. In order to correct the serial images for activity from the ^{123}I in the blood pool and interstitial space, i.e. ^{123}I not bound to the myocardial cells, a correction method was used as published by Freundlieb et al(Freundlieb, et al., 1980). Two regions of interest were used, the myocardium and a second control region over the superior vena cava. The principle is shown in Figure 3.3, which shows the count rates for each region as a function of

time. The sudden rise in count rates (in counts per minute, CPM) is due to the second injection of $Na^{123}I$. The shaded areas in the diagrams indicate the count rate related to blood pool and interstitial space, i.e. free activity, in each image region.

The total count rate from the myocardial region, T_{MY}, includes both free (background) ^{123}I and ^{123}I bound to myocardial cells, the latter shown as FA_{MY}. It is assumed that the ratio of *a*, the CPM increment in the control region, over *b*, the CPM increment in the myocardial region, is proportional to the ratio of free ^{123}I in the control and myocardial regions. The increments *a* and *b* are obtained by subtracting the count rates recorded in the last 2 minutes before the $Na^{123}I$ injection from the count rates registered in the second and third minutes after the injection.

The time course of the nonmyocardial background is given by the time activity curve T_{VC}, recorded over the control region. Therefore, the time course of the background in the myocardial region is obtained by the expression $b/a \,.T_{VC}$. Finally the counting rates related to the fatty acid bound ^{123}I in the myocardium (FA_{MY}) is calculated by the formula:

$$FA_{MY} = T_{MY} - b/a \,.T_{VC}.$$

The corrected data (FA_{MY}) was plotted and an exponential line of best fit was derived over the initial rapid clearance phase (peak to 30 minutes), allowing calculation of the IPPA half clearance time which was used as a measure of myocardial fatty acid β oxidation.

3.2.6 Statistical analysis

Paired t tests were used to compare the time to peak myocardial activity and the myocardial half clearance times between the perhexiline and/or etomoxir experiments and the corresponding control experiments within each sheep. A value of $P < 0.05$ was considered significant.

3.3 RESULTS

3.3.1 Short-term infusions

An example of a raw time-activity curve, the myocardial and background regions of interest utilised, and the background and blood-pool corrected curve for IPPA clearance are shown in Figures 3.4 and 3.5. The times to peak myocardial IPPA uptake, and half-clearance times are shown in Table 3.1. Due to a problem with venous access during one experiment, data are only available for 7 of the sheep during perhexiline infusions. Etomoxir was used as a "positive control" in 6 sheep.

There were no significant differences in time to peak myocardial uptake of the radiolabelled fatty acid between the three different infusion protocols. Nor were there any significant changes in the myocardial half clearance times between sheep receiving control vs. perhexiline infusions. The clearance of IPPA following etomoxir was highly variable, ranging from 39.1 to 166.2 minutes, but was significantly prolonged in comparison to the control values in the corresponding sheep.

The mean (± SD) plasma perhexiline concentrations at 30 (time of IPPA injection) and 60 minutes into the one hour perhexiline infusions were 0.36 ± 0.07 and 0.40 ± 0.07 mg/l respectively.

3.3.2 24 hour infusions

The results of paired 24 hour infusions (control and perhexiline) were available in 4 sheep, and are shown in Table 3.2. The plasma perhexiline concentrations at the time of IPPA injection ranged from 0.31 to 0.69 mg/l. Two sheep were sacrificed following the 24 hour infusion, and tissue perhexiline levels were measured in samples from the liver, lung, and left ventricle, shown as μg per gram of wet tissue in Table 3.3. The tissue to plasma drug concentration ratios were approximately 2 in the heart, 0.2 to 0.3 in the liver, and 20 in the lung. This marked accumulation in lung tissue has also been seen in patients on chronic oral perhexiline therapy (Dr Jennifer Kennedy, personal communication). Despite the high tissue levels of the parent compound, levels of the M1 metabolite were unrecordable except in the liver of the final sheep, where it was barely detectable (0.03 μg/g).

There were no significant differences seen in the time of peak myocardial activity or the IPPA half clearance times between the control and perhexiline infusions.

3.4 DISCUSSION

Under aerobic conditions, perhexiline administered intravenously for up to 24 hours had no demonstrable effect on myocardial utilisation of fatty acids in sheep, as evidenced by the lack of change in IPPA clearance. At the infusion rates administered, no significant haemodynamic effects would be expected on the basis of preliminary experiments.

In the only previous study of the effect of CPT-1 inhibition on myocardial fatty acid kinetics in an *in vivo* model, Dormehl et al. administered a similar dose of etomoxir (10 mg/kg) intravenously to 6 adult male baboons, which were fasted for 18 hours prior to imaging with IPPA (Dormehl et al. 1993). The myocardial half clearance times for the early rapid clearance phase were significantly prolonged following etomoxir compared to baseline values (55.0 ± 13.0 compared to 24.0 ± 2.6 respectively). This was associated with a significant delay in the time of peak myocardial activity, from 5.3 ± 1.3 minutes at baseline to 12.0 ± 2.8 minutes following etomoxir.

The time to peak myocardial activity and the myocardial half-clearance times seen in the control experiments in the current study in sheep are comparable to those seen previously in baboons (Dormehl et al. 1993) and man (Kaiser et al. 1990) under control conditions. This suggests that IPPA catabolism and clearance are similar in sheep to previous animal models studied. Unlike the study by Dormehl et al, no significant change was observed in the time to peak activity following etomoxir

administration. This early phase of the time-activity curve has been shown to be purely a reflection of perfusion in studies comparing $^{13}NH_3$ and ^{11}C-palmitic acid (Schelbert et al. 1981), and would not be expected to change significantly in the absence of a reduction in coronary flow.

The major limitation to this study may be the use of sheep as the experimental model. In ruminants such as sheep, the main source of energy are volatile fatty acids, while glucose makes only a minor contribution (4-11%) to energy metabolism. Hence inhibition of long-chain fatty acid oxidation may not alter cardiac metabolism to the same extent as in monogastric animals. For this reason, all animals were fasted for 24 hours, to maximise cardiac utilisation of the radiolabelled fatty acid. However the finding that etomoxir significantly prolonged IPPA half-clearance times indicates that this model is sensitive to CPT-1 inhibition, although the wide variability in the etomoxir results cannot be explained.

The other limitation with the use of sheep was the inability to chronically administer perhexiline (without serious toxicity). The duration of perhexiline exposure required to produce metabolic effects at the level of the cardiomyocyte is unknown. Preliminary work from our laboratory has shown a time-dependent effect of perhexiline on palmitate oxidation in cultured rat neonatal cardiomyocytes. Significant inhibition of palmitate oxidation in the presence of 1 μM perhexiline was seen at >48 hours incubation but not at <24 hours (personal communication, Dr Jennifer Kennedy). In a previous study in cultured rat hepatocytes, exposure to

perhexiline at very high concentrations (25 μ*M*) led to a reduction in β-oxidation within 24 hours, but effects of lower concentrations (5 μ*M*) were only seen at 72 hours, suggesting that there was a delay between exposure and metabolic effects (Deschamps, et al., 1994). These data suggest a considerable delay between exposure to perhexiline and mitochondrial metabolic effects. The authors postulated that the time-dependent effect may have been due to the slow accumulation of perhexiline in cultured rat hepatocytes, as had previously observed with another highly lipophilic drug, amiodarone (Honegger, et al., 1993). In addition, the accumulation of perhexiline at the subcellular level at its postulated site of action in the mitochondria may also be even slower *in vivo*.

Hence an inhibitory effect on cardiac fatty acid metabolism with longer durations of perhexiline exposure cannot be ruled out. The ideal model for future use of this technique is man, in whom the long-term administration of perhexiline is safe (with monitoring of plasma levels). Suitable subjects would be those with stable angina pectoris and normal resting coronary perfusion (as assessed with myocardial perfusion SPECT or PET imaging), to exclude regional alterations in coronary flow as a confounding variable. IPPA imaging at baseline and following oral perhexiline loading would allow correlation of effects on cardiac fatty metabolism with clinical anti-anginal effects and plasma perhexiline levels.

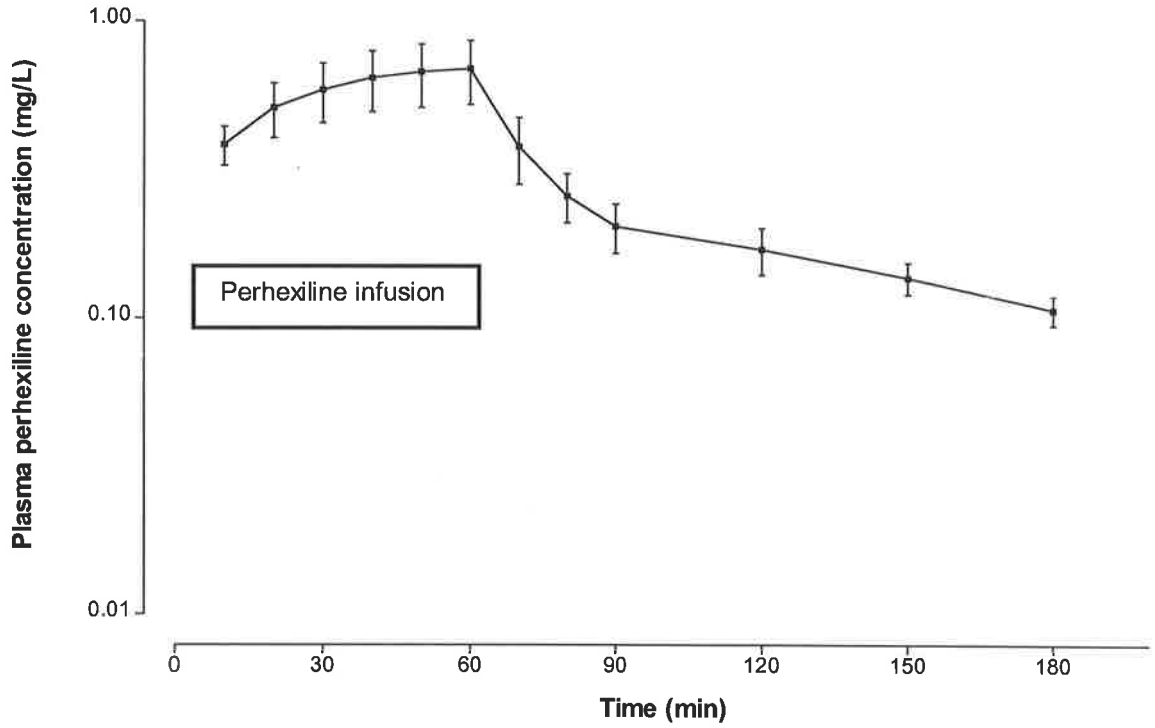


Figure 3.1: Venous plasma perhexiline concentrations (mg/L) during and after 60 minute infusions of perhexiline at 83 μg/kg/minute (total dose administered = 5 mg/kg).

Data are the means ± SEM for three sheep.

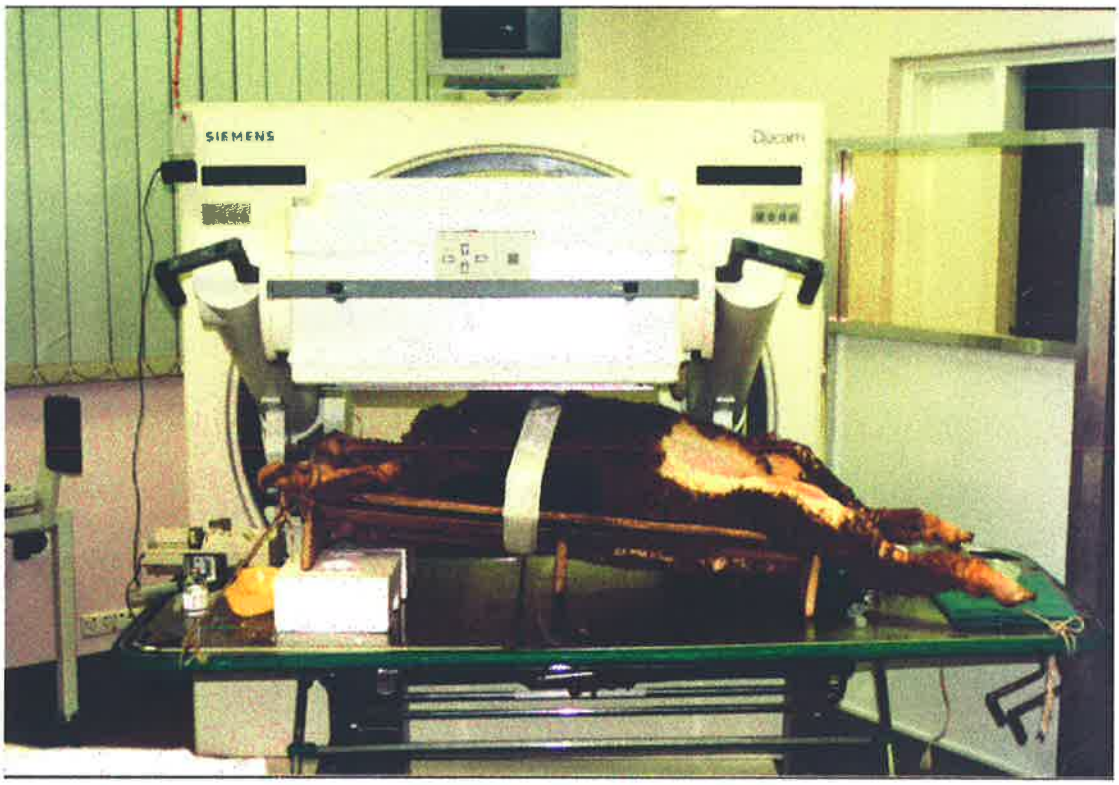
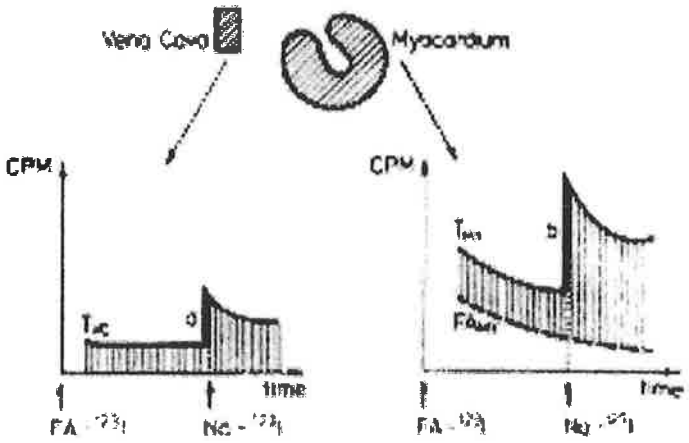


Figure 3.2 Sheep being imaged under the Siemens Diacam gamma camera, during infusion of perhexiline.



$$\frac{a}{b} = \frac{T_{VC}}{T_{MY} - FA_{MY}}$$

$$FA_{MY} = T_{MY} - \frac{b}{a} T_{VC}$$

Figure 3.3: Correction procedure for non-myocardial background activity after ^{123}I-hepta-decanoic acid injection. CPM = counts per minute; T_{VC} = count rates over control region of interest (vena cava); T_{MY} = count rates over myocardial region of interest; FA_{MY} = count rates related to organically bound ^{123}I; *a* and *b* = increments due to Na^{123}I injection. From Freundlieb et al., 1980.

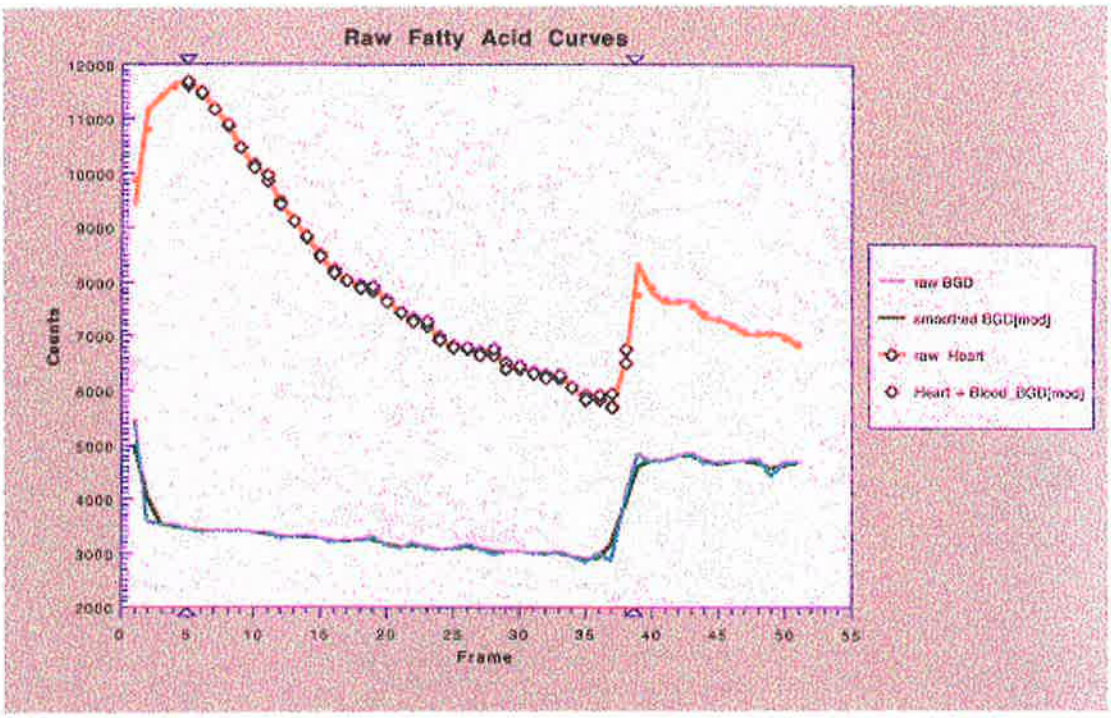


Figure 3.4. Example of the uncorrected (raw) time activity curves in one sheep imaged under the gamma camera following injection of IPPA at Frame 0 and $Na^{123}I$ at frame 37, derived from counts over the myocardium (orange) and superior vena cava (blue).

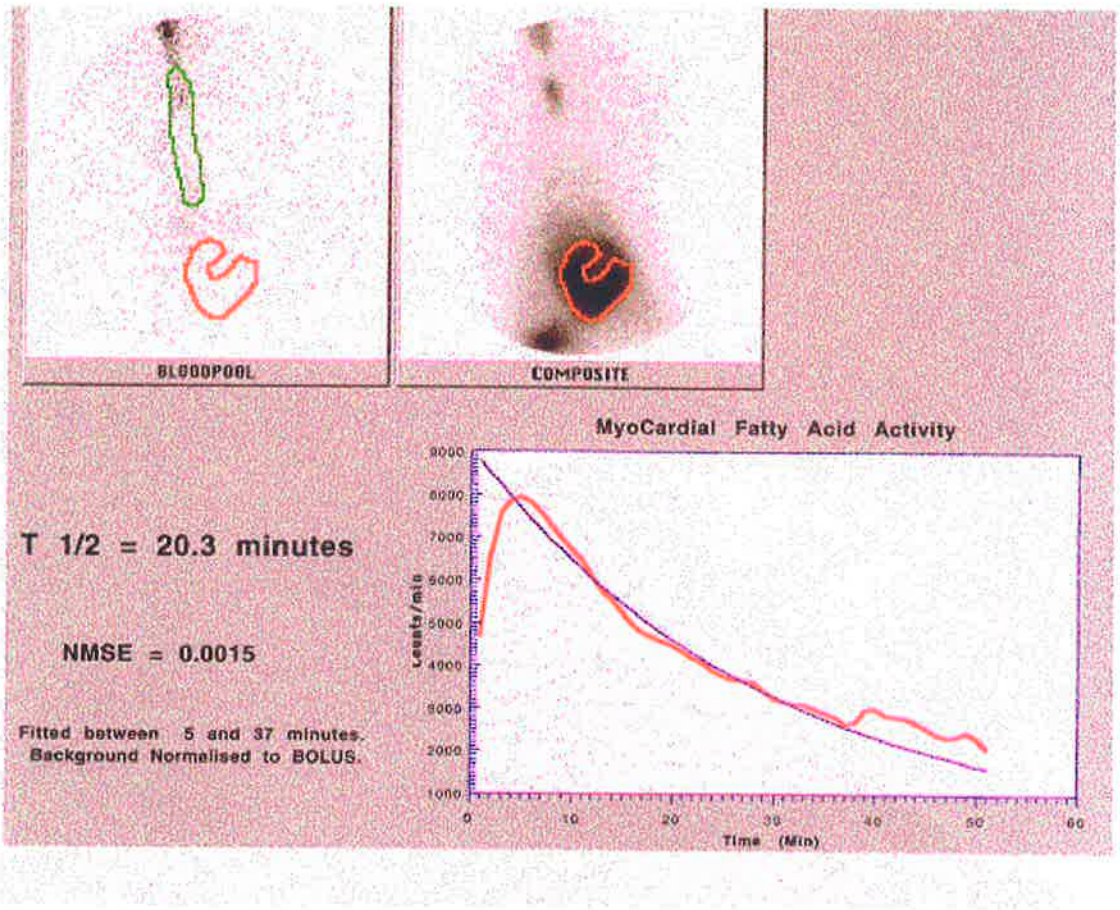


Figure 3.5: Regions of interest over the superior vena cava and myocardium, and corrected myocardial time activity curve with mono-exponential line of best fit.

	Control		Perhexiline		Etomoxir	
Sheep	**T_{max}**	**$T_{½}$**	**T_{max}**	**$T_{½}$**	**T_{max}**	**$T_{½}$**
1	5.6	14.1	6.6	13.5	4.5	39.1
2	6.1	21.4	7.5	21.4	6.6	143.3
3	7.9	22	8.2	21.9	7.2	44.3
4	4.3	28.8	3.4	18	5.3	166.2
5	5.4	24.6			5.2	41.6
6	3.0	27.9	5.6	26.8		
7	6.6	14.0	6.4	20.7		
8	5.7	21.3	4.8	22.3	4.0	45.1
Mean ± SD	5.6 ± 1.5	21.4 ± 5.5	6.1 ± 1.6	20.7 ± 4.1	5.5 ± 1.2	79.9 ± 58.4*

Table 3.1 The time (minutes) to peak uptake (T_{max}) and half clearance times ($T_{½}$) of myocardial IPPA activity in sheep treated with 60 minute intravenous infusions: control (n = 8), perhexiline 5 mg/kg (n = 7), and etomoxir 10 mg/kg (n = 6). * = significantly different from control infusions via paired t test ($P < 0.05$).

Sheep	Control		Perhexiline	
	T_{max}	T_{max}	$T_{½}$	$T_{½}$
1	6.2	39.1	5.4	21.0
2	7.1	32.3	5.9	30.7
3	4.7	23.2	6.1	34.9
4	5.1	24.6	6.2	28.0
mean ± SD	5.8 ± 1.1	29.8 ± 7.4	5.9 ± 0.4	28.7 ± 5.8

Table 3.2: The time (minutes) to peak uptake (T_{max}) and half clearance times ($T_{½}$) of myocardial IPPA activity in 4 sheep following 24 hour vehicle or perhexiline infusions. No significant differences are seen between control and perhexiline infusions.

Sheep	Plasma [P]	Heart [P]	Liver [P]	Lung [P]
1	0.50	1.05	0.10	9.50
2	0.69			
3	0.31			
4	0.45	1.32	0.15	8.70

Table 3.3: Plasma levels ([P]) in mg/l at the time of IPPA injection in each sheep following 1 gram of perhexiline administered intravenously over 24 hours. Tissue levels, in μg per gram of wet weight, are also shown for two sheep sacrificed shortly after the 24 hour infusion.

CHAPTER 4:

THE EFFECTS OF PERHEXILINE ON CARDIAC METABOLISM, FUNCTION, AND EFFICIENCY IN THE ISOLATED WORKING RAT HEART

4.1 INTRODUCTION

The metabolic effects of perhexiline, postulated as early as 1980 (Vaughan Williams, 1980), have not been investigated in detail. The first mention of inhibition of mitochondrial fatty acid metabolism was made in 1995 in a study into the possible mechanisms of toxicity of perhexiline in cultured hepatocytes (Deschamps, et al., 1994). Jeffrey et al. reported a shift in substrate utilisation from fatty acid to carbohydrate in an isolated working heart model, accompanied by an improvement in myocardial efficiency under aerobic conditions (Jeffrey et al. 1995). Finally, work from our own laboratory has demonstrated the inhibitory effects in isolated cardiac and hepatic mitochondria of perhexiline on the key regulatory enzyme in fatty acid metabolism, CPT-1 (Kennedy et al. 1996).

The purpose of the experiments in this chapter was to further investigate the metabolic and haemodynamic effects of perhexiline in the isolated working heart model, and compare these to the effects of the known CPT-1 inhibitors, oxfenicine and etomoxir. In addition, the effect of the duration of perhexiline exposure was assessed by performing *ex vivo* experiments on hearts from rats pre-treated with perhexiline.

4.2 METHODOLOGY

4.2.1 Background

4.2.1.1 Working rat heart models

The relationships between mechanical activity, oxygen consumption, and substrate utilisation by the heart can best be studied using an isolated heart preparation that does not include lungs or other tissues in the circuit. The Langendorff technique of retrograde aortic perfusion, first described in 1895 (Langendorff, 1895), has been used extensively for this purpose. Perfusate in this model is delivered to the heart in a retrograde fashion via a cannula in the aorta, perfusing the heart during diastole via the coronary arteries. However, the workload in these preparations is far below physiological levels and cardiac systolic function is difficult to quantitate.

Neely et al devised a "working" isolated rat heart preparation in 1967, which was capable of *in vitro* mechanical work (Neely et al. 1967). Perfusate was introduced into the left atrium at various filling pressures, and pumped by the left ventricle against a hydrostatic pressure head. This preparation allowed measurement of both cardiac work and oxygen consumption over a range of left ventricular and left atrial pressures. However the cardiac outputs measured using this technique were only approximately half those observed in intact animals, and cardiac performance was only constant when the perfusate was constantly changed, with a progressive decline in performance after 30 minutes.

Perfusate consisting of a physiological saline solution, such as Krebs'-Henseleit, is a practical alternative to the difficulties associated with the use of whole blood. When studying energy metabolism in the heart, the perfusate must contain physiologically relevant concentrations of the prime energy substrates such as glucose and fatty acids. The fatty acids predominantly oxidised by the heart, such as palmitate and oleate, are however extremely water insoluble. *In vivo*, they are transported to the heart in the blood either bound to albumin or as triacylglycerols contained in lipoproteins (Lopaschuk et al. 1994). Their insolubility can be overcome *in vitro* by binding them to albumin, added to the perfusate at a physiological concentration of 3%. However Neely's apparatus was unable to provide the heart with fatty acids, as the method of oxygenation of the buffer in this preparation (by bubbling gas through it) resulted in major problems with denaturing of proteins and excess frothing when albumin was used.

This problem was overcome by Taegtmeyer et al. with the use of specially designed water jacketed mulitibulb glass oxygenator columns (Taegtmeyer et al. 1980), in which the perfusate is passed over a large surface area exposed to 95% O_2-5% CO_2. Oxygen exchange between the gaseous phase and the perfusate is facilitated by the fact that albumin markedly decreases the surface tension of the aqueous perfusing solution, allowing for a thin film to form on the inner surface of the chamber. This set-up allows well-oxygenated perfusion buffer (O_2 saturation 87 ± 4%) (Taegtmeyer, et al. 1980) to reach the heart without excess foaming.

This use of fatty acids in Taegtmeyer's working heart model resulted in near-physiological performance with respect to cardiac output and aortic pressure for up to two hours. In addition, the preload and afterload could be altered, via adjustment of the pre-atrial and aortic overflow chambers respectively, to provide predictable changes in developed pressure and cardiac work (according to Starling's law). Taegtmeyer observed significant quantitative differences (up to twofold) in the rates of oxygen consumption and substrate utilisation in comparison to previous studies at subphysiological workloads. Qualitative differences were also noted, such as the greater contribution of glucose as a cardiac energy source in the presence of other substrates.

The working heart apparatus described by Taegtmeyer consisted of eight units connected by Tygon tubing (see Figure 4.1). In order to resemble the physiological *in vivo* situation as closely as possible, the temperature within the apparatus must be kept at a constant 37° Celsius. This was done by the utilisation of specially designed double jacketed glassware for all components of the working heart apparatus, with constant circulation of water warmed to 37°C by a water pump through the outer glass layer.

The heart was mounted on a stainless steel cannula assembly and held in place within a water jacketed **organ chamber (E)** by a silicane rubber bung fitted across the top of the chamber. The perfusion medium was warmed in a water jacketed glass **reservoir chamber (D)** with several openings at the top for inflow (from the organ chamber as

well as overflow from the preload and afterload chambers) and outflow of perfusate. The perfusion medium was pumped from here via an electric peristaltic roller **pump (C)** through a **Millipore filter system (B)** of 5 μm pore size and 4.5 cm diameter, to the top of the **gassing chamber (A)**, into which the gas mixture ($95\%O_2$ - $5\%CO_2$) also flowed via a separate inlet line.

At the lower end of the gassing chamber was a small reservoir containing about 10 ml of perfusate with an overflow type bubble trap. The fluid level in this trap was set at 11.5 mmHg (15 cm H_2O) above the left atrium (to simulate physiological atrial preload) via an overflow line which returned excess buffer to the reservoir chamber. From the gassing chamber the well-oxygenated perfusate flowed into the left atrium of the heart via a minimum of tubing. From the atrium, the buffer entered the left ventricle and was subsequently ejected out the aorta into a **compliance chamber (G)**, which was approximately one third filled with air to provide some elasticity into an otherwise rigid system, simulating aortic compliance. The buffer was then pumped up the afterload line by the working heart during systole, and delivered to the coronary arteries during diastole. At the top of the afterload line was another small reservoir with a bubble trap, the **aortic afterload chamber (H).** The fluid level in this chamber determined the workload of the heart, and was set a height of 80 mmHg (100 cm H_2O) to simulate physiological left ventricular afterload. The height of both the gassing chamber and the aortic overflow chamber could be adjusted over a wide range of non-physiological heights, allowing the workload of the heart to be varied. Finally, the buffer spilled over from the outlet of the aortic afterload chamber and fell

freely down to the reservoir chamber. This "recirculating" working heart system allowed the use of a constant known volume of perfusate enabling the investigator to monitor changes in the utilisation of metabolic substrates over time.

Not shown in the diagram is another gassing chamber, which was used in the initial set-up of the heart as a Langendorff perfusion. Once the aorta had been cannulated, a separate Krebs-Henseleit solution was delivered via this gassing chamber to the heart in a retrograde manner via the aortic cannula, allowing the heart to stabilise and the investigator to cannulate the left atrium and pulmonary artery. Unlike the working heart buffer, the Langendorff perfusate was not recirculated, and dripped out of the heart into a waste pan. The two buffers did not mix. Once ready, the inflow line from the Langendorff gassing chamber was clamped, and both the inflow line into the left atrium and the afterload outflow line from the aorta were unclamped, starting the working heart mode.

The final piece of apparatus in Taegtmeyer's working heart set-up was a water-jacketed Clark type **oxygen electrode (F)**, used to measure the oxygen concentration of oxygenated 'arterial' (from the bottom of the gassing chamber) or desaturated 'venous' (from a cannula in the pulmonary artery of the heart) perfusion medium. Oxygen consumption (μmol/min per gram dry weight) could be thus calculated using the formula:

$$O_2 \text{ consumption} = \frac{(\text{arterial content} - \text{venous content}) \times (\text{coronary flow in ml/min})}{\text{dry weight of the heart in grams}}$$

Estimation of cardiac output, in ml/minute, was made by addition of the aortic and coronary flow rates. Coronary flow was estimated by collection of the fluid dripping from the pulmonary artery cannula and organ chamber in a graduated cylinder for a specified time period, while aortic flow was similarly measured by collection of buffer as it overflowed from the aortic overflow chamber. A pressure transducer connected to the afterload line above the compliance chamber, via a Y tube, enabled measurement of aortic systolic and diastolic pressures.

From these measurements, it is possible to quantify the work of the heart. The main components of the work output of the heart are pressure and volume. Taegtmeyer derived an index of cardiac hydraulic work by multiplying cardiac output (the sum of aortic and coronary flow in ml/min) by the height of the aortic fluid column (cm H_2O) and the specific gravity of the perfusion medium, with final units of kg.m/min (Taegtmeyer et al. 1980). This simplified calculation ignored the kinetic work performed by the heart, as calculation of this factor is fraught with difficulties, and assumed to be constant in working rat heart experiments. However a single universal description of the pump performance of the heart is not available (Taegtmeyer et al. 1980), and various indices have been used by other investigators.

4.2.1.2 Ischaemia/reperfusion models: issue of "low-flow" ischaemia

Two models are generally used to investigate cardiac metabolism during ischaemia/reperfusion, those of no-flow and low-flow ischaemia (Depre, et al. 1999). Both models are not fully representative of the situation in vivo. In the first model,

the heart is usually perfused in a working mode, and coronary flow is directly related to the work performed. With ischaemia, the flow is totally interrupted, so that all metabolic end-products accumulate in the heart. Metabolism in this model of ischaemia is purely via anaerobic glycolysis from glycogen breakdown. When glycogen stores are exhausted, irreversible ischaemic contracture occurs (Goodwin & Taegtmeyer, 1994). In the model of low-flow ischaemia, the heart is perfused retrogradely (Langendorff technique) at constant coronary flow. Ischaemia is induced by decreasing the coronary flow to such a value that contractile activity of the heart is reduced. In this model of ischaemia, glucose uptake and glycolysis are accelerated, and residual flow allows washout of metabolic end-products such as lactate and protons. In such a model, it is possible to impose longer periods of ischaemia, so that the ischaemic damage is only partly irreversible (Bricknell et al. 1981).

Both of the major groups using the working heart model have reported attempts at introducing "low-flow" ischaemia in this model, which would be the most physiologically relevant to the investigation of anti-anginal agents assuming a setting of acute myocardial ischaemia; even more minor reductions of perfusion, combined with tachycardia, would be appropriate for models of classical angina and its therapeutics. Lopaschuk's group described attempts (Lopaschuk & Spafford, 1989; McCormack et al. 1996) to make hearts ischaemic using a "low flow" technique while still in the working mode by using a one-way ball valve technique developed initially by Neely's laboratory in 1973 (Neely et al. 1973). The one-way ball valve is used to divert aortic outflow, resulting in a 50% reduction in diastolic coronary perfusion.

However, with the more physiological set-up in use in the 1990s, it was very difficult using this methodology to obtain steady state conditions, as a progressive decline was seen in cardiac function and oxygen consumption (McCormack et al. 1996). As a result, they abandoned this approach and decided instead to switch to the Langendorff perfusion mode for assessment of drug effects during low-flow ischaemia (McCormack et al. 1996), a technique they had previously used for this purpose (Broderick et al. 1992; Finegan et al. 1993). After 15-30 minutes of normal flow in the working heart mode, hearts were switched mid-experiment to the Langendorff mode using a constant coronary flow rate of 0.5 ml/minute to simulate low-flow ischaemia. Thus their data are not a true reflection of low-flow ischaemia in a working heart model.

Taegtmeyer's group described an alternative method to reduce coronary flow in working rat hearts (Bolukoglu, et al., 1996). By altering the aortic afterload (and hence coronary perfusion pressure) in a stepwise fashion, they observed a linear relationship between coronary perfusion pressure and coronary flow. This relationship may have been the result of an artefact introduced by perfusion with a crystalline saline solution of low oxygen-carrying capacity, producing a situation of maximal coronary dilatation. Under these circumstances coronary flow becomes dependent on coronary perfusion pressure, unlike the *in vivo* situation where coronary flow remains constant over a wide range of coronary perfusion pressures.

The absence of this autoregulation in their experimental set-up enabled the investigators to create a condition of "low flow" for a specified time period via a reduction in aortic afterload from 100 to 35 cm H_2O. This resulted in a mean reduction in coronary flow of 67% and cardiac hydraulic power of 73%. These values remained stable during the period of low-flow ischaemia, and returned to pre-ischaemic baseline values on restoration of normal coronary perfusion pressure. Using glucose as the only metabolic substrate, they showed that net glucose uptake remained unchanged during low-flow ischaemia, although there was a shift from glucose oxidation to glycolysis (Bolukoglu, et al., 1996). In another report using this technique (Chen et al. 1997), it was demonstrated that glucose uptake was increased during low-flow ischaemia when a second substrate was present, such as lactate, octanoate, or beta-hydroxybutyrate.

4.2.1.3 Measurement of metabolism in the working rat heart

The working heart model described by Taegtmeyer can be used to measure cardiac metabolism using a variety of methods (see Chapter 1). The use of ^{14}C and ^{3}H labelled substrates in this physiologically relevant model has been used extensively by Lopaschuk's group (Broderick et al. 1993; Liu et al. 1996; Lopaschuk & Saddick, 1992; Lopaschuk, et al., 1992; Lopaschuk et al. 1990; Lopaschuk et al. 1988; Lopaschuk et al. 1993; Lopaschuk, 1996; Lopaschuk et al. 1986; Lopaschuk & Kozak, 1998; Lopaschuk & Spafford, 1989; McCormack et al. 1996; Saddick & Lopaschuk, 1992), Taegtmeyer's group (Bolukoglu, et al., 1996; Goodwin et al. 1996; Chen et al. 1997; Doenst & Taegtmeyer, 1999), and other investigators (Christie &

Rodgers, 1995; Tamm, 1994; Schonekess, 1997; Depre et al. 1998) to investigate the effects of various pharmacologic manipulations on myocardial metabolism during normal flow and ischaemic conditions.

The main byproducts of fatty acid and glucose metabolism in the heart are H_2O and CO_2. By labelling the glucose or fatty acids in the perfusate with either ^3H or ^{14}C, it is possible to quantitatively collect either ^3H_2O or $^{14}CO_2$ produced by the heart. By using radioisotopes that are labelled at specific hydrogen or carbon molecules on the energy substrates and knowing the specific activity of the radiolabelled substrate used, the actual rate of flux through the individual metabolic pathways can be determined (Kudo et al. 1995; Liu et al. 1996; Lopaschuk et al. 1993; Lopaschuk, et al. 1994; Saddick & Lopaschuk, 1992; Bolukoglu, et al., 1996; Goodwin et al. 1996; Chen, Goodwin, Guthrie, & Taegtmeyer, 1997; Doenst & Taegtmeyer, 1999; Christie & Rodgers, 1995; Tamm, 1994; Schonekess, 1997; Depre et al. 1998). For instance, collection of ^3H_2O from perfusate containing [9,10-3H]palmitate can be used to measure actual rates of palmitate oxidation (see Figure 1.6, Chapter 1).

Alternatively, collection of $^{14}CO_2$ from hearts perfused with [U-^{14}C]glucose allows direct measurement of glucose oxidation rates, as the heart releases the $^{14}CO_2$ during passage of acetyl CoA derived from [U-^{14}C]glucose through the tricarboxylic acid cycle (see Figure 1.7, Chapter 1). This is more difficult than collecting ^3H_2O since the $^{14}CO_2$ is either released into the air or trapped in the buffer as bicarbonate. Therefore the entire experimental set-up must be sealed, and all $^{14}CO_2$ exiting the apparatus is

captured by bubbling the exiting gas through a CO_2 trapping solution such as hyamine or sodium hydroxide. Perfusate samples must be collected simultaneously and injected into vials under mineral oil. On completion of the experiment, the $^{14}CO_2$ trapped in these perfusate samples can be released via addition of sulphuric acid and collected in sodium hydroxide traps in sealed metabolic vials.

4.2.2 Preliminary experiments: problems and solutions

Our initial apparatus was modelled on that of Taegtmeyer (Taegtmeyer et al. 1980) as described above. The main difference from Taegtmeyer's apparatus was the use of in-line electromagnetic flow probes (Zepeda Instruments, USA) both on the inlet side of the left atrium (to measure total cardiac output) and on the aortic afterload line (to measure aortic flow; coronary flow was taken to be the difference between these two) instead of graduated flow chambers. These probes were calibrated and found to be accurate down to flow rates of 2 ml/minute. The use of in-line flow probes enabled the recirculating working heart system to remain "closed", allowing collection of all CO_2 from the apparatus for the purpose of measuring glucose oxidation rates. An in-line Millipore filter of 5 μm pore size was found to be suitable for use in the recirculating system to remove any tissue matter from the circulation, preventing any microemboli from entering the coronary capillaries.

4.2.2.1 Pressure build-up in the "closed" working heart system

However, Taegtmeyer's apparatus was not designed to be a closed system. Although hearts worked well in an open system, they rapidly deteriorated as soon as the system

was closed due to a build-up of air pressure within the organ chamber. As a closed system was required for the research project, the organ chamber and perfusate reservoir were redesigned as a single water jacketed chamber, as per Lopaschuk and Barr (Lopaschuk & Barr, 1997) (see Figure 4.2). The large rubber bung used to seal the top of this chamber had four metal cannulae passing through it: the aortic cannula, left atrial cannula, the line leading from the pulmonary artery cannula to the Clark oxygen probe, and a fourth cannula for a gas outlet tube. Perfusate returning to this chamber (overflow from the left atrial preload bubble trap and the aortic afterload bubble trap, as well as fluid returning from the oxygen probe) was via three inlet holes midway down the sides of the chamber, which were situated below the heart and above the fluid level in the reservoir.

Another modification was to add a new piece of glassware, an atrial preload chamber with a bubble trap, which was separated from the gassing chamber via a short piece of Tygon tubing. The oxygenated perfusate dripped from the gassing chamber into the top of the preload chamber, and then flowed from the bottom of the preload chamber to the left atrium. Excess buffer passed from a side outlet (set at 15 cm above the heart) in the preload chamber back to the reservoir in the organ chamber. This configuration allowed a direct flow of air to pass between the gassing, preload, and organ chambers, preventing a build-up of negative pressure. Finally the organ chamber was also connected to the bubble trap in the aortic afterload chamber via an extra gas line, allowing equilibration of air pressures within all four air-spaces within

the closed set-up. Outflow gas lines from the organ chamber and gassing column led to the sodium hydroxide trap, allowing the collection of all escaping CO_2.

4.2.2.2 Foaming of the BSA buffer

Frothing within the perfusate reservoir was controlled by the addition of one drop of Antifoam-B, an aqueous emulsion of a silicone polymer containing non-ionic emulsifiers (Sigma Chemicals), to the buffer reservoir prior to cannulation of the heart. Despite this, there was enough foaming within the gassing column to cause a gradual but continuous trickle of buffer fluid down the gas outlet line leading to the sodium hydroxide trap. Although this trickle amounted to less than one millilitre over the course of a one hour experiment, this was more than enough to completely invalidate the use of ^{14}C counts in this fluid as a measure of liberated $^{14}CO_2$. This problem was solved by the use of glass tubes filled with inert silica beads (Silica Gel, 8-10 mesh, Chem-Supply, South Australia) in the air outlet lines, which absorbed any fluid while allowing air to escape freely.

4.2.2.3 Bacterial contamination

Bacterial contamination of the apparatus was an intermittent problem during early experiments, and invariably led to bradyarrhythmias and functional deterioration of the working heart. This problem was related to the use of glass-distilled water which was either not freshly distilled or distilled into contaminated glass bottles, in the preparation of the perfusate. The use of Milli-Q distilled water and meticulous wash-up of the apparatus after each day's experiments (see below) overcame this problem.

4.2.2.4 Selection of albumin source

A change in the source of supply of BSA by Boehringer Mannheim during the course of experiments led to a profound drop in measured rates of palmitate oxidation, possibly due to some contaminant in the BSA which was not removed by dialysis. Appendix A shows the measured palmitate oxidation rates from hearts perfused with four different BSA stocks. Perfusate in subsequent experiments was prepared using RIA grade BSA (Sigma Chemical Company, St. Louis, MO, U.S.A.).

4.2.2.5 Ischaemia

Attempts were also made at introducing a period of low-flow ischaemia during the working heart perfusion using the method of afterload reduction as per Taegtmeyer's group (Bolukoglu, et al., 1996; Chen et al. 1997). However, a strong correlation between aortic afterload and coronary flow was not found in our closed set-up. Furthermore, a set reduction in aortic afterload (e.g. 75%) resulted in a wide range of reductions in coronary flow rates between hearts, and this approach was abandoned.

Experiments were also performed using a period of "global no flow" ischaemia by clamping both the left atrial inlet and aortic outlet lines during a working heart perfusion (Goodwin & Taegtmeyer, 1994; Kudo et al. 1995; Liu et al. 1996; Lopaschuk, et al., 1992; Lopaschuk et al. 1993; McCormack et al. 1996; Saddick & Lopaschuk, 1992). However, the recovery of the hearts was highly variable, with 100% recovery following ischaemic periods of less than 10 minutes, 0% recovery for ischaemic periods of >15 minutes, and highly variable results (0 or 100%) for periods

of intermediate duration. This cut-off time of between 10 and 15 minutes is likely to represent the time at which endogenous glycogen stores run out, after which time irreversible ischaemic contracture occurs (Goodwin & Taegtmeyer, 1994). Given these difficulties, the experiments described in the Results section of this chapter were all performed under normal flow conditions.

4.2.2.6 Chronic dosing of rats with perhexiline

Because of the suspicion that the metabolic effects of perhexiline might only develop after prolonged exposure, methods of pre-treating rats with perhexiline were investigated to determine the optimal dosage and administration route. Previous reports of chronic treatment of rats with perhexiline have utilised various perhexiline salts, daily dosages and administration routes. These include perhexiline hydrochloride 20 mg/kg intra-peritoneally for 5 days (Sewell et al. 1989); perhexiline maleate 10 mg/kg intramuscularly for 7 days (Seth et al. 1985); oral dosing via gastric intubation in doses up to 320 mg/kg for 5 days (Hoenig & Werner, 1980) or between 80 and 160 mg/kg over 7-11 weeks (Hoenig & Werner, 1979; Hoenig & Werner, 1980); and oral dosing of 80 mg/kg daily for up to 52 weeks with perhexiline maleate pressed into food pellets (Meier et al. 1986). In none of these reports were (a) difficulty in dissolving the drug in solution or (b) overt toxic effects on the rats discussed, although in the papers from Hoenig and Werner, 80 mg/kg/day was described as the "maximum non-toxic dose" (Hoenig & Werner, 1979).

(i) Intra-peritoneal dosing

Our initial experiences with chronic dosing of rats were unsatisfactory. Intra-peritoneal administration of 25 mg/kg of perhexiline hydrochloride (Merrell Dow Pharmaceuticals Inc, U.S.A.), dissolved in 10% ethanol as per Sewell et al. (Sewell, et al. 1989), in 5 Sprague-Dawley rats resulted in unrecordable trough levels after 5 days of once daily dosing. This contrasts with trough levels of between 0.15 and 0.21 mg/L using the same methodology reported by Sewell et al (Sewell et al. 1989). Post-mortem examination revealed marked peritoneal inflammation and precipitation of the drug.

A second group of rats was treated with twice daily intra-peritoneal perhexiline (25 mg/kg daily) for 5 days, and compared to controls given ethanol vehicle. Both groups were sacrificed 15 hours after their final dose. The weight changes for both groups, and the plasma and tissue perhexiline levels in the treated group are shown in Table 4.1. Perhexiline treatment resulted in poor appetite with significant weight loss in all rats (mean weight loss 28 ± 19 grams c.f. weight gain of 19 ± 7 grams in control rats injected with 10% ethanol alone), and the death of one rat within 3 days. Peritoneal inflammation and evidence of patchy hepatic necrosis were only evident in the perhexiline-treated rats. Plasma levels obtained at 3 hours after the final dose in two of the treated rats were 0.44 and 0.30 mg/L, within the "therapeutic" range (as defined in patients on long-term oral perhexiline therapy). However trough levels, taken at the time of sacrifice 15 hours after the final dose in the 4 surviving rats, were low (0.07 ± 0.02 mg/L). Tissue perhexiline levels at this time were concentrated

approximately 3-fold in hearts and 9-fold in liver compared to plasma. This method of dosing was therefore abandoned.

(ii) Oral dosing

Attempts at oral administration of perhexiline lactate via gastric gavage also resulted in significant toxicity. Variable weight changes occurred with 5 days of 25mg/kg (-7 ± 37 grams) of perhexiline daily, with the greatest weight loss of 65 grams seen in a rat with very high tissue levels at sacrifice 3 hours after the final dose (see Table 4.3), suggesting impaired perhexiline metabolism in this rat. Post-mortem examination revealed bloated and inflamed gas-filled stomachs in all rats, most marked in those with more marked weight loss. Animals gavaged with vehicle did not display any abnormalities. Aside from the rat with "slow" perhexiline metabolism, tissue to plasma concentration ratios were approximately 10 in the heart and 20 in the liver 3 hours after the final dose. In the animal with apparent slow metabolism of perhexiline, the corresponding ratios were 25 and 110 respectively.

Another approach to administering the drug orally was to dissolve perhexiline gluconate or lactate in the drinking water, but the altered taste resulted in a marked decrease in fluid intake and dehydration. The addition of various flavourings to the water improved the amount imbibed by the rats, but also resulted in precipitation of the perhexiline out of solution. Plasma and tissue levels were variable but generally low (data not shown) and this approach was also abandoned.

(iii) Transdermal dosing

A novel approach was the use of transdermal perhexiline. The back of each animal was waxed prior to application of a paste containing sorbiline (200 mg), 20 µl of dimethylsulphoxide (DMSO), and perhexiline gluconate, which was then covered with Tegaderm adhesive film (3M Health Care, Canada). A preliminary experiment using 200 mg of perhexiline gluconate resulted in a plasma level of 0.32 mg/L and cardiac tissue level of 2.85 µg/gram after a 24 hour perhexiline "patch". However longer patch applications led to the development of hard leathery skin associated with a dense white subcutaneous deposit, presumably precipitation of perhexiline gluconate within the local tissues (not seen with "vehicle" patches). This led to a progressive decline in perhexiline bioavailability, with plasma levels of 0.41, 0.15, and 0.09 mg/L at 24, 48, and 72 hours respectively in one rat. Reapplying perhexiline on a daily basis did not help, due to the leathery skin. However, the transdermal approach did not lead to any apparent systemic toxic effects, with weight gain observed at a similar rate to controls in each animal. Given the toxic effects and variable plasma and tissue levels seen in rats treated with oral or intra-peritoneal perhexiline, 24 hour applications of transdermal perhexiline were used in the *ex vivo* experiments described in sections 4.2.3.4 and 4.3.2.

4.2.3 Experimental procedures

4.2.3.1 Animals and materials

Male Albino Wistar rats used in the experiments were obtained from the University of Adelaide Animal Service. They were housed in air-conditioned rooms maintained at

22°C on a 12 hour light-12 hour dark cycle with food and tap water available ad libitum. All experimental procedures were approved by the Institutional Animal Ethics Committee in accordance with the guidelines set by the National Health and Medical Research Council of Australia.

[9,10-^{3}H]palmitic acid and [U-^{14}C]glucose were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Bovine serum albumin (RIA grade), palmitic acid, oxfenicine (4-hydroxyphenyl glycine), perhexiline maleate, and Antifoam B solution were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Optiphase 'HiSafe 3' scintillation cocktail was obtained from Fisher Chemicals (England). Etomoxir (sodium salt) was obtained from Dr HPO Wolf (Allensbach, Germany). Perhexiline gluconate, used in the transdermal 24 hour applications, was manufactured from the maleate salt by the Victorian College of Pharmacy, while a small amount of perhexiline hydrochloride was obtained via Merrel Dow Pharmaceuticals.

4.2.3.2 Preparation of the fatty acid-containing perfusate

Preparation of the fatty acid-containing perfusate, and addition of radiolabelled substrate, was based on the methodology described by Lopaschuk and Barr (Lopaschuk & Barr, 1997). The BSA/Krebs'-Henseleit solution to be used in the working heart circulation contained a substrate mix of palmitate (0.4 m*M*), glucose (11 m*M*), insulin (100 mU/l), and either ^{14}C-glucose (200 μCi/l) or ^{3}H-palmitate (50 μCi/l).

On the day prior to planned experiments, two separate Krebs'-Henseleit solutions were prepared, one for the initial Langendorff perfusion of the hearts, and the other for the preparation of the fatty acid/albumin containing buffer to be used in the working heart set-up. The latter solution must be prepared the day before the planned experiment as the fatty acids need to be prebound to the albumin and dialysed overnight (see below). The Krebs'-Henseleit solutions contained NaCl (118 mM), KCl (4.7 mM), K_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM), $MgSO_4$ (1.2 mM), CaCl2 (2.5 mM) and EDTA (0.2 m*M*). The role of the EDTA was to chelate out any heavy metals that may contaminate the buffer.

To make a final volume of 1 litre of fatty acid/albumin containing buffer, 400 ml of Krebs'-Henseleit solution was used to dissolve the BSA. This solution was stirred at a low heat on a hot plate, and 30 g of BSA (RIA grade, cold-precipitated, Sigma Chemical Co.) was slowly added to give the final desired 3% BSA solution (at this point in time the solution was greater than 3% BSA).

For a final concentration of 0.4 mM palmitate in 1 litre, 102 mg of palmitate and 22 mg of sodium carbonate were weighed out in a small beaker. 15ml of distilled water and 10 ml of ethanol were added to the beaker, which was brought to the boil and continued to boil until all of the ethanol had evaporated. The palmitate initially bound with the sodium carbonate, which rendered the complex water soluble, allowing it to remain dissolved in the water as the ethanol was boiled off. In experiments where palmitate oxidation was to be measured, ^{3}H-palmitate was added

at this stage (50 μCi per litre of buffer). Once the ethanol had boiled off, the palmitate solution was quickly poured into the warm dissolved BSA solution.

The BSA/palmitate solution was then poured into Spectra/Por dialysis tubing (MW cut-off 6000-8000, Fisher Scientific, England) which was tied off at both ends. This tube of buffer was placed into a 5 litre beaker containing 4.6 litres of Krebs'-Henseleit solution. This beaker was sealed with Parafilm, and allowed to dialyse overnight with continuous stirring at 4°C. This allowed the buffer to equilibrate with the Krebs'-Henseleit solution, to ensure that the calcium-binding sites on the albumin became occupied, and to dialyse out any ethanol that may remain during the binding of the fatty acids to the albumin.

On the day of experiments, the BSA/palmitate solution was added to 600ml of Krebs'-Henseleit solution, making the final volume of one litre. At this stage, glucose (11 mM) and insulin (100 μU/L) were added, as well as ^{14}C-glucose (200 μCi per litre) in those experiments where glucose oxidation was to be measured. This solution was then vacuum filtered through a Millipore filter of 1.2 μm pore size to remove any particulate matter that may remain in the BSA. The perfusate was now ready for use, or for addition of drugs such as perhexiline, oxfenicine or etomoxir.

Finally, the Langendorff perfusate was prepared by adding glucose (11 mM) and insulin to the other stock of Krebs'-Henseleit solution.

4.2.3.3 Set-up of the working rat heart apparatus

To maintain constant temperature throughout the experiment and to prevent a drop in temperature as the perfusate is delivered from the gassing column to the heart, the entire apparatus was contained in a thermostatically controlled perspex chamber. This chamber, measuring 150 cm high, 80 cm wide and 70 cm deep, had an internal air temperature of 37°C maintained by a convection heater. Access doors situated frontally allowed manipulation of the heart during set-up of the working mode and the collection of samples during experiments.

Small holes were made at the side of the cabinet for inlet and outlet gas lines. In order to trap all CO_2 exiting the apparatus for the purpose of measuring ^{14}C-glucose oxidation (see below), the air outflow lines from the bottom of the gassing column, the organ chamber, and the bubble trap at the top of aortic overflow chamber all led to a glass cylinder containing a fixed volume (40 ml) of sodium hydroxide solution (Figure 4.2, **J**), through which the exiting gas was bubbled. The inflow of 95%O_2-5%CO_2 to the gassing columns was set at a rate that resulted in a slow stream of bubbles via the gas outlet line through the sodium hydroxide solution (too high a gas flow would result in saturation of the sodium hydroxide solution with CO_2 prior to the end of the experiment).

Glassware was as described above, modelled on Taegtmeyer's apparatus with a few modifications (see figure 4.2). The organ chamber/perfusate reservoir (**A**) and gassing columns (**D,H**) were double jacketed to help maintain the perfusate a 37°C.

The organ chamber had a reservoir capacity of 100 ml, the volume used for each experiment. This chamber had three glass-sleeved entry points above this fluid level (halfway up the chamber), for perfusate returning from the aortic overflow, pre-atrial reservoir overflow, and from the oxygen probe.

The heart was suspended from stainless steel cannulae within the top half of this chamber. The aortic and left atrial cannulae, the latter angulated to position within the left atrium, were grooved to accommodate the ligatures used to tie on the ascending aorta and left atrium. Two further cannulae were used for connecting the pulmonary artery cannula to another polypropylene tube leading to the oxygen probe (returning fluid re-entered the reservoir chamber as described above), and a gas outlet line leading to the sodium hydroxide trap. All cannulae passed through a rubber bung fitted to the top of the chamber, ensuring a closed system.

Glassware was connected via medical grade silastic or polypropylene tubing of 3mm internal diameter. The exception was the tubing leading from the bottom of the reservoir chamber to the top of the gassing column (via the roller pump and filter) which was 5 mm in internal diameter. This line had a section of thick walled latex rubber tubing, enabling timed perfusate samples to be removed via syringe during the experiment. A multi-channel roller pump (**B**) was used for both Langendorff and working heart circulations, with different calibre tubing in each circuit resulting in flow rates of around 100 ml/min for the working heart circulation and 20 ml/min for the Langendorff side. These high flow rates were necessary to maintain the head of

perfusate in the preload chamber (**E**). The in-line Millipore filter (**C**) had a pore size of 8μm. In-line electromagnetic flow probes (Zepeda Instruments, USA) were situated in the left atrial and aortic lines to measure total cardiac output and aortic flow respectively.

The gassing columns in both the Langendorff and working heart circulations were identical, but on the working side a separate atrial preload chamber (**E**) with a capacity of about 10 ml hung below the bottom of the column. An overflow line leading from this chamber back to the reservoir ensured a constant left atrial preload of 15 cm H_2O. The aortic compliance chamber (**F**), above the 3-way tap in the aortic line, consisted of two narrow glass columns, joined at the bottom (the inflow). Tubing from the top of one column led up to the aortic overflow chamber, while the other was open to air. The latter was clamped to trap approximately one ml of air as the working circulation was switched on. The aortic overflow chamber (**G**) had an overflow outlet, allowing the aortic output to fall back to the reservoir. The fluid level in this chamber was situated 100 cm above the left ventricle. A bubble trap at the top of this chamber was connected to the gas outlet tubing from the reservoir chamber, and both lines led via a Y-piece to the sodium hydroxide trap (**J**).

A known quantity of recirculating buffer (BSA-Krebs'-Henseleit solution containing the appropriate radioisotope) was used in each experiment. 100 ml was the usual volume utilised, as this was the minimal amount to maintain proper fluid flow during the working heart experiment, taking into account the samples of buffer removed

during the course of the perfusion. In experiments where drugs were present in the working heart buffer, these were added to the 100 ml perfusate in the appropriate concentrations before this was added to the organ chamber reservoir. The concentrations of drug in the active treatment experiments were: perhexiline 2 μM, oxfenicine 2 mM, and etomoxir 1μM or 1nM (see discussion, below). A single drop of Antifoam B was added to the buffer at the start of each experiment to prevent excess foaming.

The Langendorff Krebs'-Henseleit solution was kept in a 1 litre bottle (**I**) in a water bath, and was pumped to the top of the gassing column and subsequently ran down a line leading to a 3-way tap (**K**) above the aortic cannula. An overflow outlet at the bottom of the gassing chamber (maintaining a fluid head 60 cm H_2O above the left ventricle) led back to the litre bottle reservoir. In Langendorff mode, the 3-way tap was open to the Langendorff circulation and the rat heart aorta; with the switch to the working mode the Langendorff inflow was turned off, and the tap opened to the aortic line leading to the aortic overflow line.

The Clarke-Type oxygen electrode (Rank Brothers, England) was also water-jacketed to ensure measurement of pulmonary artery oxygen concentration was done at 37°C. Prior to setting up the heart, fully-oxygenated buffer was circulated through the oxygen electrode and the display was adjusted using the sensitivity control to read 100%. Conversion of the readout to concentration of oxygen in nmol was possible using a correction factor pertaining to the value for the concentration of oxygen in air-

saturated distilled water, (measured at 221 μmol per litre at 37°C), and the readout of the oxygen electrode for the same solution (23.7%). Thus:

$$\text{Oxygen concentration (nmol/ml)} = \frac{X*221}{23.7}$$

where X = oxygen probe readout

$$\text{and Oxygen utilisation (nmol/ml)} = \frac{(100-X)*221}{23.7} \qquad (4.1)$$

Before setting up the heart, both left atrial inflow and aortic inflow lines were clamped after ensuring no air bubbles were present, and an initial baseline sample of buffer was taken from the reservoir outlet line.

4.2.3.4 Working heart perfusions

Adult Albino Wistar rats (350-500g) were used in all experiments. In the *ex vivo* experiments, rats were briefly anaesthetised with halothane 24 hours prior to sacrifice while their backs were waxed and the perhexiline paste applied. 200 mg of perhexiline gluconate was mixed in a sorbiline (200 mg) paste containing one drop of DMSO, covered with Tegaderm film, and left in place for 24 hours. A control group underwent identical treatment with vehicle paste consisting of sorbiline and DMSO only. In these experiments, 3 ml of venous blood, for analysis of plasma perhexiline concentration, was taken via syringe from the inferior vena cava while the rat was anaesthetised immediately prior to sacrifice.

All animals were anaesthetised with halothane prior to sacrifice. Hearts were then quickly excised and placed in ice cold calcium-free Krebs'-Henseleit solution.

Excess lung, tracheal, and fatty tissue were trimmed from the heart prior to cannulation of the aorta and commencement of retrograde perfusion with the unlabelled perfusate. This perfusate was allowed to drip from the heart without being recirculated. During this initial perfusion of approximately ten minutes, the left atrial cannula was inserted in the hole where the pulmonary veins enter the left atrium, and the pulmonary artery was cut and cannulated with a fine plastic cannula which was connected to the third metal tube in the rubber bung, leading to the oxygen electrode. Photographs of hearts in the working mode are shown in Figures 4.3 and 4.4.

Following the initial equilibration period in the Langendorff mode, the heart was switched to the working mode by simultaneously turning a 3-way stopcock in the aortic inflow line and the unclamping the left atrial inflow line. Using this technique, there was minimal mixing of the two perfusate solutions. As the radiolabelled BSA-containing buffer was ejected from the left ventricle up the aortic outflow line, a clamp was applied to the side arm of the glass compliance chamber leaving an air-space of approximately 1 ml. The organ chamber was raised to enclose the heart and was sealed with the rubber bung, thus closing the system.

All perfusions in the working heart mode were performed at a 15cm left atrial preload and a 100 cm aortic afterload, and continued for 60 minutes. For measurement of palmitate oxidation, perfusate samples were taken by syringe via the silastic rubber tubing at 10 minutely intervals and frozen until analysis. When production of $^{14}CO_2$ was being measured, the perfusate samples were immediately injected into glass vials

under paraffin oil, and simultaneous samples were taken from the sodium hydroxide solution and injected into scintillation vials.

At the end of the perfusion period, hearts were snap frozen using aluminium tongs (shaped to fit over the heart while still being perfused in the working mode) cooled to the temperature of liquid nitrogen. They were clamped on their cannulae without interrupting perfusion. The frozen ventricular tissue was cut from the atria and weighed (wet weight), and then pulverised into powder under liquid nitrogen. To correct for any variations in water content, the dry tissue weight of the heart was used for calculations. A portion of the powdered tissue was dried in 5 ml plastic tubes over 2 weeks, and then reweighed to determine the dry to wet ratio of the ventricles, enabling a calculation of the total dry weight of the ventricular tissue.

At the end of each day's experiments the apparatus was cleaned and dismantled. After all perfusate was drained from the system, it was firstly decontaminated by circulating two litres of hot water containing the radioactive decontaminant Decon 90 (Selby Biolab Scientific Ltd, Australia) through the apparatus, followed by rinsing with two litres of distilled water. Finally the lines were dried with 70% ethanol, and glassware was dismantled and soaked overnight in a weak solution containing Decon 90 and detergent.

4.2.3.5 Haemodynamic measurements

The in-line electromagnetic flow probes (Zepeda Instruments, USA) situated in the left atrial and aortic lines were calibrated routinely by comparison with manual collections of fluid in measuring cylinders. Coronary flow, consisting of flow via the pulmonary artery through the oxygen probe as well as the fluid dripping from the heart into the reservoir, was calculated as the difference between cardiac output and aortic flow. A disposable pressure transducer (Transpac IV, Abbott Critical Care Systems, USA), situated at the level of the heart and connected to the aortic line via a Y-piece, allowed monitoring of aortic pressures.

Data from the flow probes, oxygen probe, and the pressure transducer in the aortic line were acquired on an 8-channel MacLab and Macintosh computer, allowing later analysis of heart rate, oxygen consumption, aortic and coronary flow rates, cardiac output, and aortic pressures. From these data, it was possible to calculate rates of stroke work (see Chapter 2, Equation 2.2), cardiac work, and myocardial efficiency.

Cardiac work (W) was calculated from the product of mean arterial pressure and cardiac output, using a conversion factor to convert the units to $g.m.min^{-1}$ (Grossman, 1980).

Oxygen consumption (MVO_2) was derived using the coronary flow rate:

$$\underset{(\mu mol/min/g.\ dry\ weight)}{MVO_2} = \frac{(O_2\ utilisation)*(coronary\ flow)}{dry\ weight\ of\ heart} \qquad (4.3)$$

Myocardial efficiency (ME) was calculated as the rate of cardiac work per μmol of myocardial oxygen consumption.

$$ME\ (gm.m.\mu mol^{-1}) = \frac{W}{MVO_2} \qquad (4.4)$$

4.2.3.6 Measurement of palmitate and glucose oxidation

The methodology used to measure rates of substrate utilisation in the isolated hearts was based on that described by Lopaschuk and Barr (Lopaschuk and Barr, 1997). Fatty acid oxidation was calculated by measurement of ^3H_2O production, which required the separation of ^3H_2O from the ^{3}H-palmitate present in the buffer. The technique described by Lopaschuk and Barr (Lopaschuk & Barr, 1997) results in a greater than 99.7% extraction and separation of ^3H_2O from ^{3}H-palmitate.

0.5 ml buffer samples were treated sequentially with 1.88 ml of chloroform/methanol (1:2 v:v) solution, 0.625 ml of chloroform, and 0.625 ml of a 2*M* KCl/0.4M HCl solution, with vortexing of the samples after each addition. Each sample was then centrifuged for 10 minutes at 3500 rpm, creating three distinct layers, the aqueous phase on top and organic phase at the bottom, separated by a white layer made up of BSA. The aqueous layer was collected via a Pasteur pipette and was subsequently treated with 1 ml of chloroform, 1 ml of methanol, and 0.9 ml of the KCl/HCl solution. Following another centrifugation for 10 minutes at 3500 rpm, three layers were again formed. Duplicate 0.5 ml samples of the top (aqueous) layer (containing ^3H_2O) were collected and transferred to scintillation vials. Following the addition of

Optiphase scintillant, the samples were counted on a liquid scintillation beta counter (Wallac 1409). The count rates, in dpm, were then multiplied by a dilution factor to correct for the total aqueous volume added, which is: 0.5 ml sample + 2.253 ml methanol + 1.525 ml KCl/HCl = 4.278 ml. Hence the 0.5 ml sample in a total volume of 4.278 ml gives a dilution factor of 8.556:1.

Glucose oxidation was measured by adding the $^{14}CO_2$ present in the simultaneously collected gaseous samples (in scintillation vials) and perfusate samples (stored under 2 ml of paraffin oil in glass vials to prevent liberation of $^{14}CO_2$).

50 ml Erlenmeyer flasks, sealed with rubber stoppers, were prepared for capture of the $^{14}CO_2$ present in the perfusate. 1 ml of 9N H_2SO_4 was placed at the bottom of these flasks, and a plastic centre well containing 250 ml of sodium hydroxide was suspended in the flask in a wire basket. Into this closed flask, the 1 ml perfusate samples were injected to mix with the H_2SO_4, and the flasks were gently shaken for one hour. The reaction between the ^{14}C-bicarbonate in the perfusate and the H_2SO_4 resulted in the liberation of $^{14}CO_2$ which was captured in the sodium hydroxide solution. Duplicate samples from the centre wells were transferred to scintillation vials. Optiphase scintillant was added to both these vials and those containing the samples collected from the sodium hydroxide solution during the experiment, and both sets were counted on a liquid scintillation beta counter (Wallac 1409).

Measurement of the specific activity of the perfusate, and correction for background (the samples taken at baseline) allowed calculation of rates of either palmitate or glucose oxidation in nmol of substrate per minute per gram dry weight of ventricle.

4.2.3.7 Biochemical tissue analysis

(i) Glycogen assay

An aliquot of tissue powder was used to determine myocardial glycogen concentration, as originally described by Walaas and Walaas (Walaas & Walaas, 1950). The technique involves extraction of glycogen by 30% KOH, precipitation with ethanol and enzymatic digestion with amyloglucosidase to form glucose, which is then assayed via spectrophotometry.

100 mg of frozen tissue powder was dissolved in 200 µl 30% w/v KOH in Eppendorf tubes, and then heated at 105°C for 60 minutes. It was then cooled to 0°C, mixed with 100µl 2% Na_2SO_4 and 100% ethanol (to give a final concentration of 75% v/v), and refrigerated at 4°C overnight. The next day, the suspension was microfuged at 13000 RPM for 2 minutes, and the supernatant was discarded. The pellet was then resuspended in 1 ml of 80% ethanol, and re-spun as before. The supernatant was again discarded , and the pellet was left to dry on a heating block at 40°C overnight. The following day, the pellet was digested by adding 125 µg of amyloglucosidase (in 250µl of 1M sodium acetate/acetic acid buffer) and 750 ml of water. After incubation at 37 °C for 60 minutes, the suspension was spun as previously, and the supernatant was assayed for glucose using reagents from the Sigma Diagnostic kit No. 510-A

(Sigma Chemical Company, St Louis, MO, U.S.A.). The assay mix, containing both colour reagent (o-dianisidine dihydrochloride) and enzyme solution (glucose oxidase), was added to a range of volumes of both standards (containing 1 mg/ml B-glucose) and the test samples, which were incubated at 37°C for 30-45 minutes before spectrophotometric analysis at 450 nm. Results were then converted to μmol glucose equivalents per gram dry weight.

(ii) Triglyceride assay

Another tissue aliquot was used for analysis of myocardial triglyceride content. Lipids were extracted from the tissue powder using the method originally described Folch et al. (Folch, et al. 1957), and the triglycerides were subsequently hydrolysed enzymatically with subsequent determination of liberated glycerol by spectrophotometry (Bergmeyer, 1974).

The tissue powder was homogenised (1 in 20 weight per volume) in methanol/ chloroform (1:2), and the solution was vortexed. 0.15M NaCl (3.75:1 chloroform/methanol to NaCl) was added prior to separation of the phases by centrifugation at 12000 g for 5 minutes. The lipid-containing chloroform layer was removed from the bottom of the tube and used for analysis. 20 μl of each sample solution was evaporated to dryness, dissolved in 10 μl of distilled water and mixed with 1 ml of reagent solution (Boehringer Mannheim, Germany) and incubated at 25°C for 10 minutes prior to spectrophotometric analysis at 500 nm. The reagent solution contained lipase, ATP, glycerol kinase (GK), glycerol phosphate oxidase

(GPO), peroxidase, 4-chlorophenol, and 4-aminophenoxazine. The test principle is as follows:

$$\text{triglycerides} + 3\ H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\ \text{R-COOH}$$

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3 phosphate} + \text{ADP}$$

$$\text{glycerol-3-phosphate} + \text{O2} \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}}$$

$$\text{4-(p-benzoquinone-mono-imino)-phenazone} + 2\ H_2O + HCl$$

The absorbance at 500 nm of each sample was measured against a Precimat glycerol curve and expressed as mmol/L, which was finally converted to μmol per gram of dry weight.

(iii) Lactate assay

Another tissue aliquot was used for analysis of myocardial lactate content. The technique involves extraction of lactate by 0.6M perchloric acid, neutralisation of the sample using 3M KOH, and then assaying via spectrophotometry as per the method described by Buttery et al (Buttery et al. 1985). The principle of this assay involves conversion of lactate to pyruvate by lactate dehydrogenase, with concomitant reduction of NAD to NADH. The NADH then reduces iodonitrotetrazolium (INT) via phenazine methosulphate (PMS) to produce a formazan colour which is measured via spectrophotometry at 510 nm.

200 grams of tissue powder was homogenised in 500 µl of 0.6M perchloric acid, followed by centrifugation at 3700 rpm for 15 minutes at 4 °C. 1 µl of methyl red was added to the 400 µl of the supernatant, followed by careful addition of 3M KOH solution until the indicator changed from pink to light yellow (taking note of the volume required). The solution was allowed to stand on an ice bath for 15 minutes, allowing the potassium perchlorate to precipitate. Following brief centrifugation, the supernatant was assayed for lactate. This involved the serial addition to both 25 µl of sample and lactate standards (0.3-10 mM) of: 500 µl AMP buffer (0.65M), 25 µl of BSA solution (Fraction V from Sigma Chemicals; 70g/L), 100 µl of colour reagent (80 mg INT, 200 mg NAD, and 5 mg PMS in 20 ml distilled water), and 10 µl of lactate dehydrogenase reagent (450 U/mg). The solutions were then vortexed and left in a water bath for 10 minutes at 37 °C. Finally 3 ml of 0.1M HCl was added to stop the reaction, and the absorbance was measured at 510 nm. The concentration of lactate in mM was calculated via calibration to the lactate standard curve, adjusted by the dilution factors involved in the pre-treatment of the sample, and finally converted to mmol lactate per gram of dry weight.

(iv) ATP assay

As ATP concentrations can alter within a fraction of a second of tissue death, all hearts were snap frozen at the end of each experiment using the molded aluminium tongs while still being perfused with buffer (as described in 4.2.3.4). ATP content was analysed using the UV-method with hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer, 1974).

The frozen tissue powder was initially deproteinised by adding $HClO_4$ (2.5 ml per gram of tissue) to tissue on dry ice, giving a total fluid volume of 3.25 ml per gram of tissue, assuming tissue water content of 75%. After warming to 2-4°C, the mixture was homogenised. Carbonate solution was added to duplicate portions of the suspension to bring the pH to 7.4. After allowing to stand for 10 minutes on ice, the solution is centrifuged and the supernatant is used for the ATP assay.

To each 100 µl sample, the following solutions were added sequentially: 2.27 ml triethanolamine buffer (50 mM), 0.10 ml NADP solution (10 mM), 0.20 ml $MgCl_2$ (0.1 M), 0.01 ml glucose-6-phosphate dehydrogenase (G6P-DH) suspension (140 kU/L), and 0.30 ml glucose solution (0.5 M). The absorbance (A_1) at 340 nm was read immediately, and then 0.02 ml of hexokinase (HK) suspension (280 kU/L) was added to start the ATP reaction. When the absorbance became constant (A_2), this value was subtracted from A1 to give ΔA_{ATP}. The concentration of ATP in mmol/L was calculated from an ATP standard curve, adjusted by the dilution factors involved in the pre-treatment of the sample, and finally converted to µmol ATP per gram of dry weight.

The principle of this assay is as follows:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADPH} + \text{H}^+$$

4.2.3.8 Statistical analysis

Samples for calculation of substrate utilisation rates were taken at 10 minutely intervals throughout each experiment. Haemodynamic parameters from the MacLab data were averaged for two minute periods around each ten minute time-point (e.g. 9-11 minutes, 19-21 minutes, etc.) Data were expressed as means ± SD. Statistical analysis of haemodynamic or metabolic changes between two or more treatment groups over the time course of a 60 minute experiment was determined using two-way ANOVA with repeated measures, using a Tukey/Kramer post-hoc analysis if the ANOVA was significant. Relationships between mean rates of palmitate oxidation and mean values for haemodynamic parameters for both control and treated hearts were plotted by linear regression, and compared by one way analysis of covariance. One-way analysis of variance was used to compare the tissue concentrations of ATP, glycogen, and triglycerides between several treatment groups in the acute experiments, and unpaired t tests were used in the comparison of these concentrations between the placebo and perhexiline-treated groups in the 24 hour experiments. A value of $P < 0.05$ was considered significant.

4.3 RESULTS

4.3.1 Acute drug exposure

In the experiments designed to assess acute exposure to perhexiline and the CPT-1 inhibitors etomoxir and oxfenicine, these drugs were added to the working heart buffer prior to starting the perfusions. Hence exposure of the isolated rat heart to the drugs began at the onset of the working heart mode and was constant throughout the

60 minute perfusion period. Palmitate oxidation was measured in 6 control hearts, 6 perhexiline treated hearts (2 μM), 6 oxfenicine treated hearts (2 mM), 6 hearts treated with high-dose etomoxir (1 μM), and 4 hearts treated with low-dose etomoxir (1 nM). Glucose oxidation was measured in 6 control hearts, 6 perhexiline treated hearts, and 6 oxfenicine treated hearts.

The uptake of perhexiline into the heart was assessed in one experiment, where the heart was snap frozen after 30 to determine the tissue concentration of perhexiline at the mid-point of the perfusion. This level was 2.45 μg per gram of wet tissue, an approximate 4-fold increase over the perhexiline concentration in the perfusate.

4.3.1.1 Haemodynamic effects

For the purposes of comparing haemodynamic parameters, data from both sets of experiments (palmitate and glucose oxidation) were combined as all experiments were performed under identical conditions. Haemodynamic data was available for 5 of the hearts exposed to 1 μM etomoxir in the palmitate oxidation experiments.

The time-courses of various haemodynamic and contractile parameters during the 60 minute working heart perfusions are plotted as mean ± standard deviations in Figures 4.5 to 4.11. The mean values for each treatment group are shown in Table 4.3.

Four of the oxfenicine-treated hearts were initially bradyarrhythmic, accounting for the low mean heart rate in this group over the initial 20 minutes. However there were no overall significant differences in heart rate between the four groups ($P = 0.26$;

Figure 4.5). Nor were the cardiac output (P = 0.42; Figure 4.6), double product (P = 0.07; Figure 4.7), stroke work (P = 0.17; Figure 4.8) or cardiac work (P = 0.30; Figure 4.9) significantly different between the groups.

Cardiac function in the working heart model was not constant over time, with a gradual decline in stroke work (Figure 4.8), an index of myocardial contractility, in all groups after 30 minutes (effect of time, F=9.8, P<0.0001, ANOVA; P < 0.01 for control hearts and P < 0.05 for perhexiline-treated hearts at 60 minutes vs. 10 minutes, Tukey/Kramer). This occurred at a similar rate in all groups, suggesting an inherent instability in the working heart model rather than any pharmacologic effect.

Cannulation of the pulmonary artery was not always successful in the early experiments. Hence measurement of oxygen consumption was only possible in 5 control hearts, 8 perhexiline-treated hearts, 8 oxfenicine-treated hearts, 2 hearts treated with etomoxir 1 μM and 2 hearts treated with etomoxir 1nM. In these hearts, there were no significant differences between the groups with respect to rates of oxygen utilisation (Figure 4.10), nor in overall myocardial efficiency (Figure 4.11).

4.3.1.2 Metabolic effects

Figure 4.12 shows the amounts of palmitate oxidised in nmol/gram dry weight in 5 groups of rat hearts during 60 minute working perfusions: control (n=6), perhexiline 2 μM (n=6), oxfenicine 2 mM (n=6), etomoxir 1 μM (n=6) and etomoxir 1 nM (n=4). Oxfenicine-treated hearts had significantly reduced rates of palmitate oxidation in

comparison to control hearts (P = 0.0005). This difference was apparent within 20 minutes and became more marked with increasing duration of exposure. No significant differences were seen in rates of palmitate oxidation between control hearts and those exposed to perhexiline or either concentration of etomoxir.

Similar results were observed in those hearts in which glucose oxidation was measured. The results of these experiments are shown in Figure 4.13. Three groups were studied: control (n=6), perhexiline 2 μM (n=6), and oxfenicine 2 mM (n=6). Glucose oxidation rates were significantly increased in hearts exposed to oxfenicine in comparison to control hearts (P = 0.03). Again the effect was observed at 20 minutes, and became more marked with increasing duration of exposure. However, there was no significant difference seen between control hearts and those exposed to perhexiline. Table 4.4 summarises the mean rates of substrate utilisation in the acute experiments.

The relationships between cardiac work (averaged over 60 minutes for each experiment) and mean rates of palmitate utilisation were plotted for each treatment group via linear regression, shown in Figures 4.14 to 4.16, and compared via analysis of covariance. There were no significant differences in cardiac work performed per unit consumption of palmitate between control hearts and those exposed to either etomoxir or perhexiline, although there was a trend (P = 0.19) towards a favourable effect with perhexiline. The relationship between work and fatty acid oxidation was

significantly different in the oxfenicine-treated hearts compared to controls ($P < 0.05$).

The myocardial tissue concentrations of ATP, glycogen, lactate, and triglycerides are shown in Table 4.5. These assays were performed on the ventricular tissue which was snap frozen at the end of each 60 minute aerobic working heart perfusion. There were no significant differences in these biochemical parameters between any of the treated groups and the control group.

4.3.2 24 hour perhexiline exposure

In this set of experiments, rats were exposed to either 200 mg perhexiline (n = 5) or placebo (n = 5) (sorbiline applied to the skin and covered with Tegaderm) *in vivo* for 24 hours prior to sacrifice (see Section 4.2.2.6 for methods). By 24 hours, the perhexiline-treated rats had developed a white precipitate under the skin on their backs (not present in the placebo group). The rats treated with perhexiline also seemed less active, but continued to eat and drink. In the treated group, plasma perhexiline concentrations were determined at the time of sacrifice, and are shown in Table 4.6. In this group, perhexiline 2 μM was also present in the working heart perfusate.

The plasma perhexiline concentrations varied considerably between the 5 rats in the treated group, ranging between 0.25 mg/L (0.83 μM) and 0.94 mg/L (3.1 μM) with a mean of 0.53 ± 0.28 mg/L (see Table 4.6). The rates of hepatic metabolism were also unpredictable, with the levels of hydroxyperhexiline varying between 10 and 62% of

the corresponding parent compound levels. The variations in plasma perhexiline levels seem to be related to differences in bioavailability (transdermal absorption) rather than metabolism of the drug, as those rats with the higher perhexiline levels also had higher metabolite to parent drug ratios.

4.3.2.1 Haemodynamic effects

Mean haemodynamic effects following 24 hours of transdermal placebo or perhexiline patches are shown in Table 4.7. Heart rate, cardiac output, and double product are plotted in Figures 4.17, 4.18, and 4.19 respectively. The perhexiline-treated rat hearts had a significantly higher heart rate ($P < 0.05$), cardiac output ($P < 0.001$), and double product ($P < 0.005$) than the placebo hearts. This may have been partly related to significantly lower heart rates ($P < 0.05$) in the 24 hour placebo-treated rat hearts compared to the control hearts used in the acute drug exposure experiments (Section 4.3.1) although cardiac outputs in the two control groups did not differ significantly. In addition, there were non-significant trends towards higher heart rates ($P = 0.25$) and cardiac outputs ($P = 0.06$) in the hearts from rats pre-treated with 24 hours of perhexiline compared to those exposed to perhexiline *in vitro* only.

The increase in cardiac output in the perhexiline pre-treated group was due mostly to the increase in heart rate, as there was no significant change in stroke work (Figure 4.20) per gram of dry weight between the two groups. Again, there was evidence of eventual deterioration in the working heart preparation over time with respect to myocardial contractility, from peak stroke work at 20 minutes in the control group

(effect of time, F=8.6, P<0.0001, ANOVA; control hearts at 20 min compared with 40, 50 and 60 min, each P<0.01, Tukey/Kramer). By comparison there was no significant decline in stroke work with time in the perhexiline pre-treated group (Tukey/Kramer). Cardiac work (Figure 4.21) also was higher in the perhexiline pre-treated group, although this did not achieve statistical significance (P = 0.08).

Pulmonary artery cannulation was successful in all ten of these experiments, allowing calculation of myocardial oxygen consumption. Despite the differences in cardiac output, there was no difference in utilisation rates of oxygen between the two groups (Figure 4.22). This resulted in a significant improvement in calculated myocardial efficiency (Figure 4.23) in those hearts pre-treated with perhexiline compared to the control group (P < 0.05).

4.3.2.2 Metabolic effects

Palmitate oxidation rates for the rats treated with 24 hour placebo or perhexiline patches are shown in Figure 4.24 and Table 4.8. The rate of palmitate oxidation was constant during the 60 minute working heart perfusions in both groups. There was no difference in overall palmitate oxidation rates between the rats which had been pre-treated with perhexiline and those with placebo, with mean rates of 761 ± 112 and 729 ± 138 nmol per gram dry weight per minute respectively. However, when these data were related to the amount of work performed (Figure 4.25), a significant increase (P = 0.02 by analysis of co-variance) in cardiac work per nmol of palmitate utilised was observed in the hearts pre-exposed to perhexiline compared to the control

hearts ($P = 0.006$). In addition, there was a significant increase in work performed per unit palmitate oxidation in the *ex vivo* perhexiline pre-treated hearts compared to those exposed acutely to the drug *in vitro* only (see Figure 4.26).

To assess this effect further, the rates of mean palmitate oxidation in the *ex vivo* perhexiline pre-treated hearts and controls were also related to heart rate (Figure 4.27) and cardiac output (Figure 4.28). These data showed that the rate of palmitate oxidation was not dependent on heart rate, but was strongly correlated to cardiac output, particularly in the perhexiline pre-treated hearts ($r^2 = 0.98$ by linear regression). The difference in the relationship between cardiac output and palmitate oxidation rate was highly significantly different between the control and perhexiline pre-treated groups ($P < 0.0001$ by analysis of covariance). This suggests that stroke volume rather than heart rate accounted for the difference observed in the relationship between cardiac work and rate of palmitate oxidation between the two *ex vivo* groups. The relationships between heart rate and stroke volume in the control and perhexiline groups were therefore plotted for both the acutely exposed hearts (Figure 4.29) and the hearts pre-treated for 24 hours (Figure 4.30). Pre-treatment with perhexiline for 24 hours induced a significant change in this relationship, with an increased stroke volume per unit heart rate compared to control hearts ($P < 0.0001$). This effect was not seen in the acutely exposed hearts.

The myocardial ATP levels at the end of the perfusions are also shown in Table 4.8. These was no significant difference in the tissue ATP levels between perhexiline pre-treated hearts (14.0 ± 2.6) and control hearts (18.3 ± 5.9).

4.4 DISCUSSION

The initial objective of the experiments in this chapter was to compare the metabolic and haemodynamic effects of perhexiline and two other CPT-1 inhibitors, oxfenicine and etomoxir, in working rat hearts, and to deduce from the data the relative effects on efficiency of myocardial function. Because of emerging evidence that the metabolic effects of perhexiline develop relatively slowly, experiments were performed both on working hearts exposed acutely *in vitro* to perhexiline and on hearts from rats which had been pre-treated with perhexiline or vehicle *(ex vivo* experiments).

4.4.1 Summary of results

The most important results arising from these experiments can be summarized as follows:

(a) It has been possible to develop a model of 24 hour perhexiline pre-treatment in the rat for *ex vivo* studies. Importantly it was possible to devise a treatment regimen

which resulted in plasma concentrations equivalent to the "therapeutic " range in human subjects.

(b) This pre-treatment model has been used together with the working heart to demonstrate major differences in the effects of the drug between acute *in vitro* and 24 hour *in vivo* exposure. The experiments utilising acute exposure indicated that perhexiline, at a concentration of 2μM, which is similar to the upper therapeutic range, produced no significant effects on myocardial metabolism or haemodynamics in the working heart preparation. In contrast to the acute exposure, 24 hour *in vivo* exposure to perhexiline induced an increase in myocardial performance without incremental oxygen consumption. This effect translated to an improvement of myocardial efficiency in the working heart prepartion under normal flow conditions of approximately 50% compared with control hearts. Furthermore, *in vivo* perhexiline pre-treatment appeared to delay the process of haemodynamic deterioration in the working heart preparation which otherwise appeared after approximately 20 minutes. In this preparation, palmitate oxidation was directly related to cardiac output and cardiac work. The increased myocardial efficiency following 24 hours pre-treatment with perhexiline was accompanied by a significant upward shift in the relationship between cardiac work and palmitate oxidation. Although this represented a relative decrease of palmitate oxidation per unit cardiac work, absolute rates of palmitate utilisation were unchanged relative to controls.

(c) Some unexpected findings may be summarised as follows:

i) Surprisingly, 24 hour pre-treatment with perhexiline increased heart rates of the *ex vivo* working heart preparations compared with those from placebo animals.

ii) In addition to perhexiline, the CPT-1 inhibitor etomoxir also failed to alter myocardial metabolism following acute exposure in the working heart model. Nevertheless the significant shift in cardiac metabolism from fatty acid to glucose utilisation observed in the presence of oxfenicine confirms that this model is suitable for the assessment of alterations in cardiac metabolism.

iii) Although producing a significant shift in myocardial substrate utilisation, no significant improvements in myocardial haemodynamics or efficiency were observed with oxfenicine.

4.4.2 Comparison with published studies using the working rat heart model.

In published studies using the same technique of substrate labeling with ^{14}C or ^{3}H as used in the current experiments and similar substrate concentrations, the rates of both glucose and palmitate oxidation are approximately equivalent in their contribution to aerobic myocardial energy requirements, approximately 300-600 $nmol.min^{-1}$ per gram of dry weight (Henning et al. 1996; Lopaschuk & Barr, 1997; McCormack et al. 1996; Saddick & Lopaschuk, 1992). In the current series of experiments, palmitate oxidation rates in the control hearts were approximately 650 $nmol.min^{-1}$ per gram of dry weight, but glucose oxidation rates were significantly higher at approximately

2400 nmol.min^{-1} per gram of dry weight. The lower calculated glucose oxidation rates reported by Lopaschuk's group may in part be due to the contribution of lactate (present at 0.5 mM lactate in their perfusate) to overall carbohydrate oxidation, with reported lactate oxidation rates of approximately 1250 nmol.min^{-1} per gram of dry weight (Lopaschuk & Barr, 1997). The current metabolic data are incomplete given that glycolysis was not measured, but would be expected to occur at a similar rate to glucose oxidation from the data of Kantor et al. (2000) for the working rat heart perfused under almost identical conditions.

The present studies failed to replicate an acute effect of 2μM perhexiline on substrate metabolism and performance in the working rat heart previously demonstrated by Jeffrey et al. (1995). These authors demonstrated a 35% reduction in fatty acid utilisation accompanied by a non-significant increase in lactate utilisation in the presence of 2μM perhexiline in the perfusate. This was accompanied by a 43% increase in cardiac output but only a small (11%) rise in oxygen consumption, suggesting an improvement in myocardial efficiency. These investigators employed ^{13}C NMR spectroscopy of neutralised tissue samples at the end of 30 minute perfusions to analyse the shifts in cardiac metabolism, and used a more varied substrate mix which included (in mM) glucose 5.5, lactate 1.2, acetoacetate 0.17, and long-chain fatty acids 0.35. Insulin was not present, which may have led to their unusual finding of 0 % glucose utilisation for both control and perhexiline-treated hearts. Another non-physiological aspect was the use of only 1% BSA in the perfusate. Hence the different results with respect to cardiac substrate utilisation may

have related to the different experimental technique for assessing substrate utilisation, the lack of insulin, and/or the lower concentration of BSA.

The concentration of perhexiline used in the acute study was based on the limited published experimental work. Barry et al. (Barry et al. 1985) reported an EC_{90} of 4.8 μM for negative inotropic effects (corresponding to 30% inhibition of ^{45}Ca uptake) in cultured chick ventricular cells. Similarly, Fleckenstein-Grün et al. showed significant negative inotropic effects in isolated guinea pig papillary muscles and significant vasodilatory effects in isolated pig coronary arteries only with concentrations of perhexiline of over 5 μM (Fleckenstein-Grün et al. 1978), much higher than the "therapeutic range" used to guide chronic perhexiline therapy in patients with angina (Cole, et al., 1990; Horowitz et al. 1986). Hence the concentration used in these experiments was unlikely to have led to significant calcium channel-blocking effects on the myocardium or coronary arteries. In fact, there was a non-significant trend towards an increase in stroke work in the hearts acutely exposed to perhexiline (Figure 4.8).

The concentration of oxfenicine (2mM) was based on previous studies in isolated rat hearts which demonstrated both a reduction in fatty acid oxidation and a decrease in myocardial LCAC content (Bielefeld et al. 1985; Liedke et al. 1984; Madden, et al. 1995) with this dose. The current study confirmed a shift from palmitate to glucose utilisation with oxfenicine in aerobic working heart perfusions. This shift in substrate utilisation was not accompanied by a significant change in tissue levels of glycogen,

lactate, triglycerides, or high energy phosphates. From a previous study (Jodalen et al. 1988), some enhancement of triglyceride content may have been expected with inhibition of fatty acid oxidation by oxfenicine, but none was observed in the present study. Despite the shift in substrate utilisation observed in the present study, there was no suggestion of any resulting improvement in cardiac function or reduction in oxygen consumption as a result of this metabolic shift, suggesting that inhibition of CPT-1 alone does not improve myocardial efficiency under aerobic conditions. This contrasts with the study by Jeffrey et al. (1995) where the shift in metabolism induced by perhexiline 2μM was accompanied by an increase in cardiac output compared to control hearts.

The published data on etomoxir are more complex. Previous work from Lopaschuk's group has reported dose-related qualitatively different effects in working rat heart preparations subjected to a period of global ischaemia (Lopaschuk & Saddick, 1992; Lopaschuk et al. 1990; Lopaschuk et al. 1988; Lopaschuk et al. 1989). Higher concentrations of etomoxir (1 μM) significantly increased both glucose oxidation and post-ischaemic recovery of function, but had no effect on LCAC levels in comparison to controls. In contrast, lower concentrations (1 nM) did not affect palmitate or glucose oxidation nor prevent post-ischaemic dysfunction, but significantly decreased LCAC levels. A decrease in oxygen consumption per unit work and increased ATP levels during reperfusion were only observed with the higher concentration of etomoxir, suggesting a dissociation between etomoxir's protective effect and its effect on myocardial LCAC levels.

Hence the effects on palmitate oxidation of both doses of etomoxir were investigated in the current study. Surprisingly, neither dose had any significant effect on palmitate oxidation rates, nor on cardiac function, suggesting a lack of significant CPT-1 inhibition at these concentrations. Interestingly, given etomoxir's action as a CPT-1 inhibitor, Lopaschuk et al. stated that the increase in glucose oxidation was not accompanied by a parallel decrease in fatty acid oxidation, a finding they were unable to adequately explain (Lopaschuk & Saddick, 1992; Lopaschuk et al. 1990; Lopaschuk et al. 1988; Lopaschuk et al. 1989). Hence a metabolic effect of etomoxir can not be entirely excluded in the current study, as its effects on glucose oxidation were not assessed.

4.4.3 Establishment of an ex vivo model

The establishment of a pre-treatment model for perhexiline is important given the evidence which emerged during the course of work for this thesis that there was likely to be a delay in the onset of the effects of perhexiline. Firstly the experiments in the working rat heart demonstrated a lack of acute effects of perhexiline. Secondly, additional data from our laboratory indicated that reversal of nitrate resistance in patient platelets which resulted from 3 days of perhexiline therapy was not demonstrable by *in vitro* exposure of platelets to perhexiline (Willoughby, 1999). Thirdly, as discussed in Chapter 3, incremental inhibitory effects of perhexiline on palmitate oxidation in cultured cardiomyocytes were obtained after 48 hours exposure as opposed to acute exposure to the drug (JA Kennedy, experiments in progress). The

latter observation in cardiomyocytes agrees with the data of Deschamps et al. (1994) who demonstrated inhibition of palmitate oxidation by 5μM perhexiline in hepatocytes following 72 hours of exposure but not with short term treatment. At odds with these studies, is the single report of acute effects of perhexiline in the working rat heart model by Jeffrey et al. (1995).

The finding that heart rate was greater in the 24 hour perhexiline-pre-treated rat hearts is superficially surprising, as perhexiline does not induce tachycardia in humans, nor did it do so acutely in the sheep (Chapter 2). There is no obvious pharmacological basis for a heart rate increasing effect of perhexiline, raising the possibility that this may be limitation of *ex vivo* reduction in heart rate. *In vivo* comparisons of heart rates between perhexiline-pre-treated and control rats would resolve this question and remain to be carried out. An alternative reason for the higher levels of heart rate and other haemodynamic parameters in the *ex vivo* perhexiline experiments may be an amelioration of the "metabolic insult" inherent in the working heart model. In the acute experiments, a small but progressive deterioration was noted in several haemodynamic indices over the 60 minute perfusions. There are many reasons why this model may not be stable; many were encountered during the preliminary experiments (Section 4.2.2). They include an artificial perfusion medium and method of oxygenation used, and the possibility of contaminants given the recirculating nature of the working heart system. Previous studies have suggested that trace levels of endotoxin in the perfusion system stimulate a low-level expression of inducible nitric oxide synthase (iNOS) (Schulz et al., 1995), mediated by the endogenous

production of pro-inflammatory cytokines (Panas et al., 1998). The enhanced generation of nitric oxide and its product with superoxide, peroxynitrite, lead to an uncoupling of ATP synthesis from mechanical work, and hence a progressive decline in mechanical function and cardiac efficiency (Ferdinandy et al., 1999, Panas et al., 1998). Interestingly, in the present study, there was less evidence of deterioration in the perhexiline pre-treated hearts, raising the possibility of a cytoprotective effect with longer durations of perhexiline exposure.

Interpretation of the heart rate results in these experiments is complicated somewhat by the differences in heart rates of the control hearts from the acute and pretreatment experiments. However the haemodynamic parameters observed in the current experiments are similar to those reported in the literature for the working rat heart preparation. As in the current studies, there is considerable variation in reported heart rates under identical control conditions between papers from the same group. For example, mean heart rates (± SD) during aerobic working heart perfusions have been variously reported as 246 ± 14 (McCormack et al. 1996), 299 ± 7 (Wambolt, et al., 1999), and 183 ± 17 (Lopaschuk, et al., 1992) beats per minute in papers from the same laboratory. This potential for variability in the working rat heart model may account for the different mean heart rates observed between the control rat hearts in the acute and 24 hour pre-treatment experiments.

The development of a pre-treatment model proved difficult in terms of establishing a model which was reproducible and not associated with untoward side effects. The

transdermal preparation in the rat proved satifactory for these purposes but is probably not suitable for more than 24 hours of treatment. The onset of the cardio-protective effect of perhexiline in the isolated heart model is unknown as is the time for optimal effect. Hence, an absolute metabolic shift induced by more prolonged periods of perhexiline exposure cannot be ruled out. The equivalent tissue concentrations of perhexiline were not assessed in hearts from the animals pre-treated for 24 hours. During the acute experiments, four-fold accumulation of perhexiline was detected in the heart at 30 minutes of perfusion. However, from the studies in sheep (see Chapter 2), myocardial uptake of the drug is relatively slow following parenteral administration *in vivo*. Nevertheless the "lag phase" for onset of effects of perhexiline has been observed in tissue culture as well as whole organ preparations and is more likely to be related to the time required for perhexiline to reach peak concentrations in subcellular sites of action, e.g. mitochondria. Perhexiline has been shown to accumulate in isolated mitochondria from hepatocytes (Deschamps et al., 1994), but mitochondrial concentration relative to total tissue concentration, and the time course of mitochondrial accumulation in intact tissues, is unknown.

In our experience, the rat is not ideally suited to chronic perhexiline administration beyond 24 hours in so far as it is difficult to maintain steady state plasma levels. The combination of weight loss and rapid elimination kinetics were not mentioned in early reports of chronic oral dosing in rats (Hoenig & Werner, 1979; Meier et al. 1986). The exception is the dark Agouti rat, a mutant in which females show poor debrisoquine hydroxylation (Al-Dabbagh et al. 1981). In one study, the effects of

long-term oral perhexiline maleate administration were compared in dark Agouti and Sprague Dawley rats (Meier et al. 1986). After 4 to 14 weeks of 80 mg/kg daily, perhexiline concentrations in the liver were almost 1000-fold the plasma concentrations in the dark Agouti rats, but unrecordable in most of the Sprague Dawleys. Histological changes of marked lipid accumulation were only seen in the neurons of the dorsal root and sympathetic ganglia of the dark Agouti rats. Other signs of toxicity however, such as weight loss, were not reported in these animals, and biochemical indices of liver function were unchanged. Further pharmacokinetic studies of oral perhexiline administration to the dark Agouti are required to determine whether this may be a more suitable model for the *in vivo* or *ex vivo* investigation of the metabolic effects of chronic perhexiline administration, provided dosing can be maintained at sub-toxic levels.

4.4.4 Changes in metabolic efficiency

24 hours pre-treatment of rats with perhexiline produced a dramatic increase in *ex vivo* myocardial efficiency of approximately 50 % in comparison to matched control hearts. This was associated with a clear change in the relationship between long-chain fatty acid metabolism and performance when compared both to control hearts and to hearts treated acutely with perhexiline. However there was no absolute change in palmitate oxidation between perhexiline pre-treated hearts and control hearts. Such a large change in myocardial efficiency was unexpected, even if associated with an absolute shift in substrate utilisation given the theoretical 13 to 15% difference in oxygen utilisation efficiency between glucose and fatty acids. Moreover, no similar

increase in myocardial efficiency in normal flow has been described for other metabolic anti-anginal agents such as trimetazidine, ranolazine or glucose/insulin. In the case of trimetazidine, Kantor et al. (2000) reported no change in cardiac work and no change in oxygen consumption in working rat hearts exposed to 1μM trimetazidine, a concentration which inhibited fatty acid oxidation. Ranolazine10μM produced no change in cardiac work during normal flow in the working rat heart but did significantly increase cardiac work in reperfusion following a period of global no-flow ischaemia (McCormack et al., 1996). However, myocardial oxygen utilisation was not estimated in this study. In dogs, myocardial efficiency was unaffected by insulin infusion during normal flow, although the fall in myocardial efficiency accompanying decreased coronary perfusion pressure was largely prevented by insulin (Tune et al., 1998).

With respect to known CPT-1 inhibitors, acute exposure to oxfenicine produced a significant shift in myocardial substrate utilisation consistent with its CPT-1 inhibitory effect. Nevertheless this metabolic effect, while producing a leftward shift in the relationship between cardiac work and palmitate oxidation, did not lead to a significant increase in myocardial efficiency. In comparison, a previous study in a Langendorff preparation reported an approximate 20% increase in cardiac performance with no change in oxygen consumption associated with increased carbohydrate oxidation in the presence of 2 mM oxfenicine (Higgins, 1980). It is difficult to relate these results to the present data since no measure of cardiac work, and therefore no measure of efficiency, is available in the Langendorff model. The

present data with oxfenicine suggest a dissociation between overall pattern of substrate utilisation and myocardial haemodynamics under aerobic conditions in the working rat heart. Although neither concentration of etomoxir produced an acute *in vitro* effect on palmitate oxidation in the current study, a metabolic effect of etomoxir cannot be excluded since effects of etomoxir on glucose oxidation were not estimated. Insufficient data on oxygen utilisation were obtained to allow an estimate of effects on myocardial efficiency. Nevertheless there was no significant improvement in cardiac performance for either concentration of the drug under the normal flow conditions of the present study. Amiodarone, which alters myocardial metabolism via CPT-1 inhibition, but is somewhat less potent than perhexiline (Kennedy et al., 1996), also has been reported to increase myocardial metabolic efficiency in non-ischaemic working rat hearts (Moreau et al., 1999), although the magnitude of this change was less than that seen with perhexiline pre-treatment in the current study.

These data raise the question as to the mechanism of the increased myocardial efficiency produced by perhexiline pre-treatment. Does this effect involve CPT-1 inhibition? The current data provide no direct evidence for CPT-1 inhibition. However, this question cannot be answered unequivocably. Since cardiac work and palmitate oxidation were proportional in the control hearts, one would need to address this question in a model in which cardiac work was standardised. However, CPT-1 inhibition appears unlikely to be a sole mechanism for the substantial change in myocardial efficiency given the negative result with acute oxfenicine treatment in the current studies, and the fact that the metabolic efficiency gain to be expected would

be of the order of 13 to 15%. Assuming that there is an additional mechanism underlying the marked increase in myocardial efficiency with perhexiline, this is likely to reflect changes other than a pure shift in substrate utilisation. The current experiments reveal no significant differences in ATP levels induced by perhexiline. Potential sites for the additional mode of action might include incremental availability of calcium at the level of excitation-contraction coupling, or increased efficiency of energy generation in association with electron transfer. The results, above all, suggest that changes in myocardial substrate utilisation may not be the sole mechanism available for altered myocardial efficiency.

No previous studies have reported the magnitude of increase in myocardial metabolic efficiency induced by perhexiline, and no previous studies using other agents have demonstrated increases in efficiency of a magnitude similar to that seen in the current experiments with perhexiline, especially under normal flow conditions. Jeffrey et al. (1995) demonstrated an apparent increase of approximately 30% in myocardial efficiency with acute exposure to perhexiline. Also, although this may not reflect purely increased efficiency of oxygen utilisation, Daniell et al. (1977) documented a 40% decrease in acute infarct size in dogs pretreated with perhexiline.

A limitation to the present studies is that the effect of perhexiline pre-treatment on myocardial performance was not examined during ischaemia. The small decline in function in the working heart preparation may indicate that this model undergoes some changes in common with ischaemia especially as regards recent evidence for

reactive oxygen species generation (Ferdinandy et al., 1999). However, further studies should address this issue in specific ischaemic models to determine the relative magnitude of the effect of perhexiline pre-treatment under defined ischaemic conditions compared with aerobic conditions. Measurement of efficiency, combined with substrate utilisation, is likely to prove difficult in the *in vitro* ischaemic rat heart models at our disposal. A combined approach of low-flow ischaemia in the Langendorff perfusion mode, followed by reperfusion in the working mode, using atrial pacing to control for the potentially confounding effect of heart rate, appears to be the best option available for *ex vivo* models.

Finally the present results do not provide any information on the time course nor optimal dose for the effect of perhexiline pre-treatment on myocardial efficiency. Although the dose used in the present experiments resulted in plasma concentrations spanning the "therapeutic" range, further studies should aim to examine several dose levels for perhexiline pre-treatment. The use of an animal model such as the dark Agouti rat would allow *ex vivo* assessment of metabolic and haemodynamic effects at serial time-points during chronic administration of perhexiline at various dosages.

Thus it has been possible to develop a "chronically treated" rat model and to demonstrate that perhexiline markedly increases efficiency of oxygen utilisation in this model. There is likely to be at least one other underlying mechanism beyond CPT-1 inhibition to account for this. It is likely, but not yet tested, that similar changes would occur during ischaemia/reperfusion in this model. The question

arises: how can this be related to the clinical setting? Clinically, perhexiline markedly prolongs exercise duration without significant effects on heart rate (Lyon et al., 1971, Brown et al., 1976, Mir & Kafetzakis, 1978, Horgan et al., 1981). In order to determine whether similar shifts occur in myocardial efficiency in patients with angina, experimental protocols would require measurement of myocardial oxygen consumption and performance before and after perhexiline loading. The time constraints of oral drug loading imply the need to perform such a protocol on a non-paired basis.

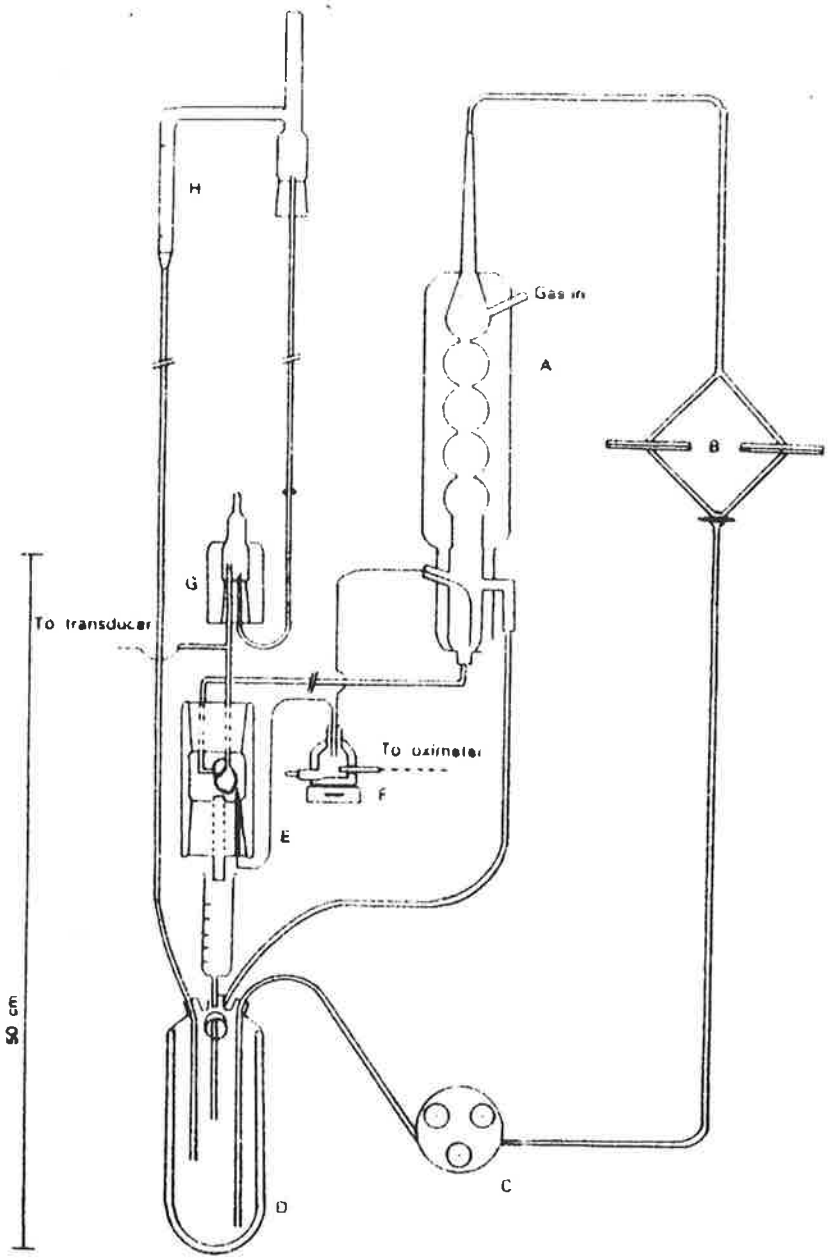


Figure 4.1: Set-up of the working heart apparatus of Taegtmeyer et al. The apparatus consists of 8 units: a multi-bulb oxygenator with a 10 ml reservoir at the bottom (A), a Millipore filter system (B), a roller pump (C), a perfusate reservoir (D), an organ chamber (E), an oxygen electrode (F), a compression chamber (G), and an aortic overflow chamber (H). All glassware is water-jacketed. The height of A and H can be varied over a wide range (broken lines), allowing the workload of the heart to be varied. Preliminary retrograde perfusion is carried out via the side arm of the aortic cannula (labelled 'to transducer'). During the working mode, perfusate reaches the heart directly from A via the left atrial cannula and is expelled through the aorta. (Reproduced from Taegtmeyer et al., 1980.

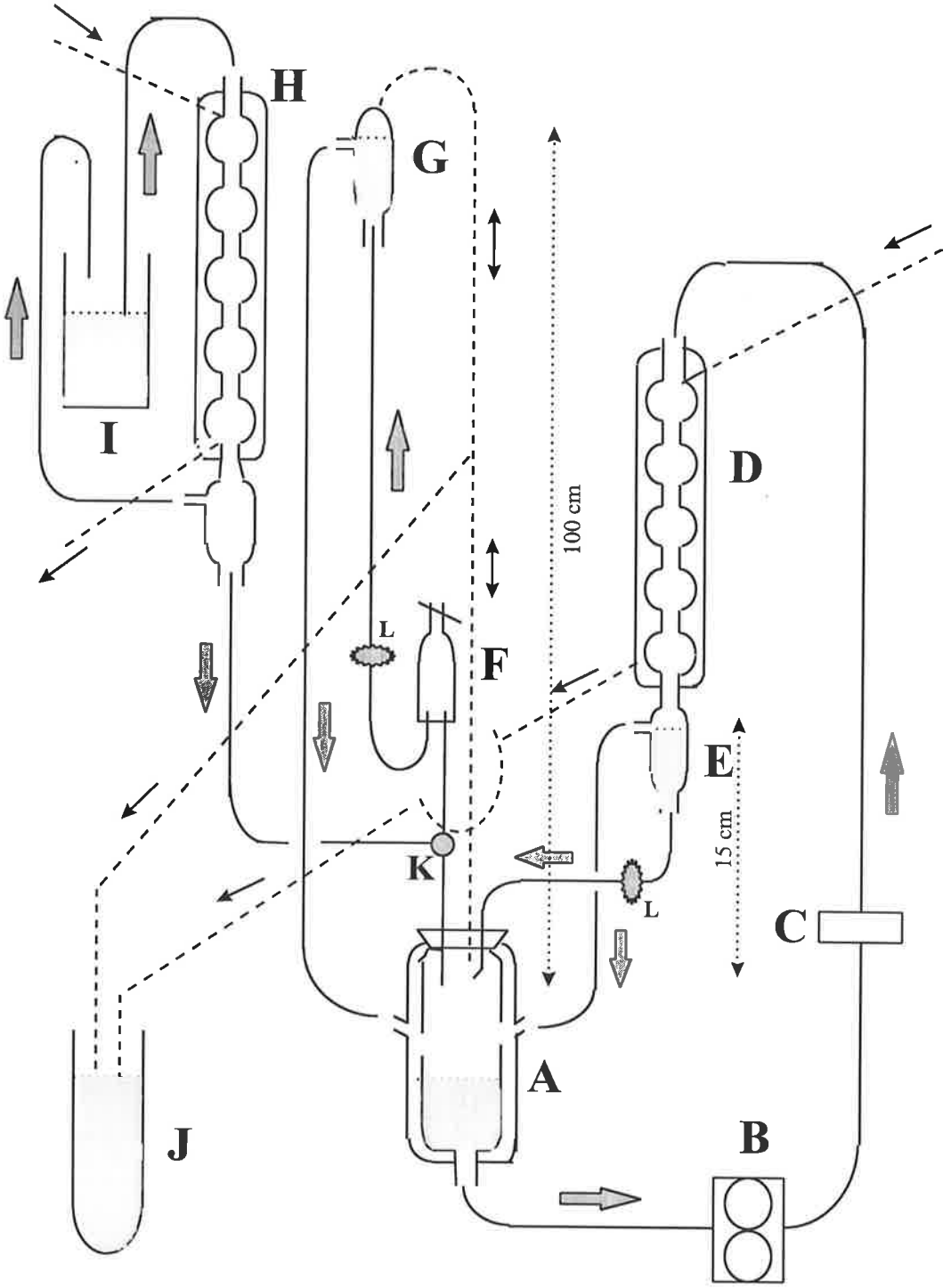


Figure 4.2: Set-up of the working rat heart apparatus. A = organ chamber; B = multi-channel roller pump; C = Millipore filter; D = gassing column; E = atrial preload chamber; F = aortic compliance chamber; G = aortic overflow chamber; H = gassing column for Langendorff perfusate; I = Langendorff reservoir; J = sodium hydroxide trap; K = 3-way tap; L = in-line electromagnetic flow probes. Dotted lines represent gas input and output lines; arrows show direction of flow. Not shown is the pulmonary artery cannula leading to the oxygen electrode.

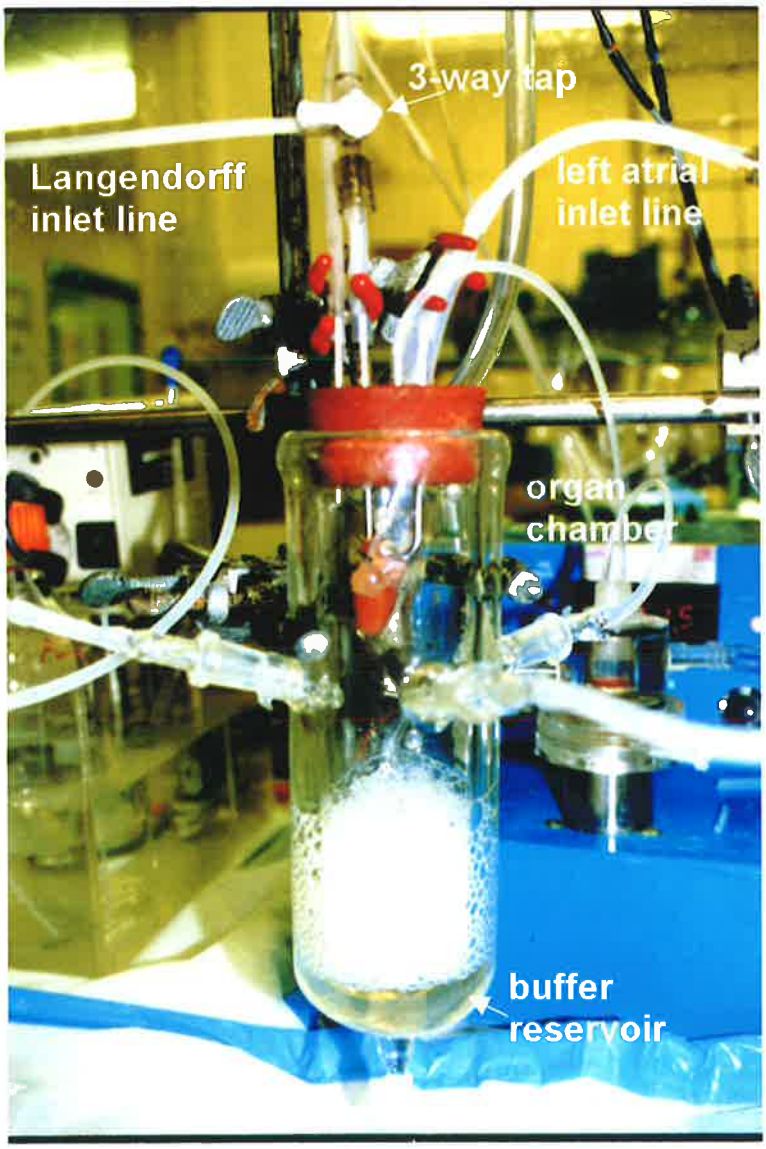


Figure 4.3 Photograph of the isolated working rat heart, showing the combined organ chamber/buffer reservoir, as well as the inlet lines from the Langendorff gassing column to the aortic cannula (via 3-way tap) and from the working heart gassing column to the left atrial cannula. In the working mode, the 3-way tap is switched (as shown) to close the Langendorff inlet line, and the recirculating buffer is ejected by the left ventricle up the aortic line to the aortic overflow chamber (not shown).

Figure 4.4 Rat heart set up in working mode, showing position of left atrial, pulmonary, and aortic cannulae.

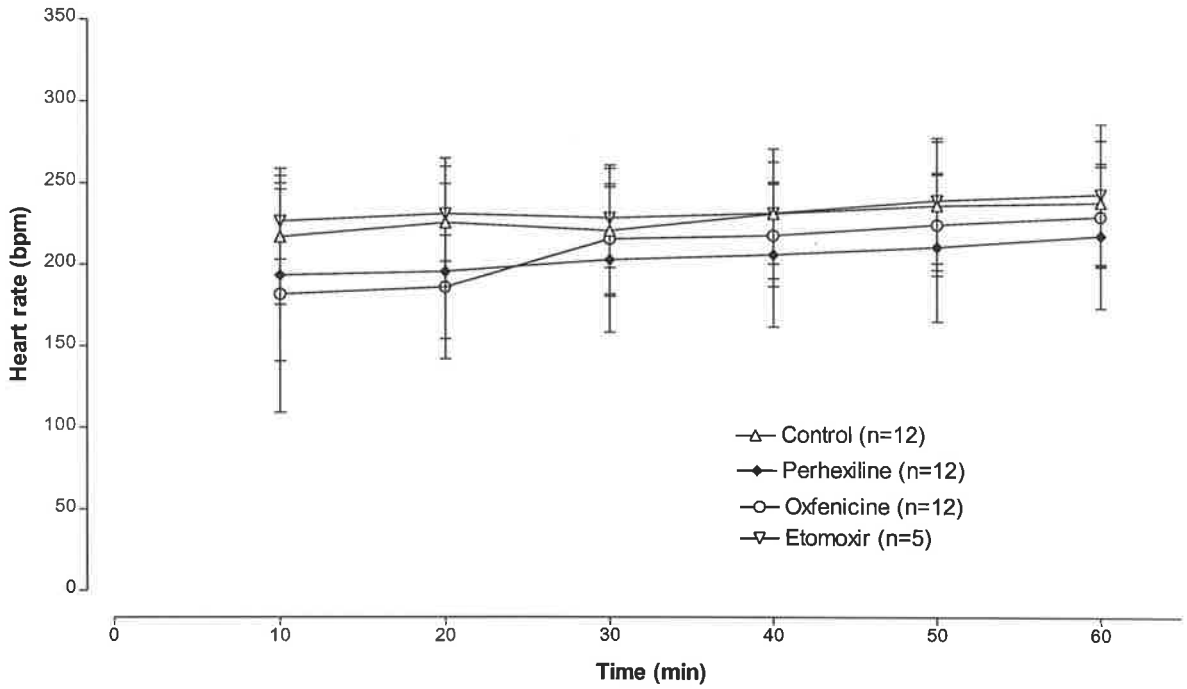


Figure 4.5: Mean (± SD) heart rates during 60 minute perfusions of working rat hearts in the four different acute treatment groups: control (Δ; n = 12), perhexiline 2 μ*M* (♦; n = 12), oxfenicine 2m*M* (O; n = 12), and etomoxir 1 μ*M*(∇; n = 5). There were no significant differences between the four groups by ANOVA with repeated measures (F = 1.41; P = 0.26).

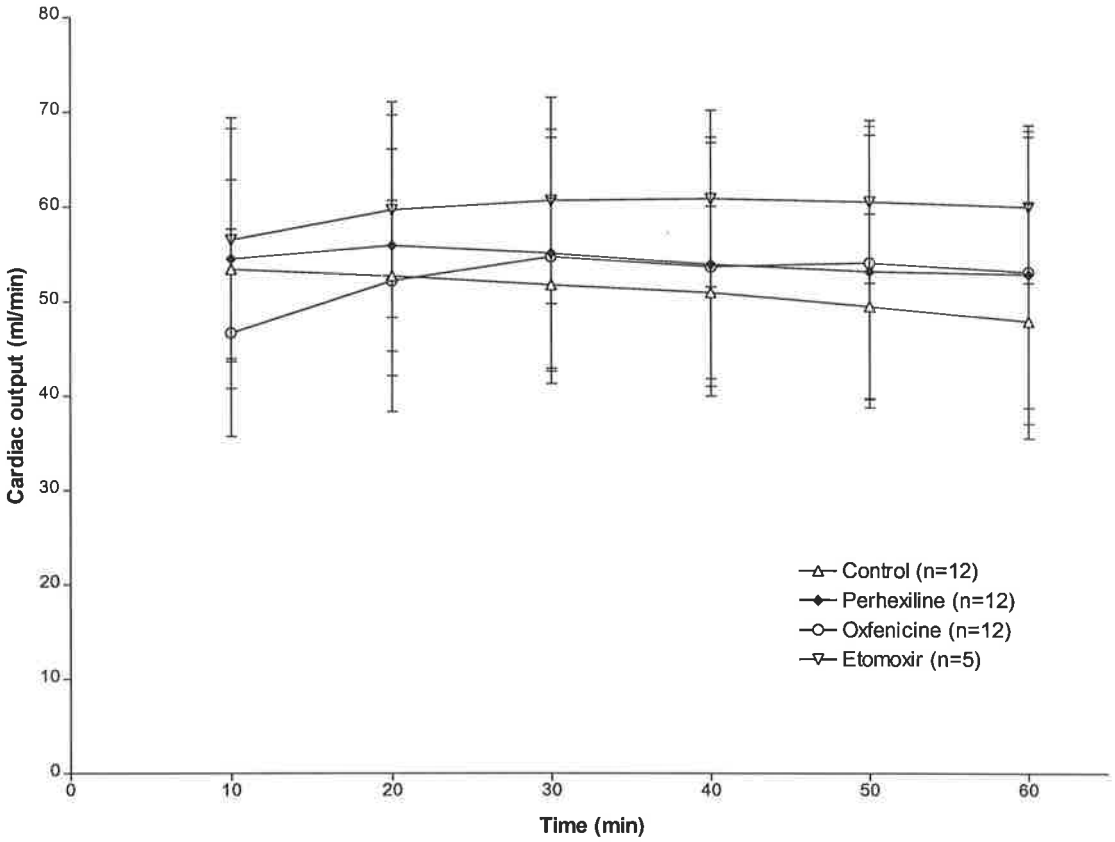


Figure 4.6: Mean (± SD) cardiac outputs during 60 minute perfusions of working rat hearts in the four different acute treatment groups: control (Δ; n = 12), perhexiline 2 μ*M* (♦; n = 12), oxfenicine 2m*M* (O; n = 12), and etomoxir 1 μ*M* (∇; n = 5). There were no significant differences between the four groups by ANOVA with repeated measures (F = 0.97; P = 0.42).

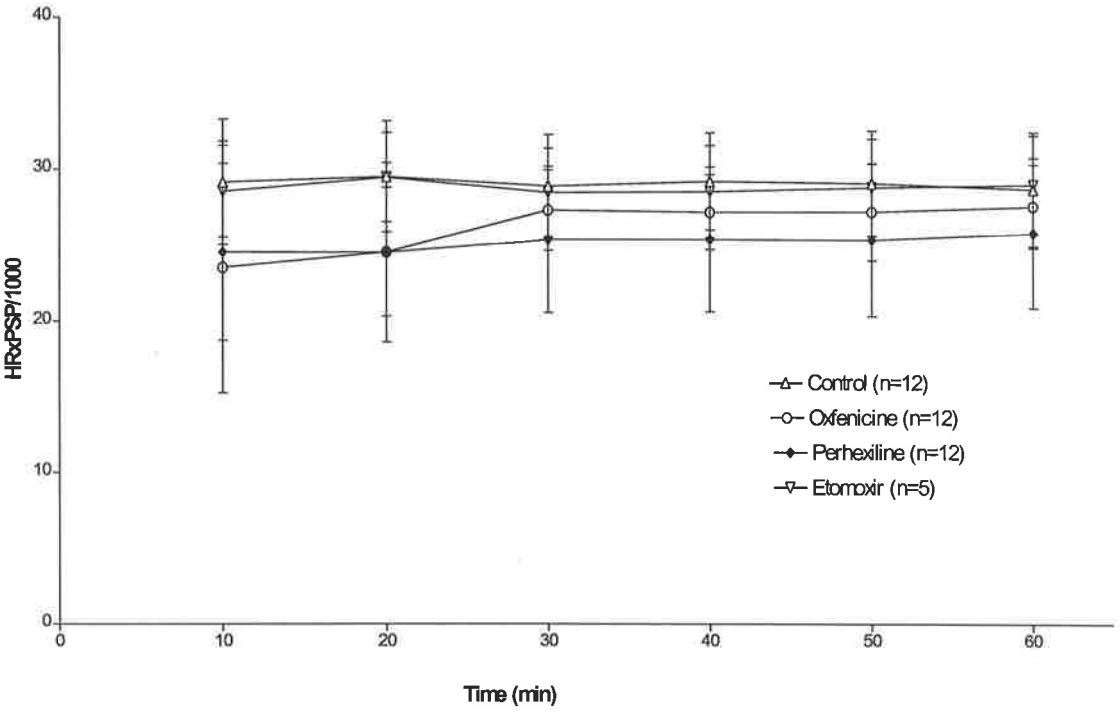


Figure 4.7: Time courses of rate pressure-product (mean ± SD) during 60 minute perfusions of working rat hearts in the four different acute treatment groups: control (Δ; n = 12), perhexiline 2 μ*M* (♦; n = 12), oxfenicine 2 m*M* (O; n = 12), and etomoxir 1 μ*M* (∇; n = 5). There were no significant differences between the four groups by ANOVA with repeated measures (F = 2.51; P = 0.07).

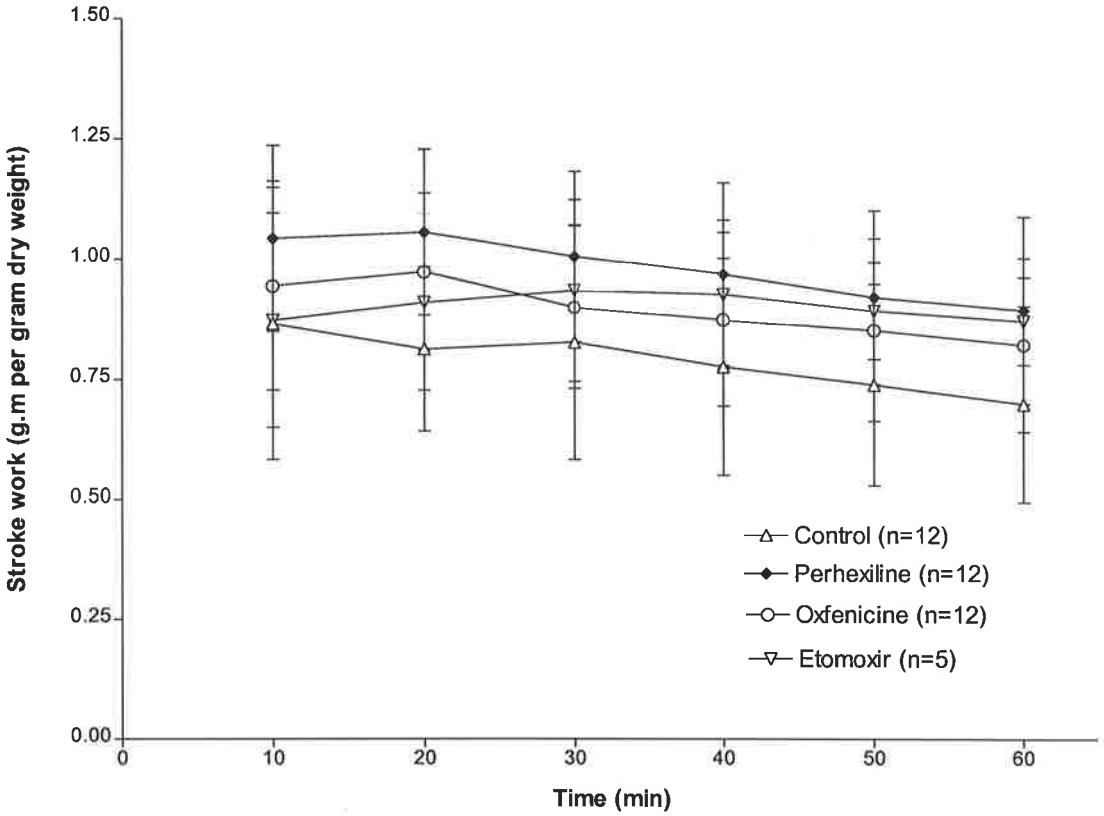


Figure 4.8: Time courses of stroke work per gram dry weight (mean ± SD) during 60 minute perfusions of working rat hearts in the four different acute treatment groups: control (Δ; n = 12), perhexiline 2 μ*M* (♦; n = 12), oxfenicine 2m*M* (O; n = 12), and etomoxir 1 μ*M* (∇; n = 5). There was no significant difference between the groups by ANOVA with repeated measures (F = 1.78, P = 0.17).

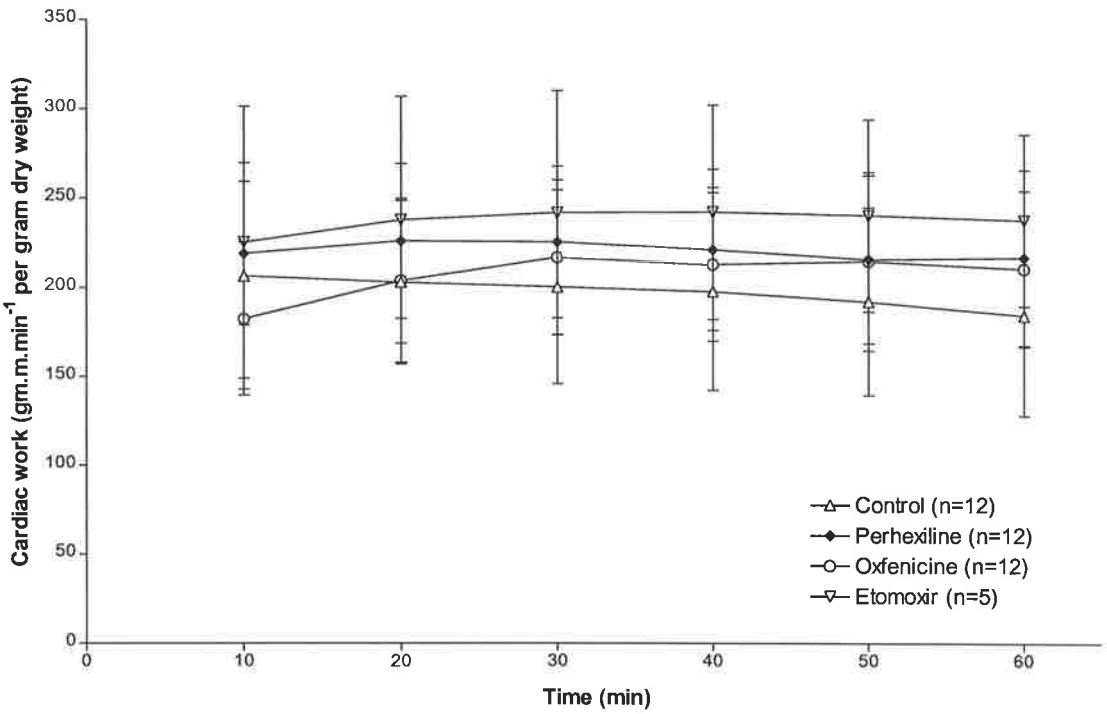


Figure 4.9: Time courses of cardiac work (mean ± SD) during 60 minute perfusions of working rat hearts in the four different acute treatment groups: control (Δ; n = 12), perhexiline 2 μ*M* (♦; n = 12), oxfenicine 2 m*M* (O; n = 12), and etomoxir 1 μ*M* (∇; n = 5). There were no significant differences between the four groups by ANOVA with repeated measures (F = 1.27; P = 0.30).

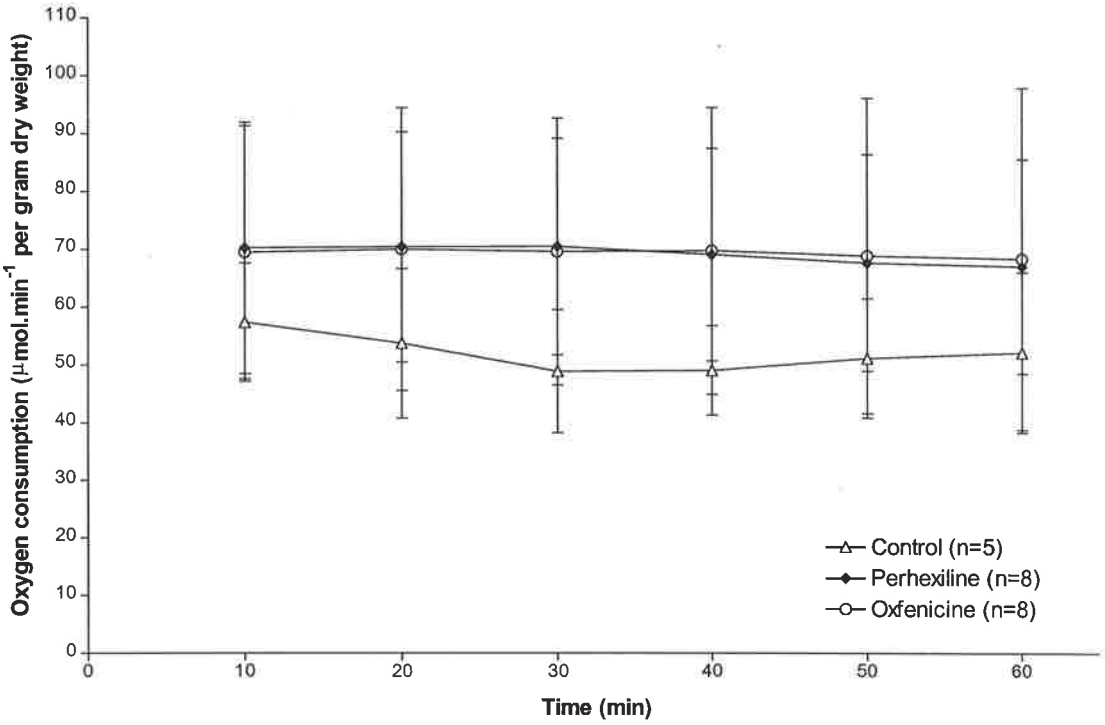


Figure 4.10: Oxygen consumption (mean ± SD) during 60 minute perfusions of working rat hearts in the three different acute treatment groups: control (Δ; n = 5), perhexiline 2 μ*M* (♦; n = 8), and oxfenicine 2m*M* (O; n = 8). There were no significant differences between the three groups by ANOVA with repeated measures (F = 1.78; P = 0.20).

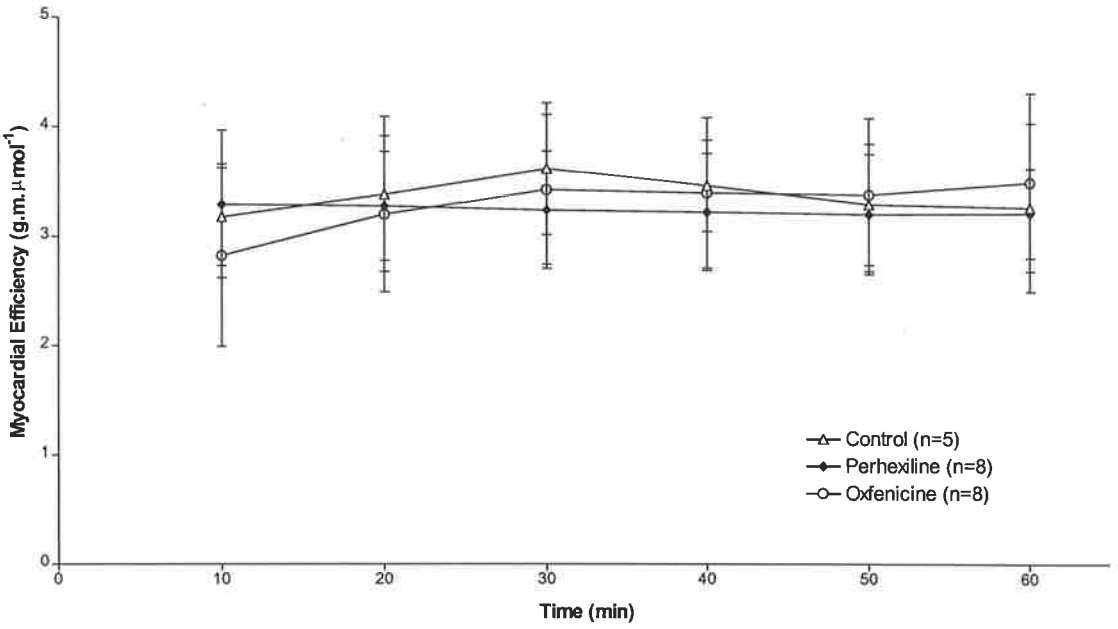


Figure 4.11: Myocardial efficiency (mean ± SD), calculated as cardiac work performed per μmol of oxygen utilised, during 60 minute perfusions of working rat hearts in the three different acute treatment groups: control (Δ; n = 5), perhexiline 2 μ*M* (♦; n = 8), and oxfenicine 2m*M* (O; n = 8). There were no significant differences between the three groups by ANOVA with repeated measures (F = 0.03; P = 0.97).

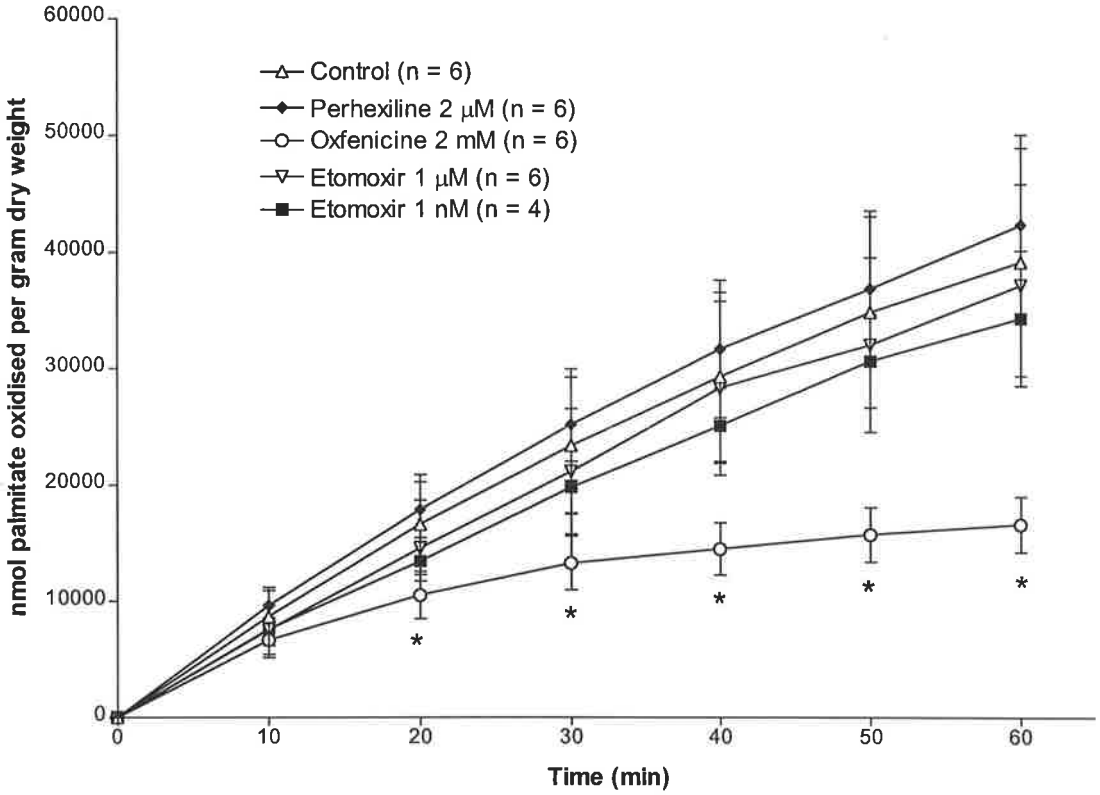


Figure 4.12: Cumulative nmol of palmitate oxidised (mean ± SD) during 60 minute working heart perfusions with acute exposure to drugs as shown in legend. *significantly different from control values via 2-way ANOVA with repeated measures (F = 7.58, P = 0.0005).

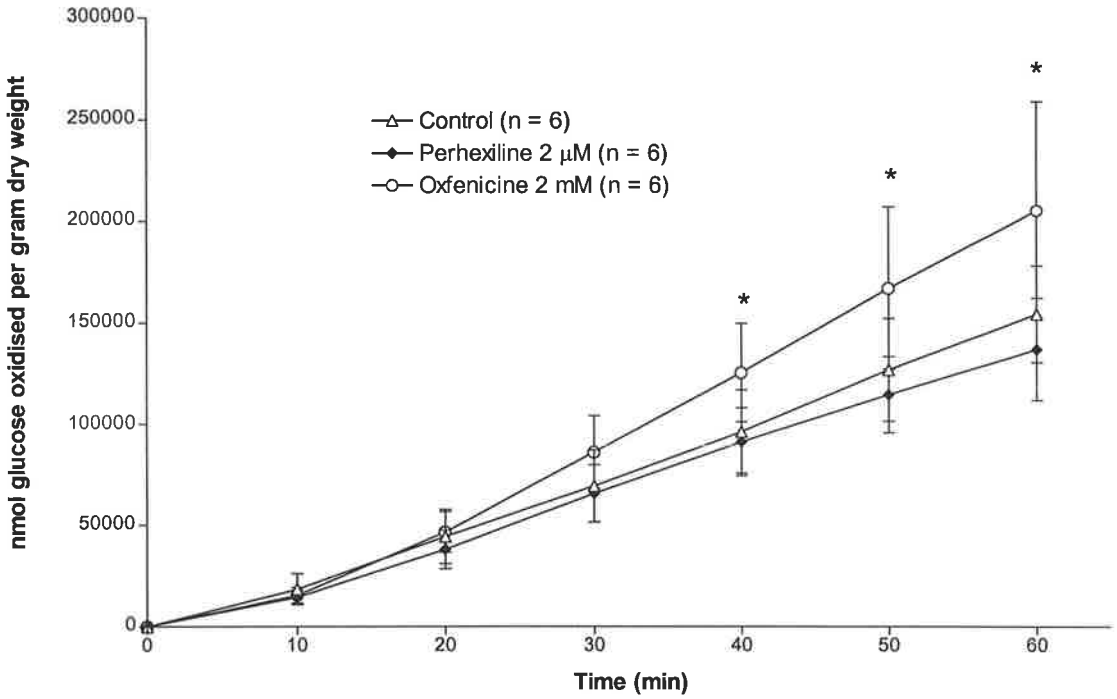


Figure 4.13: Cumulative nmol of glucose oxidised (mean ± SD) during 60 minute working heart perfusions with acute exposure to drugs as shown in legend. *significantly different from control values via 2-way ANOVA with repeated measures ($F = 4.35$, $P = 0.03$).

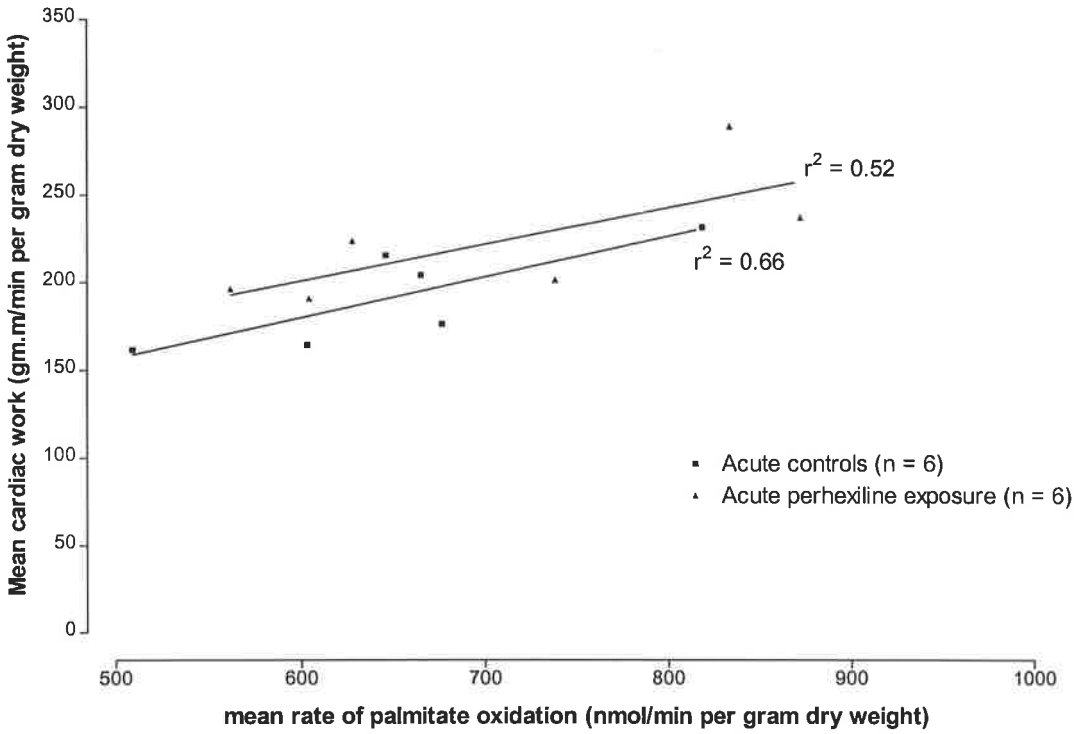


Figure 4.14: Relationship between mean cardiac work and mean rates of palmitate oxidation in working hearts exposed to perhexiline *in vitro* and control hearts. The relationships did not vary significantly between the two treatment groups by analysis of covariance ($F = 2.01$; $P = 0.19$).

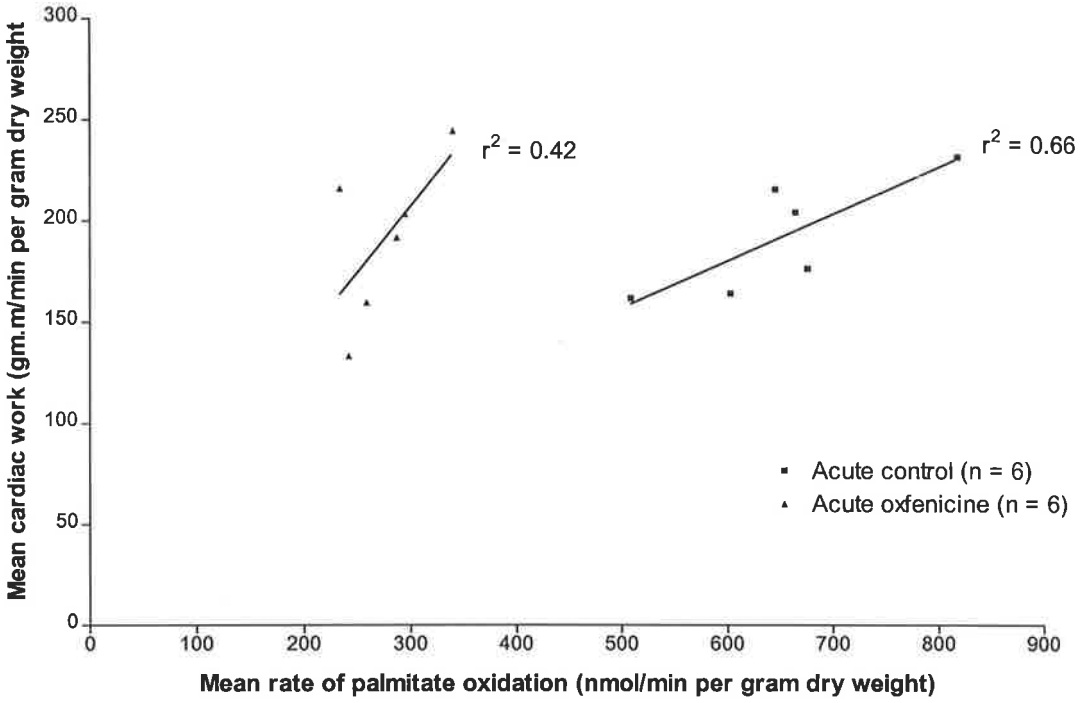


Figure 4.15: Relationship between mean cardiac work and mean rates of palmitate oxidation in working hearts exposed to oxfenicine *in vitro* and control hearts. The relationships varied significantly between the two treatment groups by analysis of covariance ($F = 5.24$; $P = 0.05$).

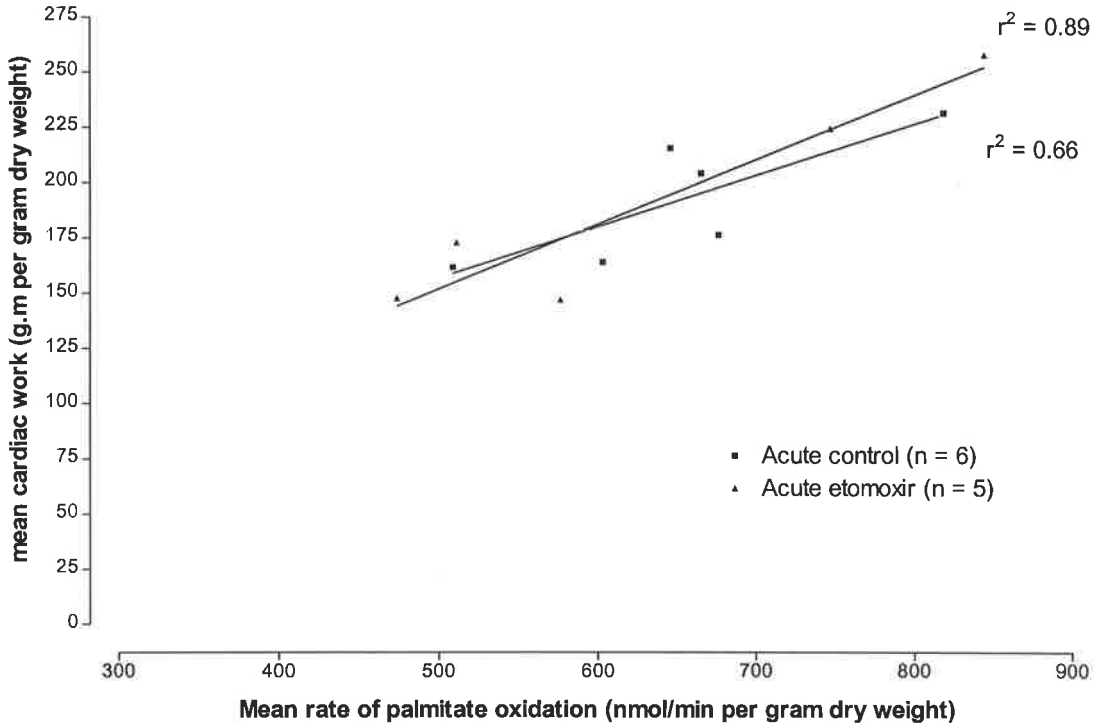


Figure 4.16: Relationship between mean cardiac work and mean rates of palmitate oxidation in working hearts exposed to etomoxir *in vitro* and control hearts. The relationships did not vary significantly between the two treatment groups by analysis of covariance ($F = 0.12$; $P = 0.73$).

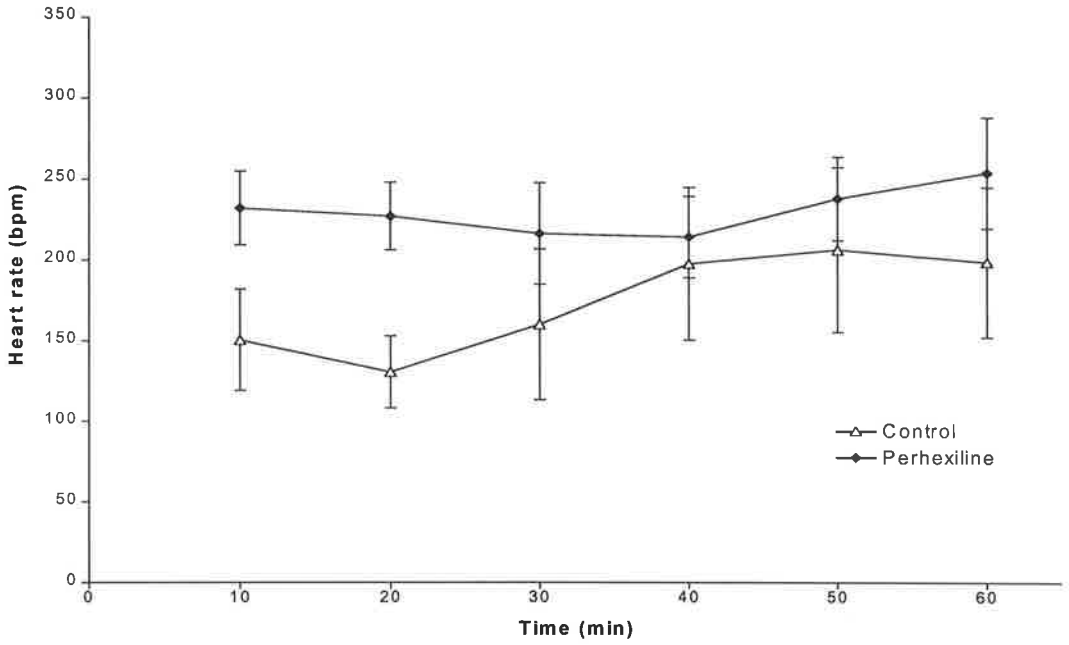


Figure 4.17: Heart rates (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). Heart rates were significantly higher in the perhexiline group by ANOVA with repeated measures (F = 9.51; P = 0.02).

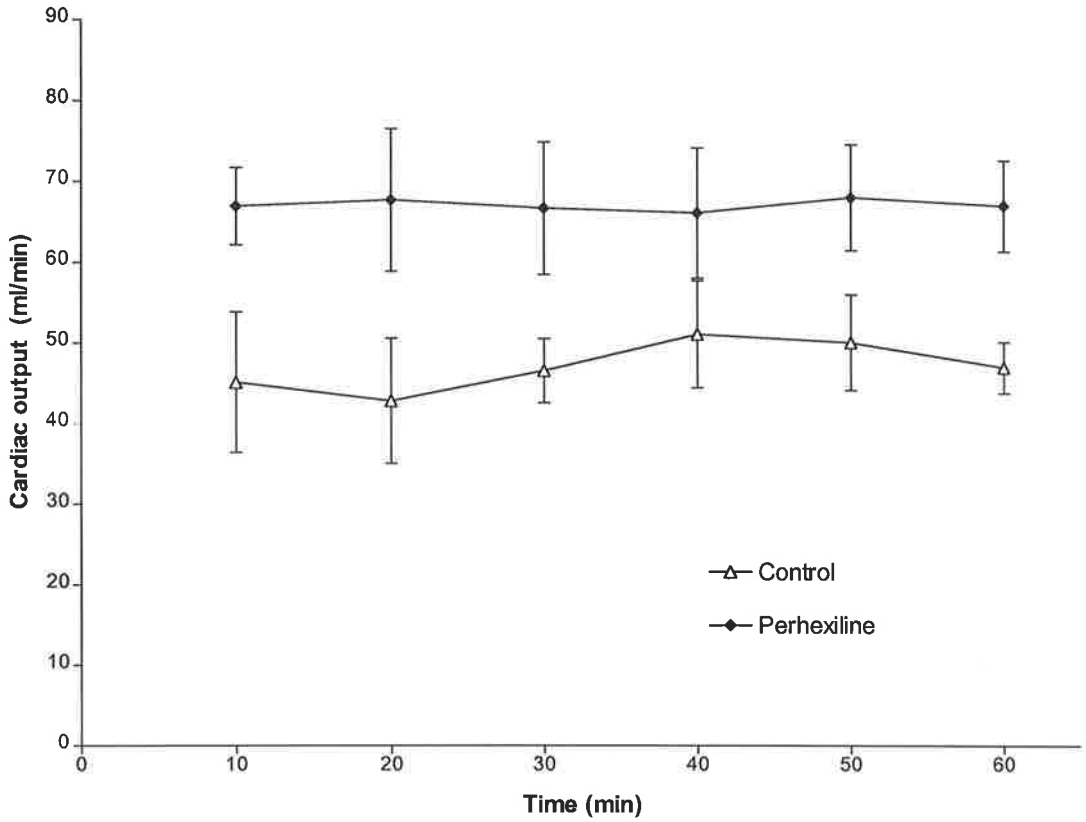


Figure 4.18: Cardiac output (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). Cardiac outputs were significantly higher in the perhexiline group by ANOVA with repeated measures (F = 31.2; P = 0.0005).

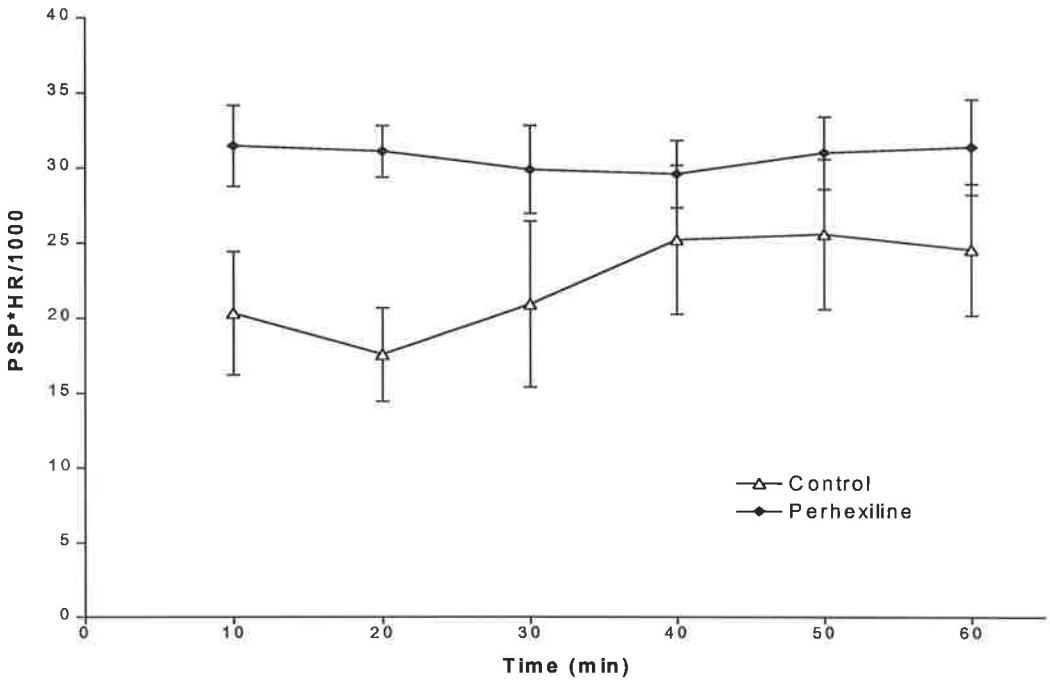


Figure 4.19: Time courses of rate-pressure product (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). Rate-pressure products were significantly higher in the perhexiline group by ANOVA with repeated measures (F = 19.11; P = 0.002).

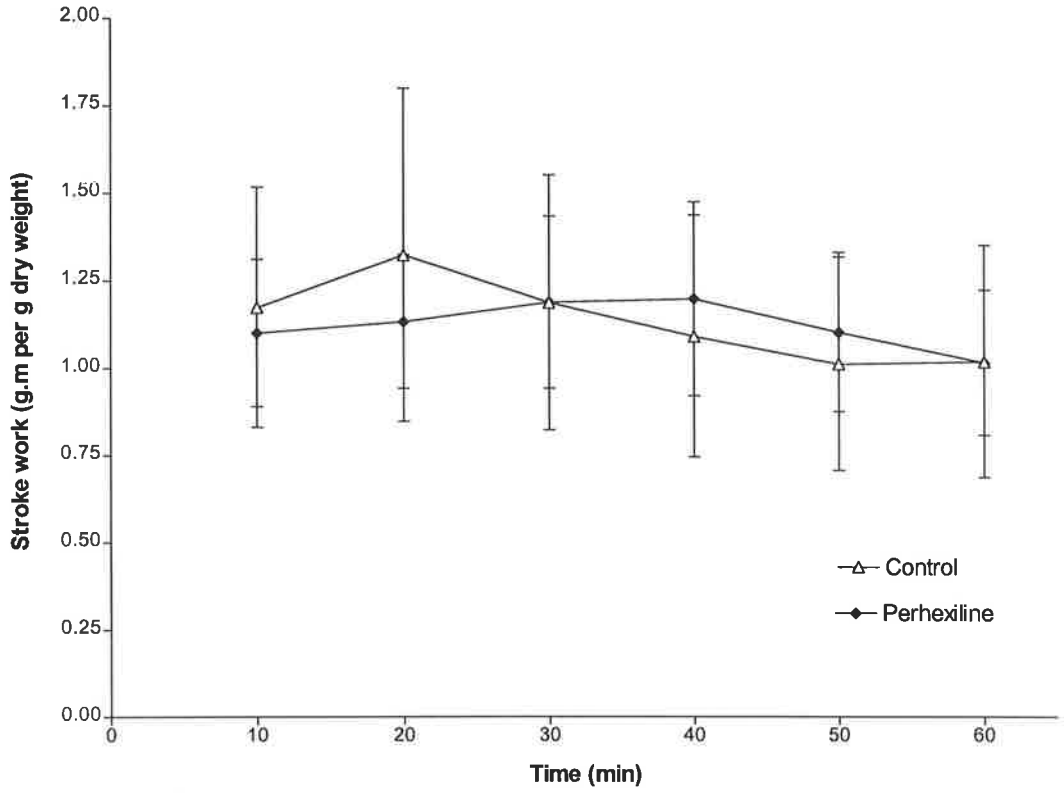


Figure 4.20: Time courses of stroke work (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). There were no significant differences between the two groups by ANOVA with repeated measures (F = 0.003; P = 0.96).

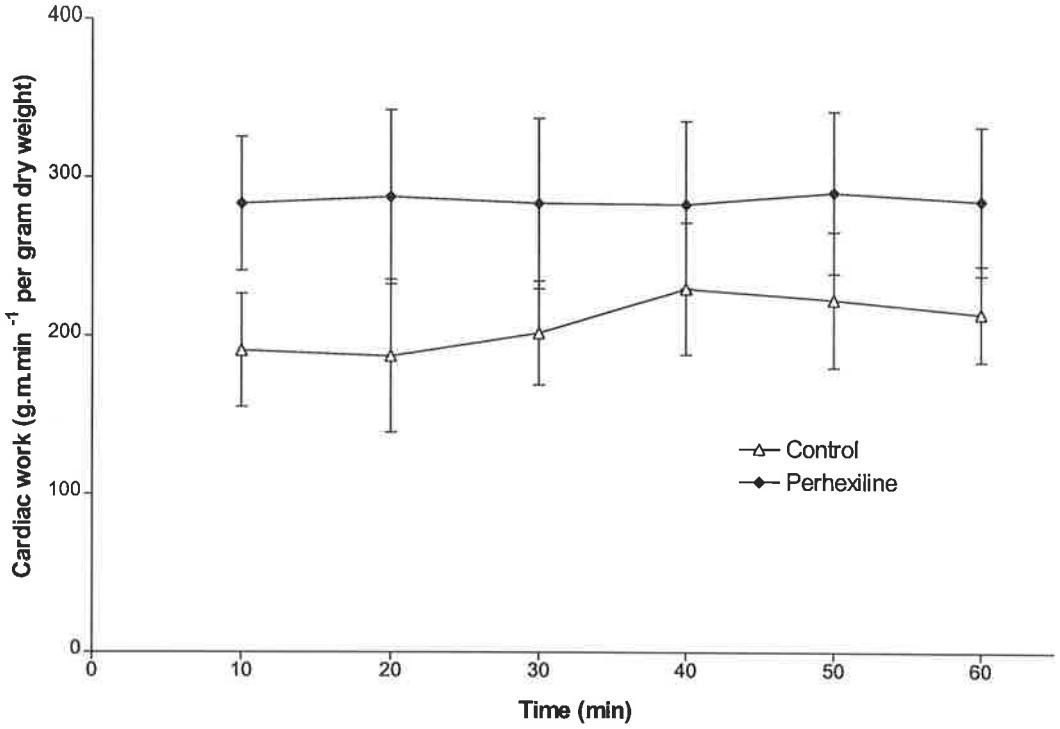


Figure 4.21: Time courses of cardiac work per gram dry weight (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). Cardiac work was significantly higher in the perhexiline group by ANOVA with repeated measures (F = 9.0; P = 0.02).

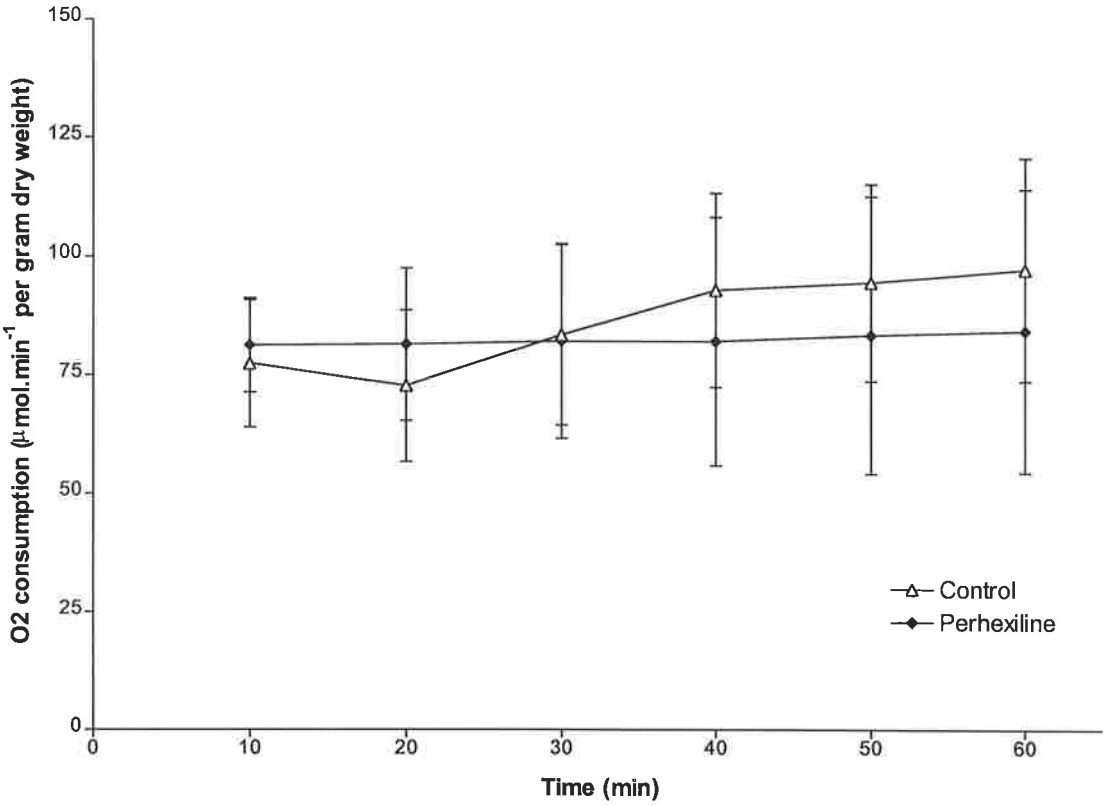


Figure 4.22: Oxygen consumption per gram dry weight (mean ± SD) during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2µ*M* (♦; n = 5). There were no significant differences between the two groups by ANOVA with repeated measures (F = 0.10; P = 0.76).

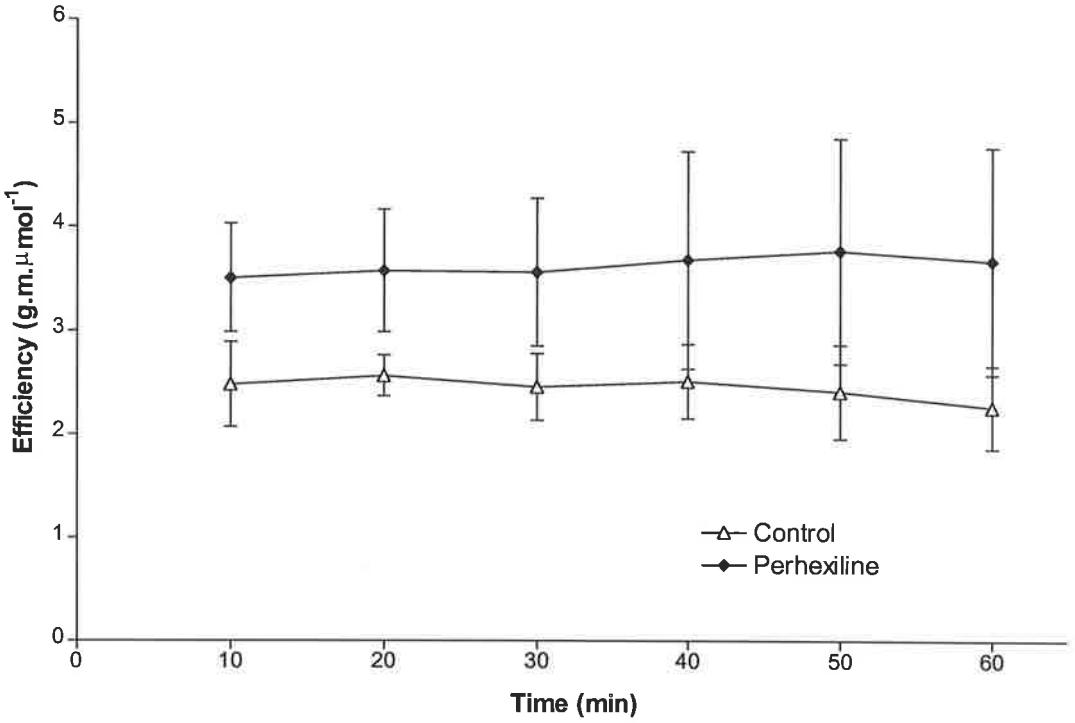


Figure 4.23: Time courses of cardiac efficiency (mean ± SD), calculated as cardiac work performed per μmol of oxygen utilised, during 60 minute perfusions of working rat hearts pre-treated with placebo patches (Δ; n = 5) or perhexiline both via 24 hour patch and *in vitro* at 2μ*M* (♦; n = 5). Cardiac efficiency was significantly higher in the perhexiline group by ANOVA with repeated measures (F = 11.12; P = 0.01).

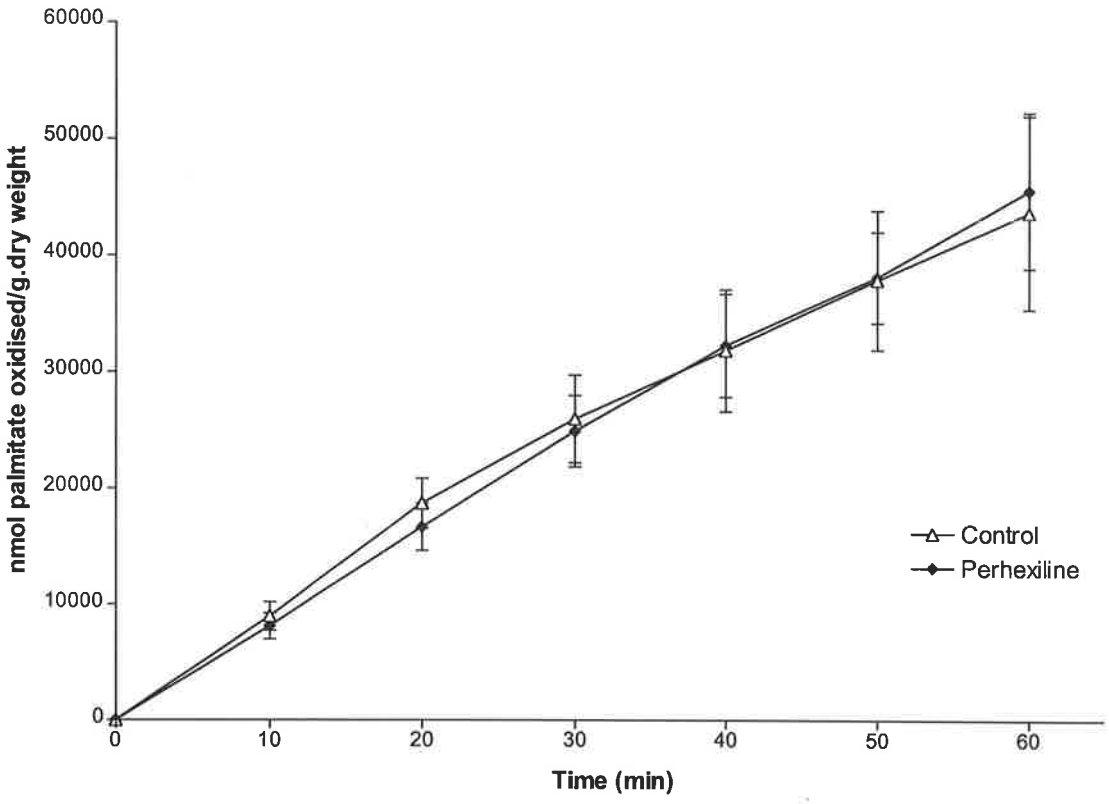


Figure 4.24: Cumulative palmitate oxidation, in nmol per gram dry weight, during 60 minute working heart perfusions following 24 hour transdermal administration of perhexiline (♦, n = 5) or vehicle (Δ, n = 5). There was no significant difference in the rate of palmitate oxidation between the two groups by two-way ANOVA with repeated measures.

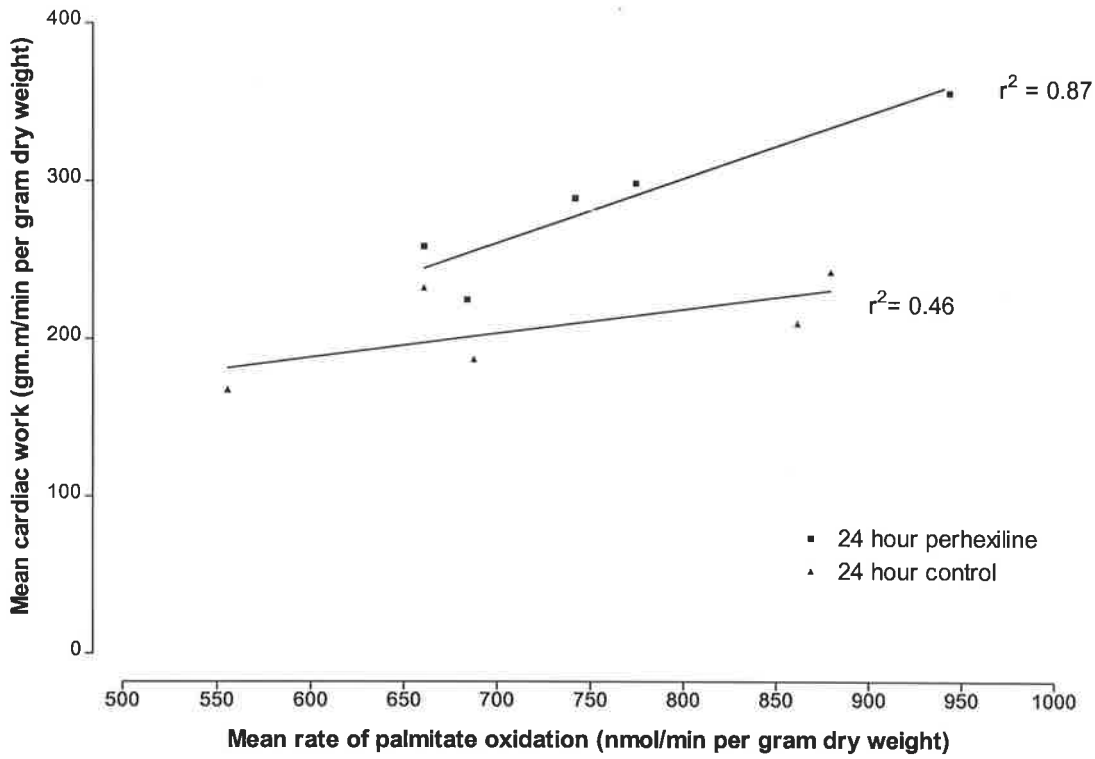


Figure 4.25: Relationship between mean cardiac work and mean rates of palmitate oxidation in working hearts of rats pre-treated with perhexiline for 24 hours (n = 5) compared to the control group (24 hours of vehicle; n = 5). The relationships varied significantly between the two treatment groups by analysis of covariance (F = 15.56; P = 0.006).

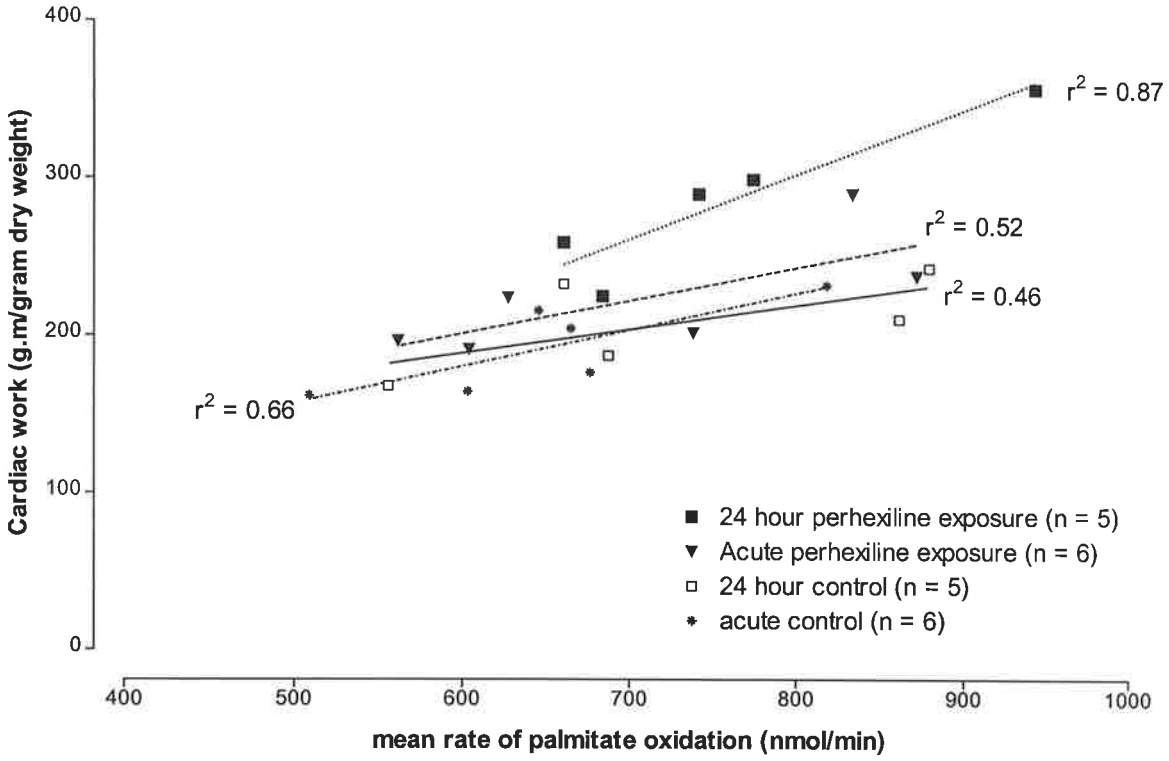


Figure 4.26: Relationship between cardiac work and rates of palmitate oxidation in working hearts exposed to perhexiline acutely and in matched controls, and in those pre-treated with either perhexiline or placebo patches for 24 hours. The relationships vary significantly between the hearts exposed to perhexiline acutely and those exposed for 24 hours ($F = 7.94$, $p = 0.02$ by analysis of covariance).

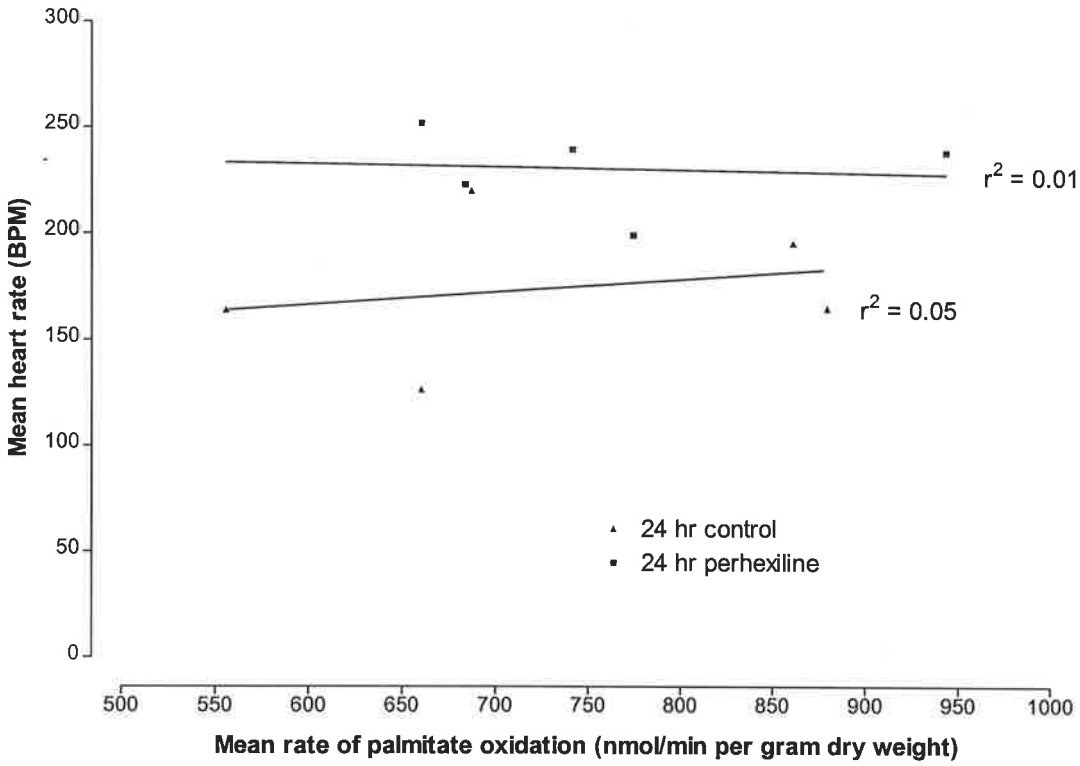


Figure 4.27: Relationship between mean heart rate and mean rates of palmitate oxidation in working hearts of rats pre-treated with perhexiline for 24 hours (n = 5) compared to the control group (24 hours of vehicle; n = 5). The relationships varied significantly between the two treatment groups by analysis of covariance (F = 8.02; P = 0.03).

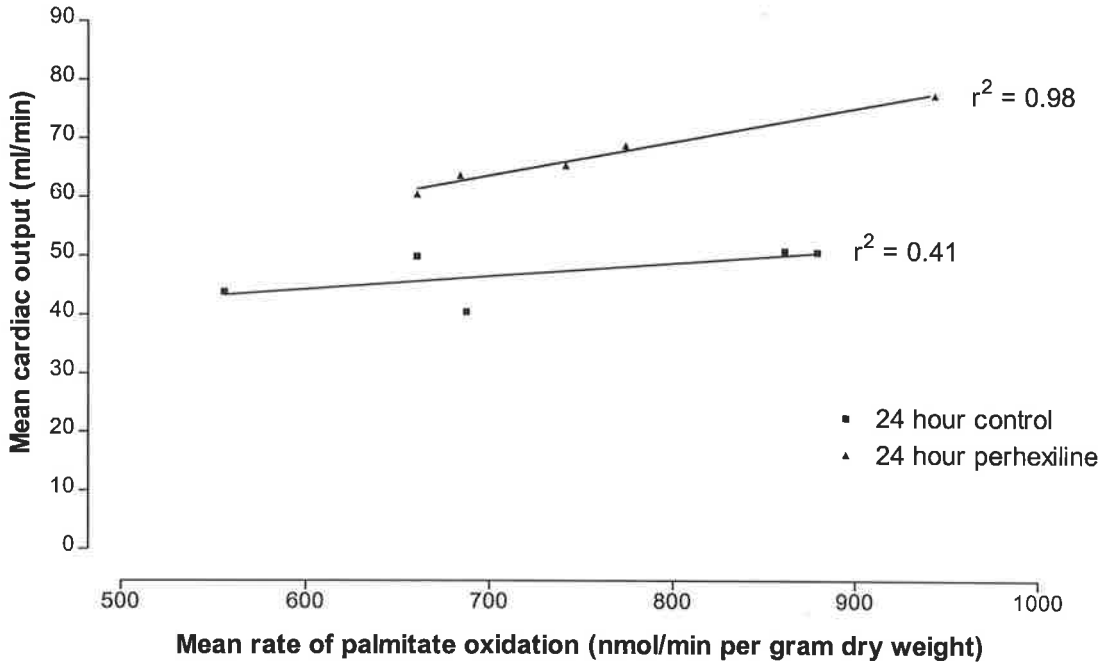


Figure 4.28: Relationship between mean cardiac output and mean rates of palmitate oxidation in working hearts of rats pre-treated with perhexiline for 24 hours (n = 5) compared to the control group (24 hours of vehicle; n = 5). The relationships varied significantly between the two treatment groups by analysis of covariance (F = 64.73; P = 0.03).

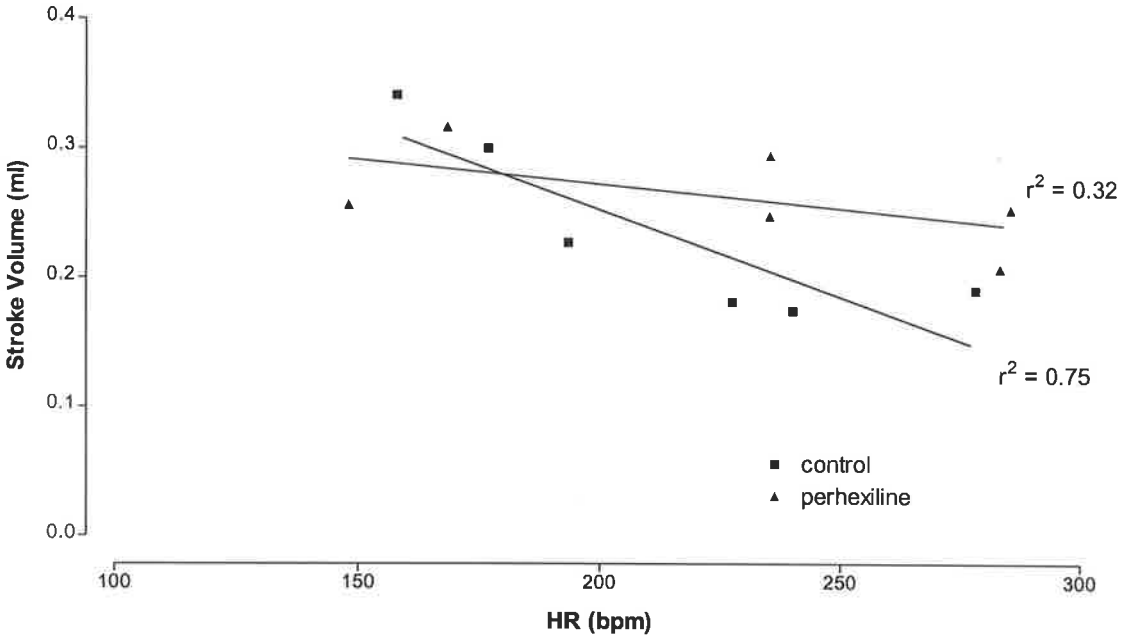


Figure 4.29: Relationship between stroke volume and heart rate in working hearts exposed to perhexiline acutely, and in control hearts. The relationships between the two treatment groups do not vary significantly ($F = 2.15$, $p = 0.18$ by analysis of covariance).

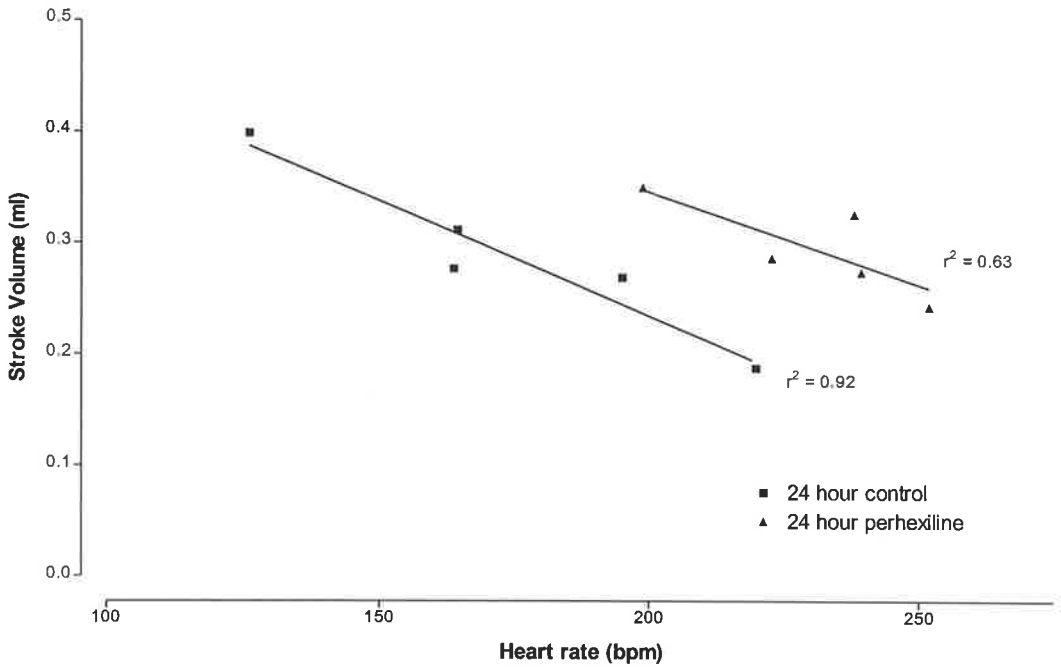


Figure 4.30: Relationship between stroke volume and heart rate in working hearts pre-exposed to either perhexiline (n = 5) or control (n = 5) patches for 24 hours. The relationships vary significantly between the two treatment groups ($F = 60.9$, $p < 0.0001$ by analysis of covariance).

	Controls		Perhexiline-treated animals (25 mg/kg IP for 5 days)					
	baseline weight (g)	weight change (g)	baseline weight (g)	weight change (g)	plasma [P] at T3 (mg/L)	plasma [P] at T15 (mg/L)	heart [P] at T15 (μg/g)	liver [P] at T15 (μg/g)
1	400	+20	450	0	0.44	0.08	0.35	0.35
2	470	+20	350	-30		0.06	0#	0.35
3	460	+30	440	-40	0.30	0.05	0.20	1.00
4	410	+10	410	-40		0.09	0.35	0.70
5	385	+15	380	*				
Mean ± SD	425 ± 28	+19 ± 7	406 ± 42	-28 ±19		0.07 ± 0.02	0.23 ± 0.17	0.60 ± 0.31

Table 4.1: Weight changes in rats treated with 25 mg/kg of perhexiline intra-peritoneally (n=5) or vehicle (n = 5) for 5 days. Plasma and tissue levels at the time of sacrifice 15 hours after the final dose (T15), and plasma levels 3 hours after the final dose (T3) in two rats, are also shown. # = unrecordable level; * = rat died on Day 3.

	Controls		Perhexiline treated animals (25 mg/kg orally for 5 days)				
	baseline weight (g)	weight change (g)	baseline weight (g)	weight change (g)	plasma [P] at T3 (mg/L)	heart [P] at T3 (μg/g)	liver [P] at T3 (μg/g)
1	320	+55	320	+5	0.07	0.98	1.39
2	310	+30	275	-65	0.25	6.36	28.5
3	290	+30	320	0	0.10	1.15	2.08
4	320	+45	320	+35	0.04	0.40	1.30
5	280	+35	295	-10	0.04	0.33	1.00
mean ± SD	304 ± 18	+39 ± 11	306 ± 20	-7 ± 37	0.10 ± 0.09	1.84 ± 2.55	6.85 ± 12.11

Table 4.2: Weight changes in rats treated with 25 mg/kg of perhexiline orally (n=5) or vehicle (n = 5) for 5 days. Plasma and tissue levels at the time of sacrifice, 3 hours after the final dose (T3) are also shown.

	Control	Perhexiline (2 μM)	Oxfenicine (2 mM)	Etomoxir (1μM)	F	P
Heart rate (BPM)	227 ± 39 (12)	204 ± 47 (12)	209 ± 44 (12)	233 ± 31 (5)	1.41	0.26
Cardiac output (ml/min)	51.0 ± 9.6 (12)	54.2 ± 13.4 (12)	52.4 ± 13.3 (12)	59.7 ± 9.5 (5)	0.97	0.42
HR x PSP (mmHg/min/1000)	29.1 ± 3.5 (12)	25.2 ± 5.1 (12)	26.2 ± 4.5 (12)	28.8 ± 2.8 (5)	2.51	0.07
Stroke work (g.m)#	0.79 ± 0.23 (12)	0.98 ± 0.19 (12)	0.90 ± 0.19 (12)	0.90 ± 0.15 (5)	1.78	0.17
Cardiac work (g.m./min)#	197 ± 53 (12)	245 ± 77 (12)	207 ± 45 (12)	237 ± 58 (5)	1.27	0.30
O_2 consumption (μmol/min)#	52.1 ± 10.6 (5)	69.2 ± 18.4 (8)	70.7 ± 26.8 (8)		1.78	0.20
Cardiac efficiency (g.m/μmol)	3.37 ± 0.56 (5)	3.24 ± 0.51 (8)	3.26 ± 0.77 (8)		0.03	0.97

Table 4.3: Mean values (± SD) for haemodynamic values during 60 minute working heart perfusions, with acute exposure to drugs as indicated. Numbers in brackets refer to the number of hearts included in each analysis. Statistical comparison between groups was performed using two-way ANOVA with repeated measures. #per gram dry weight.

	Rate of oxidation (nmol/min per gram of dry weight)	
	Palmitate	**Glucose**
Control	653 ± 163	2461 ± 627
Perhexiline 2μM	706 ± 128	2250 ± 410
Oxfenicine 2mM	276 ± 40*	3423 ± 893*
Etomoxir 1 μM	620 ± 144	
Etomoxir 1 nM	572 ± 97	

Table 4.4: Average rates of palmitate and glucose oxidation during 60 minute working rat heart perfusions. N = 6 in each group except etomoxir 1 nM (n = 4). Values are the mean ± SD. *Significantly different from controls by two-way ANOVA with repeated measures.

	Control	Perhexiline 2 μM	Oxfenicine 1 mM	Etomoxir 1 μM	
ATP (μmol/gram)	7.1 ± 4.0	9.2 ± 4.4	5.2 ± 2.4	7.2 ± 4.6	F = 1.01
					P = 0.41
Triglycerides (μmol/gram)	60.8 ± 18.8	45.2 ± 17.8	49.2 ± 20.9	62.7 ± 21.7	F = 0.89
					P = 0.46
Glycogen (μmol/gram)	96.3 ± 35.0	83.0 ± 34.6	57.2 ± 10.6	88.3 ± 25.0	F = 2.21
					P = 0.12
Lactate (μmol/gram)	8.2 ± 4.6	8.3 ± 3.4	9.1 ± 3.1	11.9 ± 7.5	F = 0.73
					P = 0.55

Table 4.5: Myocardial tissue levels of ATP, triglycerides, glycogen, and lactate in μmol per gram of dry tissue weight at the end of 60 minute working heart perfusions. Values are mean ± SD of 6 hearts in each group. No significant differences are seen between the 4 groups by one-way ANOVA.

Rat	Plasma perhexiline (A) (mg/L)	Plasma OH-perhexiline (B) (mg/L)	B:A ratio
1	0.25	0.07	.28
2	0.40	0.04	.10
3	0.94	0.51	.54
4	0.69	0.35	.51
5	0.39	0.24	.62
mean ± SD	0.53 ± 0.28	0.24 ± 0.19	.41 ±. 21

Table 4.6: Plasma perhexiline and mono-hydroxyperhexiline concentrations at time of sacrifice following 24 hours of transdermal perhexiline (200 mg perhexiline in sorbiline + DMSO) in 5 rats.

	Control	Perhexiline	F	P
Heart rate (BPM)	174 ± 48	230 ± 28*	9.51	0.02
Cardiac output (ml/min)	47.1 ± 6.4	67.0 ± 2.8*	31.20	0.0005
HR x PSP (mmHg/min/1000)	22.4 ± 5.1	30.7 ± 2.5*	19.11	0.002
Stroke work (g.m)#	1.1 ± 0.4	1.1 ± 0.2	0.003	0.96
Cardiac work (g.m./min)#	208 ± 39	285 ± 46*	9.00	0.02
O_2 consumption (μmol/min)#	86.3 ± 19.7	82.4 ± 21.1	0.10	0.76
Cardiac efficiency (g.m/μmol)	2.4 ± 0.3	3.6 ± 0.8*	11.12	0.01

Table 4.7: Mean values (± SD) for haemodynamic values during 60 minute working heart perfusions, following 24 hours of transdermal perhexiline (n = 5) or vehicle (controls; n = 5). Statistical comparison between groups was performed using two-way ANOVA with repeated measures.

#per gram dry weight.

*statistically different from control group.

Rat	Control		Perhexiline	
	Palmitate oxidation rate (nmol/min)	**ATP (μmol/gm dry weight)**	**Palmitate oxidation rate (nmol/min)**	**ATP (μmol/gm dry weight)**
1	687.6	11.21	684.2	16.40
2	861.6	27.08	774.6	16.52
3	879.6	19.28	741.9	10.67
4	660.8	15.01	943.6	14.20
5	555.9	18.69	660.9	12.17
Mean ± SD	729 ± 138	18.3 ± 5.9	761 ± 112	14.0 ± 2.6

Table 4.8 Mean rates of palmitate oxidation and tissue levels of ATP in isolated working hearts of rats pre-treated with perhexiline (200 mg) or placebo transdermally for 24 hours. No significant differences are seen in the rates of palmitate oxidation (2-way ANOVA with repeated measures) or tissue ATP levels (paired t test) between the two groups.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY: MAJOR EXPERIMENTAL FINDINGS

The experiments described in chapters 2-4 essentially constitute three distinct strategies to examine the cardiac effects of perhexiline in animal models (intact sheep and working rat heart). The starting point for these experiments has been the unique clinical efficacy of perhexiline as a prophylactic anti-anginal agent, coupled with major deficiencies in relevant background data as regards haemodynamic effects and precise effects on myocardial performance, metabolism and metabolic efficiency.

Superficially, the major conclusions to be drawn from these three chapters are:

1. Brief parenteral administration of perhexiline in conscious animals (sheep) results in peripheral vasoconstriction associated with negative chronotropy and no significant change in myocardial contractility (Chapter 2). These findings suggest that perhexiline does not have typical L-type calcium channel blocking activity in the concentrations used in the present study. While these results are consistent with the proposed "metabolic" anti-ischaemic effect of perhexiline, no anti-ischaemic effects were evaluated. Furthermore the mechanism(s) of vasoconstriction/negative chronotropy were not delineated.
2. Short term (up to 24 hours) parenteral adminsitration of perhexiline does not have any significant effects on myocardial β-oxidation of long-chain fatty acids in sheep, as assessed by *in vivo* imaging of the myocardial kinetics of radio-labelled fatty acid substrate (Chapter 3). On the other hand, etomoxir significantly inhibits myocardial β-oxidation of long-chain fatty acids in the same model.

3. Acute *in vitro* exposure to perhexiline does not have any major metabolic or haemodynamic effects in an isolated working rat heart model under normal flow conditions (Chapter 4). However, *ex vivo* experiments using hearts from rats pre-exposed to 24 hours of transdermal perhexiline indicated an increase in cardiac work and myocardial efficiency, accompanied by a decrease in palmitate utilisation per unit work performed relative both to controls, and to hearts exposed acutely to perhexiline *in vitro*. However, absolute rates of palmitate oxidation were unchanged relative to control hearts. Acute treatment with oxfenicine induced a shift from fatty acid towards glucose oxidation (the "Randle effect") while acute treatment with etomoxir had no effects on metabolism.

5.2 ISSUES ARISING FROM MAJOR EXPERIMENTAL FINDINGS

Comparison of the results from these chapters raises a number of important issues;

5.2.1 Is there an "appropriate" animal model for the anti-ischaemic effects of perhexiline?

None of the experiments were performed during ischaemic conditions (although there is evidence that progressive deterioration in performance in the working heart model is analogous to ischaemia in some respects, by virtue of the role of increased oxidative stress [Ferdinandy et al. 1999, Panas et al. 1998]). However, it would have

been expected that perhexiline might have induced changes in tissue metabolism irrespective of the presence/absence of ischaemia.

Two potential areas of concern might have been raised about the sheep and rat models utilised. Firstly, in ruminants such as sheep, it might have been expected that long-chain fatty acid metabolism might have accounted for a relatively small proportion of metabolism, and that it might have been difficult to detect a "metabolic shift" due to CPT-1 inhibition. This proved not to be the case, as was evident from the results with etomoxir. Secondly, there was a possibility that both sheep and rats would clear perhexiline extremely rapidly, making it difficult to achieve tissue 'loading" with the drug; this was likely given the extremely high activity of cytochrome P-450 2D6 (CYP2D6) in most non-primate mammalian species (Ingelman-Sundberg et al., 1999). This actually proved to be a major problem in both sheep and rats. Indeed, it is impossible to be entirely sure that the dosing of sheep for 24 hours was "adequate" for optimal uptake of perhexiline into cardiac mitochondria. In the case of rats, a drug administration regime suitable for 24 hour treatment with perhexiline was devised. The fact that this regimen resulted in marked changes in myocardial metabolic efficiency offers a basis for assumed efficacy; this regimen is likely to be utilised in future studies in the rat.

As regards the anti-ischaemic effects of acute perhexiline in *in vitro* models, these have recently been the subject of experiments with Langendorff-perfused rat hearts in our laboratory. The results, which have been submitted for publication, suggest

modest protection against ventricular dysfunction during low-flow ischaemia, with no protection during reperfusion. More importantly, perhexiline did not improve cardiac performance during moderate ischaemia (70% flow reduction). These essentially negative results are surprising and raise the possibility that the models may have been sub-optimal in some respect. The most likely deficiency of the above models, in light of the results of experiments in Chapter 4, is lack of chronic pre-exposure to perhexiline.

5.2.2 Is there a "lag phase" prior to onset of effects of perhexiline on myocardial efficiency, and what is the basis for this phase?

The results in Chapter 4 clearly indicate different effects of acute *in vitro* versus 24 hour *ex vivo* perhexiline exposure. This implies that perhexiline does not acutely alter myocardial metabolic efficiency, but does not provide a basis for this phenomenon. Two possible categories of explanation might be offered: pharmacokinetic and biochemical.

The process of uptake of perhexiline into tissues has been studied in a number of models. The current studies demonstrate concentration of perhexiline in the myocardium relative to perfusate, but do not permit elucidation of the time course of uptake into the mitochondria, the probable major site of action of the drug. Deschamps et al. (1994) have demonstrated *in vitro* concentration gradients of perhexiline of approximately twenty fold from mitochondria to buffer in hepatocytes, however this provides little indication of uptake kinetics. Reciprocally, wash-out of

perhexiline from its sites of negative inotropic effect in monolayers of embryonic chick heart cells was disproportionately slow relative to calcium antagonists such as verapamil and diltiazem, suggesting extensive intra-myocardial binding (Barry et al., 1985). Therefore it is quite likely that equilibration of perhexiline with tissue sites of action may be slow.

However, an alternative hypothesis would be that perhexiline either has relatively slowly developing inhibitory effects on metabolising enzymes such as CPT-1, or that it acts indirectly during chronic administration, for example by changing expression of genes affecting myocardial metabolic efficiency. The finding that *in vitro* incubation of isolated mitochondria with perhexiline induces maximal CPT-1 inhibition in 15 minutes (Kennedy et al., 1996) argues against a purely "biochemical lag" basis.

It must be stated that there are considerable data favouring the fact that the effects of perhexiline develop slowly. Although not part of this thesis, such experiments include:

(1) Potentiation of platelet responsiveness to NO during chronic therapy *in vivo* in humans subjects, but not *in vitro* (Willoughby, PhD thesis, 1999).

(2) Accentuation of inhibition of palmitate metabolism in cultured rat heart cells after 48 hours pre-exposure to perhexiline relative to acute exposure (Dr J Kennedy: experiments in progress). These latter data are in accord with those of

Deschamps et al. (1994) who demonstrated a considerable "lag phase" (72 hours) in the ability of perhexiline to inhibit palmitate oxidation in hepatocytes.

These data have important implications: effects of perhexiline cannot be expected necessarily to parallel the acute attainment of "therapeutic levels". If perhexiline is to be developed as an agent for emergency cardioprotection in man, the relevant issue is time to onset of cardioprotective effect. This being the case, the experiments in Chapter 4 beg important questions:

(a) How rapidly does the "metabolic" effect appear (e.g. in 1 to 2 hours)?

(b) What is the time course of the magnitude of this effect relative to plasma perhexiline concentrations? The latter important question would require many additional data points, but would be useful for effect modeling.

The transposition of "lag" data to the clinical setting poses considerable challenges. The intravenous preparation of perhexiline may not be completely safe, as indicated from morbidity among sheep studied. If rapid oral dosing is to be utilized, the methodology for assessing onset of effects on the myocardium (as distinct from circulating platelets) would probably involve assessment of metabolism after 12 to 24 hours of therapy.

5.2.3. How does perhexiline work? Can animal models help to decide?

The number of "negative" experiments in the current thesis offers strong testimony as regards the difficulty of this issue. However, a number of definite conclusions are available (along with several speculative ones):

(a) Perhexiline in "therapeutic" concentrations does not behave as a typical L-channel calcium antagonist in either sheep or rat, in that it induces neither vasodilatation nor negative inotropy. This is certainly consistent with clinical impression in human subjects, although careful haemodynamic studies are lacking (see Chapter 1).

(b) In the rat, pretreated for 24 hours with perhexiline, the drug exerts a marked and potentially beneficial effect on efficiency of myocardial oxygen utilisation. Perhexiline also produced a marked shift in the relationship between palmitate oxidation and myocardial performance. Nevertheless, there was no absolute change in palmitate oxidation, and therefore no evidence of CPT-1 inhibition. However the lack of effect on absolute rates of palmitate oxidation is potentially at least in part due to variable cardiac work between perhexiline-treated and control hearts.

Clinical studies have in general shown a large beneficial effect of perhexiline therapy on anginal frequency and exercise performance in comparison to other "metabolic" agents such as trimetazidine and ranolazine (Chapter 1). The precise mechanism(s) of perhexiline's metabolic effects remain unclear; in particular, the current results do not show any association between CPT-1 inhibition and the

improvement in myocardial efficiency seen in hearts pre-treated with perhexiline. On the other hand, despite the clear-cut metabolic shift induced by acute oxfenicine exposure, no associated effects on efficiency were observed.

5.3 FUTURE EXPERIMENTS

There are several priorities for future experiments. These can be summarised as follows:

(1) The development of a suitable animal model for long-term administration of perhexiline, such as the dark Agouti rat. The potential advantage of this model over the 24-hour dosing schedule developed in the Albino Wistar rat extends beyond convenience of perhexiline administration: it represents the only possible model to test the hypothesis that the effects of perhxiline on efficiency reach peak values beyond 24 hours of treatment. However, if further experiments on Wistar rats indicate plateau effects developing earlier, the priority of a further rat model would be lower.

(2) Definition of major sites of "therapeutic" effect of perhexiline.

The current experiments do not define the basis for increased myocardial metabolic efficiency with perhexiline: while the demonstration of this change is important, it would be desirable to delineate the precise mechanism(s) involved.

The first series of experiments in this regard should attempt to determine the relationship between CPT-1-sensitive and CPT-1-independent processes and

myocardial efficiency effects of perhexiline. Thus, in rats pre-treated with perhexiline (compared with controls) the relationship between myocardial performance and metabolic rates for oxidation of the long-chain fatty acid, palmitate (CPT-1 substrate), should be compared with that of the short-chain fatty acid, butyrate (CPT-1 independent). If analysis of covariance indicates equivalent shifts in the work : metabolism relationship for both substrates, this would suggest that CPT-1 inhibition by perhexiline was not involved in the change in efficiency. Even if there was a differential shift of the work : fatty acid oxidation relationship, this would causally implicate the long-chain fatty acid uptake and β-oxidation pathway as a whole, with CPT-1 being merely the most likely site.

This raises the issue of whether increased myocardial efficiency might arise from bases other than a shift in metabolic substrate utilisation. In theory, it might be suggested that agents which induce oxidative stress might reduce the proportion of high energy phosphates utilised in excitation-contraction coupling, and therefore any cytoprotective/anti-oxidant effect might improve efficiency. In this regard, reduced cardiac efficiency of working rat hearts due to cytokine exposure has been postulated to involve the activation of energy wasting repair cycles and uncoupling of oxidative phosphorylation (Panas et al., 1998). However, studies to this effect are currently lacking. *Ex-vivo* experiments related to putative anti-oxidative effects of perhexiline have not yet been performed, but perhexiline lacks acute effects as a free-radical scavenger.

(3) Evaluation of perhexiline's effects in ischaemic models.

Effects of perhexiline need to be examined on *ex vivo* cardiac metabolism and efficiency in a model of low-flow ischaemia, such as

(i) a combined approach of graduated flow reduction in a Langendorff isolated heart preparation during continuous pacing of the atria, followed by reperfusion in the working mode (using techniques to analyse ^{14}C and ^{3}H labelled substrate utilisation described in Chapter 4) or

(ii) an *in vivo* large animal model of partial occlusion of the left anterior descending artery, with analysis of cardiac metabolism using coronary sinus sampling or imaging with iodinated fatty acids.

(4) Human studies remain a critically important objective for this agent. It is clear that there is little purpose in administering perhexiline via intravenous injection (given the slow onset of effect) and thus rapid oral loading (Horowitz et al., 1986) represents a satisfactory means for drug administration.

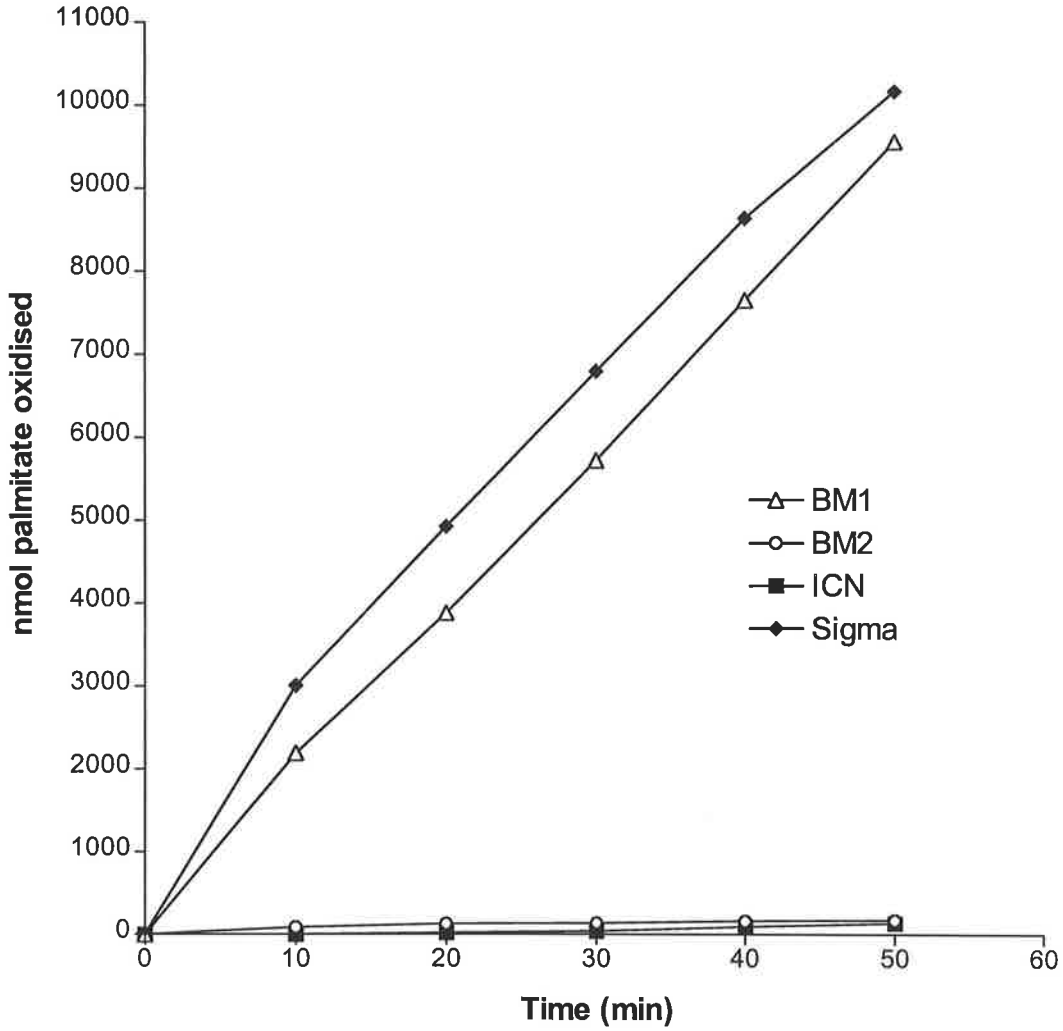
It might be important to perform IPPA-imaging experiments in patients with ischaemia on the grounds that (a) sheep were not ischaemic, and (b) humans may behave differently to sheep. Ideally such a study would be carried out in patients with chronic stable angina at baseline and after achievement of steady-state perhexiline levels, correlating the metabolic data with myocardial perfusion data (e.g. with ^{99m}Tc-sestamibi imaging), clinical effects, and plasma perhexiline

concentrations. However, this would involve some difficulties, such as determining whether it would be advisable ethically to perform paired (pre/post perhexiline) uptake studies, and whether it would be possible to image uptake under similar cardiac workloads, to obviate the difficulties encountered in Chapter 4.

The most definitive available technology for relating myocardial performance to metabolism in humans would utilise cardiac catheterization with measurement of coronary sinus blood flow and trans-myocardial clearance of oxygen and selected metabolites. This could be performed in patients with stable angina pectoris; effects of perhexiline could be evaluated with heart rates standardised via atrial pacing. The difficulties inherent in this approach relate to inability to introduce perhexiline rapidly: this would therefore require non-paired methodology, with relatively large patient numbers.

For these reasons, it is likely that maximal information in human studies might be available from patients randomly treated with perhexline or placebo while awaiting coronary revascularization. The examination of trans-coronary concentration gradients of glucose relative to long- and short-chain fatty acids at operation (prior to, and after, aortic cross-clamping) would provide evidence of metabolic shifts induced by perhexiline, which could be correlated with preoperative anti-anginal effect and perioperative effects on myocardial function.

In conclusion, the experiments in this thesis have ultimately provided further evidence to support the concept that perhexiline has a uniquely beneficial effect on myocardial metabolic efficiency, without completing the delineation of the mechanism of effect. Although there is little doubt (from falls in blood glucose levels, Stewart et al., 1996) that perhexline produces metabolic effects in human subjects consistent with its CPT-1 inhibitory effects in isolated mitochondrial preparations, it remains uncertain whether this is the sole mechanism of its beneficial effect. As with amiodarone, an agent for which CPT-1 inhibition represents only one of at least five previously described clinically relevant effects, it may prove difficult to precisely apportion beneficial mechanisms. Given the beneficial effects of perhexiline on cardiac efficiency, an additional, somewhat empirical, research objective might be to attempt modifications of the agent's structure, in order to ensure more homogeneous metabolism, while maintaining impact on efficiency of cardiac performance.



Appendix 1. Plots of palmitate oxidation in working rat perfusions, using different sources of BSA (n = 1 for each).

BM1 = original stock from Boehringer Mannheim (Germany), from European beef; BM2 = new stock from Boehringer Mannheim (Germany), from U.S. beef; ICN = stock from ICN Biomedicals Inc. (Aurora, Ohio, U.S.A); Sigma: RIA grade stock from Sigma Chemical Company (St. Louis, MO, U.S.A.).

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CHAPTER 1

1. P. 25, Para 1, line 1 (insert after "effects"):
[on platelets]

2. P. 26, Para 1, line 5 (insert after "(Opie & Messerli, 1995)."):
[In recent years amlodipine, a dihydropyridine with minimal reflex tachycardia, has been proven effective in the treatment of angina, and safe in the presence of patients with chronic heart failure (Packer et al., 1996).]

3. P. 25, Para 1, line 2 (insert after "disease."):
[Nicorandil, a nicotinamide nitrate with both vasodilatory and potassium-channel opening activity, has equivalent antianginal efficacy to isosorbide di- and mono-nitrate (Doring 1992). More recent studies have focused on the potential cardioprotective effects of nicorandil via its activation of the mitochondrial ATP-sensitive potassium-channel ($K+_{ATP}$), which is involved in the phenomenon of ischaemic preconditioning (Knight et al., 1995). In patients undergoing primary PTCA during acute myocardial infarction, administration of nicorandil reduces infarct size, as assessed by regional left ventricular wall motion, compared to controls (Ito et al., 1999; Sakata et al. 1997).]

4. P. 27, Para 3, line 7 (insert after "lesion."):
[Recent studies have shown that the use of intracoronary gamma-radiation therapy may play an adjunctive role with repeat angioplasty in patients with in-stent restenosis (Waksman et al, 2000; Terstein et al, 2000).]

5. P. 28, Para 2:
Section heading should be "1.4.2.2"

6. P. 28, Para 2, line 12 (insert after "Cohen et al. 1975)"):
[Diabetes is an independent predictor of lesion progression, occlusion, and reduced long-term survival following CABG (Alderman, et al., 1993). However, two recent randomised trials have shown that long-term survival in diabetic patients with multivessel coronary disease is improved with CABG compared to PTCA as the initial revascularisation procedure (BARI investigators 2000; King et al., 2000).]

7. P. 29, Para 2:
Section heading should be "1.4.2.3"

8. P. 29, Para 2, line 8 (insert after "1999)."):
[More recently, laser ablation from the endocardial surface of the left ventricle via a less invasive catheter-based technique (percutaneous myocardial laser revascularisation) has also shown significant antianginal benefits (Lauer et al, 1999).]

9. P. 72, Para 3, line 4:
Should read "Figures 1.6 and 1.7"

10. P. 86
Page heading should be "Chapter 1"

CHAPTER 2

1. P. 95, Para 2, line 7 (insert after "man"):
[The objective was not to fully characterise the pharmacokinetics of intravenous perhexiline in sheep, as the impetus for clinical use of intravenous preparations of the drug has diminished with recent data showing a considerable "lag-time" between administration and metabolic effects (see Chapter 3, P. 138).]

2. P. 105, Para 1, line 5 (insert after "perhexiline"):
[In a single sheep receiving the 75 mg dose of perhexiline, arterial samples taken at baseline, mid-infusion, and 10 minutes post-infusion were immediately frozen and transported to the Biochemistry Department at Flinders Medical Centre for analysis of catecholamine (adrenaline and noradrenaline) levels.]

3. Page 106, Para 1, line 7:
Replace "5 mg and 20 mg of perhexiline (in 5 ml 5% dextrose)" with [37.5 mg in 10 ml 5% dextrose and 150 mg of perhexiline in 30 ml 5% dextrose]

4. Page 106, Para 1, line 9:
Replace "5mg" with [37.5 mg]

5. Page 106, Para 1, line 10:
Replace "20 mg" with [150 mg]

6. P.106, Para 2, line 6 (insert after "phase"):
[The plasma clearance of the 37.5 mg bolus dose was largely linear, while that of the 150 mg dose was not. Although this was only a single experiment, the data suggest the development of zero order kinetics with higher doses of perhexiline in sheep, as is the case in man.]

4. P. 107, Para 2, line 3 (insert after "cava"):
[(see also Appendix 2)]

5. P. 107, Para 2, line 7 (insert after "period"):
[In these group data, initial clearance of perhexiline with the lower dose was slightly faster than with the larger dose, but the differences were not marked.]

6. P. 107, Para 3, line 4 (insert after "Figure 2.3A"):
[and Appendix 2]

4. Page 117, Figure 2.1 Legend:
Replace 5 mg and 20 mg with [37.5 mg and 150 mg]

CHAPTER 3

1. P. 129, Para 1, line 1 (insert before "initial"):
[Preliminary experiments were performed with the aim of developing a non-toxic administration regime which would result in venous plasma perhexiline

concentrations within or just above the "therapeutic range" as described in man (i.e. 0.15-0.60 mg/L) (Cole et al. 1990; Horowitz et al. 1986).]

2. P. 129, Para 1, line 4 (insert after "days"):
[(see Appendix 2)]

3. P. 129, Para 3, line 5 (insert after "Figure 3.1"):
[and Appendix 2. During the final 30 minutes of these infusions, levels ranged between 0.5 and 0.7 mg/L, and no acute toxicity or haemodynamic effects were observed.]

4. P. 132, Para 2, line 11 (insert after "IPPA injected"):
[as a bolus]

5. P. 132, Para 2, line 14 (insert after "apart")
[, and the order of experiments (baseline, perhexiline, etomoxir) was the same for each sheep].

6. P. 133, Para 1, line 5 (insert after "solution"):
[The one litre infusions were administered via a Baxter infusion pump at 42 ml/hour. The control experiment was performed one week before the perhexiline infusion. For both the control and perhexiline infusions, IPPA was administered as a bolus via the jugular vein catheter during the final hour of the 24 hour infusion.

7. P.142
Page heading should be "Chapter 3"

8. P. 146, Table 3.2:
The Control data should be headed "T_{max}" and $T_{1/2}$", as should the Perhexiline data.

CHAPTER 4

1. P. 165, Para 2, line 4:
Should read " see Table 4.2"

2. P. 207, Para 1, line 10 (insert after "models"):
[A relatively small number of animals was studied in the 24 hour pretreatment experiments, five in each group. However, the large difference observed between perhexiline-treated rats and control rats (approximately 4 SD) meant that five per group was sufficient to obtain a statistically very significant result. Nevertheless, considering the small numbers, it is desirable that these findings are confirmed and extended in future studies.]

3. P. 253, Para 3, line 3 (insert after "studied"):
[Given the data demonstrating the considerable lag phase between administration of perhexiline and onset of metabolic effects, the current impetus for the development of a safe intravenous preparation of perhexiline has diminished, as there would appear to be minimal advantages over a rapid oral dosing schedule.]

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Appendix 2: plasma perhexiline levels in all sheep experiments.

Minutes	1	2	3	Mean ± SD
5	1.58	1.02	1.17	1.26 ± 0.29
10	0.38	0.30	0.36	0.35 ± 0.04
15	0.25	0.17	0.18	0.20 ± 0.04
30	0.16	0.09	0.11	0.12 ± 0.04

Table 1: Plasma perhexiline levels (mg/L) following 5 minute intravenous infusions of 37.5 mg perhexiline (see Figure 2.2).

Minutes	1	2	3	Mean ± SD
5	1.59	1.81	1.98	1.79 ± 0.20
10	0.67	1.04	0.54	0.75 ± 0.26
15	0.34	0.37	0.25	0.32 ± 0.06
30	0.20	0.31	0.19	0.23 ± 0.07

Table 2: Plasma perhexiline levels (mg/L) following 5 minute intravenous infusions of 75 mg perhexiline (see Figure 2.2).

	Art	CS
1	0.62	0.12
2	1.30	0.32
3	1.60	0.41
4	1.77	0.56
5	1.98	0.71
6	0.92	0.58
8	0.60	0.41
10	0.54	0.42
12	0.39	0.34
15	0.25	0.37
20	0.25	0.35
30	0.19	0.31

Table 3: Femoral artery (Art) and coronary sinus (CS) plasma perhexiline levels taken simultaneously in one sheep during and after a 5 minute intravenous infusion of 75 mg perhexiline (see Figure 2.3).

	1	2	3	Mean ± SD
10	0.32	.58	0.28	0.39 ± 0.16
20	0.40	.79	0.32	0.50 ± 0.25
30	0.51	.91	0.35	0.59 ± 0.29
40	0.64	1.00	0.39	0.68 ± 0.31
50	0.75	1.10	0.31	0.72 ± 0.40
60	0.75	1.25	0.40	0.80 ± 0.43
70	0.36	0.68	0.22	0.42 ± 0.24
80	0.23	0.44	0.19	0.29 ± 0.13
90	0.17	0.40	0.15	0.24 ± 0.14
120	0.12	0.31		0.22 ± 0.13
150	0.08	0.19	0.10	0.12 ± 0.06
180	0.07	0.12		0.10 ± 0.04

Table 4: Venous plasma perhexiline levels (mg/l) during and after 60 minute intravenous infusions of 5 mg/kg perhexiline in 3 sheep (see Figure 3.1).

	Sheep A	Sheep B	Sheep C
24 hours	0.0	0.0	0.0
48 hours	0.0	0.0	0.0
72 hours	0.0	0.0	0.0

Table 5: Venous plasma perhexiline levels (mg/l) following oral administration of 100 mg bd (Sheep A), 400 mg bd (Sheep B), and 600 mg bd (Sheep C) over 3 days.