A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

A Pharmacological Approach towards Myocardial Protection: New Perspectives in Acute and Chronic Cardiac Disease

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To my family

with love and admiration

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Abstract

Several cardiac diseases include myocardial ischaemia (acute or chronic), heart failure (systolic or diastolic) and left ventricular hypertrophy (either as a “primary” cause or developed secondary to other diseases) share the commonality of myocardial energetic deficiency or suboptimal myocardial metabolism. Therefore, approaches to modify myocardial metabolism in order to improve energetics present as an attractive therapeutic option. This is particularly useful when other options are limited: for example, lack of optimal symptom control with “maximal” treatment, or contraindications to other pharmacological treatment (by virtue of impairment of left ventricular systolic function and/or hypotension).

The objective of this thesis is to examine the biochemical effects of various pharmacological agents towards modulation of myocardial metabolism, both in the acute (e.g. acute coronary syndrome) and chronic cardiac disease settings (e.g. diabetic heart). In particular, the effects of perhexiline, an interesting drug known to possess not only metabolic effects (by virtue of inhibiting carnitine palmitoyl transferase-1 [CPT-1], thereby shifting myocardial fatty acid oxidation towards glycolysis) but also anti-inflammatory effects, will be further explored.

First, the pharmacokinetics and myocardial uptake profile of the individual perhexiline enantiomers were examined. This study showed that the myocardial uptake of both perhexiline enantiomers in patients were slow; and that in multivariate backward stepwise analysis, (-)-perhexiline was inversely correlated with on-treatment heart rate. This finding suggested that the weak calcium

antagonist effect of perhexiline may potentially lie predominantly within the (-)-enantiomer.

Additionally, other aspects of myocardial metabolism, including the nexus between inflammatory activation and metabolic effect, were investigated. In a study involving 12 patients presenting with acute coronary syndrome and hyperglycaemia, rapid reversal of hyperglycaemia with insulin infusion in 12 hours improved the anti-aggregatory effect of platelets, independent of the platelet content of the pro-inflammatory marker thioredoxin-interacting protein (TXNIP).

Furthermore, this thesis also investigated the potential insulin sensitization effect of perhexiline in diabetic patients. This is a corollary of increased glucose utilization, which appears to be relevant even against the background of concomitant therapy with other insulin-sensitizing agents such as AMPK activators or ACE-inhibitors. Furthermore, platelet content of TXNIP tended to fall slightly (but not significantly) after perhexiline treatment, implying its lack of significant critical role in the improvement of both nitric oxide responsiveness and insulin sensitization. However, its overall contribution still cannot be completely ruled out.

Lastly, in an in vitro experiment, the potency of inhibition of CPT-1 by both perhexiline enantiomers was investigated. It was found that the 50% inhibitory concentrations of both enantiomers were not significantly different. This provided evidence that the (differential) toxicity seen with the individual enantiomers (in previous studies) might be independent of CPT-1 inhibition. The CPT-1 inhibitory potency of several other cardiac drugs, including fluorinated perhexiline (developed by collaborators in Aberdeen, UK) and dronedarone (a benzofluranyl

compound, structurally similar to amiodarone) was also determined in this thesis, and it was shown in particular that dronedarone was a potent CPT-1 inhibitor.

The overall thrust of this work reinforces the concept that CPT-1 inhibition is seen with a large number of cardiovascular drugs, and is retained by enantiomers and structural analogues of perhexiline. The myocardial uptake of perhexiline and its enantiomers indicates a relatively slow process of equilibration with its primary sites of action.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Date: 28th April 2017

Signature:

Cher-Rin Chong

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"We dance for laughter, we dance for tears,
we dance for madness, we dance for fears,
we dance for hopes, we dance for screams.
We are the dancers, we create the dreams."

Albert Einstein

As a mindful wanderer and a faithful stargazer, it was beyond my own imagination that I would (or even could) begin the journey of a PhD. Admittedly, such wild dreams had been previously masked by clouds of fear and waves of insecurities. I am still puzzled, but in 2011 Professor John Horowitz (who later became one of my supervisors, by chance or by luck) discovered some of my deeply hidden "potentials". At the darkest of times when rejections mounted and self-doubts were unavoidable, he gave me a helping hand and led me towards a brighter path. Whether it was a gamble on his part or not, he took me on board, and assisted me in gaining entry into the program. Over the subsequent years, he injected me with infusions of caffeine and confidence that had guided me through countless failed experiments and “negative results”. He constantly patted me on my back, taught me how to learn from my mistakes, showed me how to earn one’s respect, and be persevering. Now that I am about to embark on another chapter of my life, a few layers of gastric mucosa have been shed and many invisible scars are freshly formed. But instead of being defeated by failures and negativities, I

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List of abbreviations

ACC	Acetyl-coA carboxylase
ADMA	Asymmetric dimethylarginine
AMPK	Adenosine monophosphate kinase
ATP	Adenosine triphosphate
CK	Creatine kinase
CPT-1	Carnitine palmitoyl transferase-1
DM	Diabetes mellitus
EF	Ejection fraction
EPC	Endothelial progenitor cells
FFA	Free fatty acid
GLUT	Glucose transporter
IC_{50}	50% inhibitory concentration
MCD	Malonyl-coA decarboxylase
MPO	Myeloperoxidase
MVO2	Myocardial oxygen consumption
NO	Nitric oxide
NOS	Nitric oxide synthase
$ONOO^-$	Peroxynitrite

PARP	Poly(ADP)-ribose polymerase
PCr	Phosphocreatine
PDH	Pyruvate dehydrogenase
PGC-1α	Peroxisome proliferator-activated receptor-γ co-activator 1α
Px	Perhexiline
UCP	Uncoupling protein
ROS	Reactive oxygen species
TSP-1	Thrombospondin-1
TXNIP	Thioredoxin-interacting protein

Chapter 1: Introduction

1.1 Myocardial energetics: physiological considerations

"…all living things must be continuously fed with energy…"

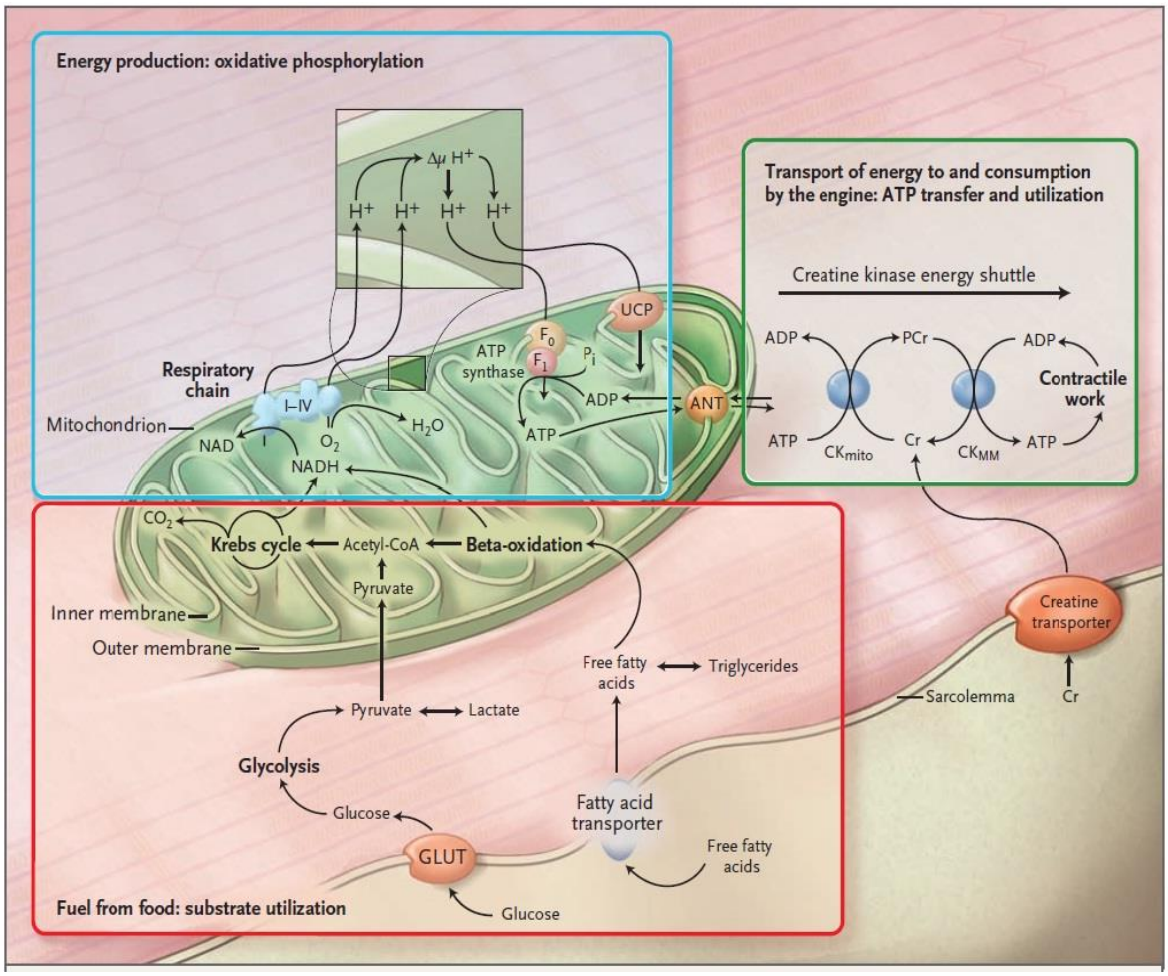
Hans Krebs, Nobel banquet speech, Stockholm, 1953.

The heart is a predominantly aerobic organ which relies heavily on the aerobic oxidation of substrates, such as glucose, fatty acids, lactate, and even some amino acids, to generate adenosine triphosphate (ATP), a major cellular source of energy. The process of substrate selection is dynamic and is dependent on fetal/adult status, fed/fasting cycles, normoxic/hypoxic condition, or (sudden increase in) myocardial workload demand. These dynamic processes are integrated to ensure that the myocardial contractile performance and housekeeping functions are maintained. Derangements in cardiac energy metabolism occur in many diseases, including angina, acute coronary syndrome, heart failure, cardiomyopathy, and aortic stenosis [1].

The process of cardiac energy metabolism is divided largely into three components (see Figure 1-I). The first process constitutes of cellular uptake of substrate (mainly free fatty acids and glucose), and subsequent production of intermediary metabolites for entry into the Krebs cycle, either via glycolysis of carbohydrates or β-oxidation of free fatty acids [1]. The second component is the oxidative phosphorylation of ADP by the mitochondrial respiratory chain into ATP. The third component is the ATP transport and utilization by myofibrils, via the creatine kinase energy shuttle [1]. The creatine kinase energy shuttle acts like an

energy buffer and includes the transfer by creatine kinase of a phosphate group from ATP to creatine to form phosphocreatine and ADP. Phosphocreatine then diffuses into the myofibrils, where the myofibrillar creatine kinase catalyses conversion of phosphocreatine back into creatine and ATP. The free creatine then diffuses back into the mitochondria [1]. During conditions when myocardial energy demand outweighs its supply, the level of phosphocreatine decreases to keep ATP at a constant level, but the free ADP level rises. The raised free ADP then inhibits the function of many cellular enzymes leading to impairment of cardiac contractility. Therefore even when ATP level remains unchanged, metabolic derangement still occurs when phosphocreatine level falls and ADP rises [1,2].

Figure 1-I: Physiology of myocardial metabolism in relation to high energy phosphate generation.



Adapted from reference [1].

Abbreviations: GLUT = glucose transporter; NAD = nicotinamide adenine dinucleotide ; NADH = nicotinamide adenine dinucleotide ; ANT = adenine nucleotide translocator ; PCr = phosphocreatine; Cr = creatine; CKmm = myofibrillar creatine kinase isoenzyme; CKmito = mitochondrial creatine kinase isoenzyme.

1.1.1 Metabolic pathways responsible for ATP generation

1.1.1.1 Extra-mitochondrial

The generation of ATP is a series of complex oxidation-reduction reactions occurring within the cell. Such reactions require several co-enzymes, including nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and flavin adenine dinucleotide (FAD).

Generation of ATP in the cytoplasm is minimal, contributing to <2% of the total ATP generated [3]. Cytoplasmic generation is predominantly from glycolysis, which involves ten enzyme-mediated steps to breakdown six-carbon monosaccharides (e.g. glucose, fructose) into two molecules of pyruvate, two molecules of ATP, two molecules of NADH, two molecules of water, and two hydrogen ions [4].

1.1.1.2 Intra-mitochondrial

1.1.1.2.1 Citric acid cycle: Carbohydrate metabolism

Upon entry into the mitochondria matrix, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase, before entering into the citric acid cycle (also known as tricarboxylic acid cycle or Krebs' cycle). This cycle produces all the intermediaries that are required for the subsequent

generation of ATP. They include one molecule of $FADH_2$, three molecules of NADH, and one molecule of guanosine triphosphate (see [5] for review).

The subsequent metabolic pathway, oxidative phosphorylation, occurs exclusively within the inner mitochondrial membrane, utilizing the differences in product concentrations from the citric acid cycle to form ATP [6]. Such process involve the respiratory chain (which includes four complexes: complexes I-IV and a Q protein to transport H^+ from the inner mitochondria to the intermembrane space) and ATP synthesis via ATP synthase [5,6].

Glucose and lactate supply 10 to 40% of the energy requirement of the heart under normoxic conditions. Lactate, readily extracted from the blood, can be converted to pyruvate in the cytosol and further metabolized to acetyl-CoA for ATP generation [5,6].

1.1.1.2.2 Long-chain fatty acids

Fatty acids supply about 60-90% of the energy requirement for the heart and are oxidized in the mitochondria. Upon entry into the cytoplasm, either via passive diffusion or uptake by fatty acid translocase or fatty acid binding protein (FABP), the non-esterified fatty acids are then esterified to fatty acyl-CoA by fatty acyl-CoA synthase [7]. Following this, fatty acids with less than 12 carbons in length can cross the mitochondrial membranes readily. However, saturated fatty acids longer than 12 carbons are transported via the carnitine shuttle [4].

Carnitine palmitoyltransferase-1 (CPT-1), located at the outer mitochondrial membrane, is responsible for catalyzing the metabolism of water-soluble fatty acyl-CoA to lipid-soluble fatty acylcarnitine in the compartment between the inner and outer mitochondrial membranes [5,6]. Then this long-chain fatty acylcarnitine is transported by carnitine acyltranslocase across the inner mitochondrial membrane in exchange for free carnitine. Finally, carnitine palmitoyltransferase-2 (CPT-2) regenerates the long-chain fatty acyl-CoA in the mitochondrial matrix. Out of the three enzymes involved in the “carnitine shuttle”, CPT-1 is the rate-limiting enzyme in controlling fatty acid uptake into the mitochondria [5,6].

Once inside the mitochondria, fatty acid breakdown continues via β-oxidation, a four-step reaction with specific enzymes involved in each stage: acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. The reactions, removing 2 carbons each cycle, continue until the entire fatty acid chain is degraded into acetyl-CoA, which then enters the citric acid cycle (see section above) to generate ATP [5,6].

Very long chain fatty acids (> 22 carbons), cannot be broken down by the mitochondria. They are broken down by peroxisomes in the cytoplasm into medium or short chain fatty acids, before entering β-oxidation and undergoing degradations as described above [4].

1.1.2 Interactions between pathways (Randle Cycle): checks and balances

The flux between fuel utilization by mammalian organs is governed by the "Randle cycle", a biochemical process described by Philip Randle, Peter Garland, Nick Hales and Eric Newsholme in 1963 [8]. This process described a reciprocal and non-dependent metabolic relationship between glucose and fatty acids: that the utilization of one nutrient inhibited the uptake of another directly and without hormonal mediation. Such dynamic adaptation to nutrient availability applies to the interaction between adipose tissue and muscle tissue.

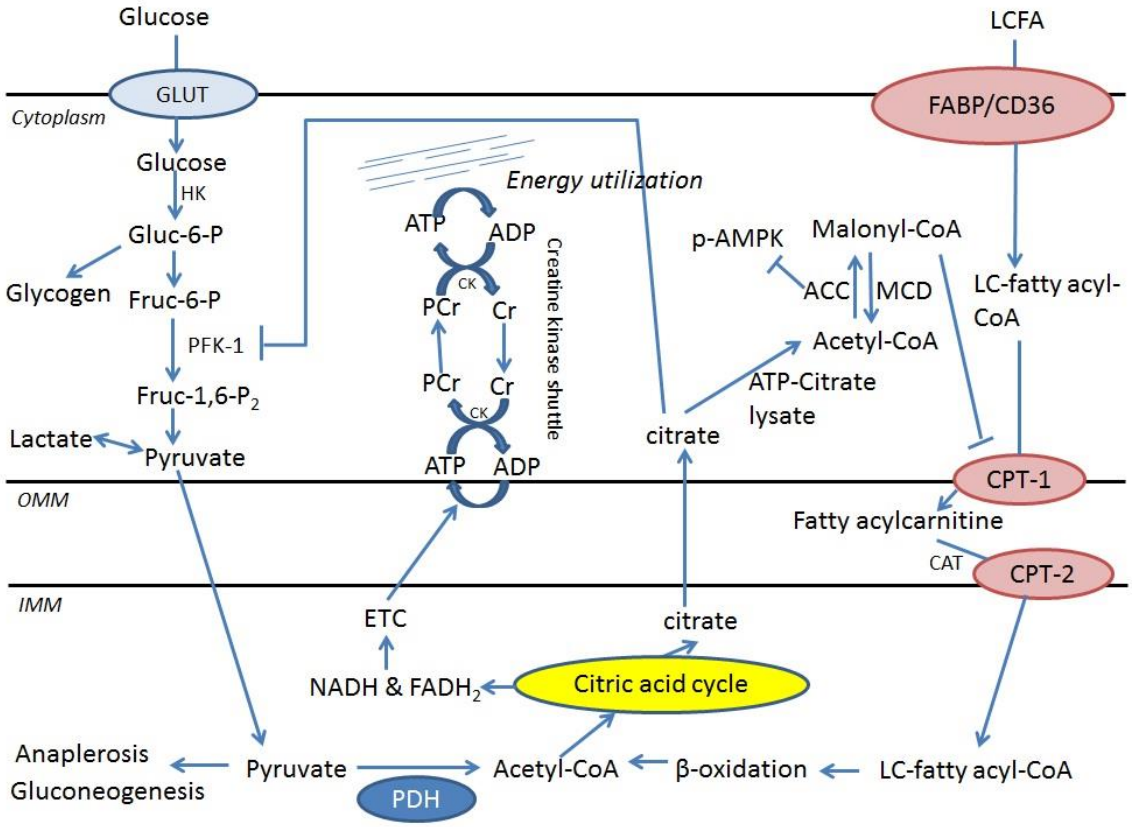
As components of the Randle cycle, fatty acid utilization inhibits glucose metabolism by inhibiting several glycolytic steps, most significantly at the level of pyruvate dehydrogenase (PDH). Oxidation of fatty acid triggers an initial increase in the mitochondrial ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD+], both of which will inhibit the activity of PDH complex by activating PDH kinase causing phosphorylation. Such changes then lead to the accumulation of cytosolic citrate, which inhibits phosphofructokinase-1 (PFK-1), leading to an increase in glucose-6-phosphate, which inhibits hexokinase eventually [5,6] (see Figure 1-II).

Conversely, the rate-limiting enzyme of long-chain fatty acid oxidation, CPT-1, is strongly inhibited by malonyl-CoA, a key physiological regulator of fatty acid oxidation. An increase in malonyl-CoA suppresses fatty acid oxidation by inhibiting CPT-1 and the "carnitine shuttle"; and a reduction in malonyl-CoA increases fatty acid uptake and oxidation. Malonyl-CoA is formed from the carboxylation of extramitochondrial acetyl-CoA by acetyl-CoA carboxylase (ACC), and is degraded by malonyl-CoA dehydrogenase (MCD) [5,6]). Extramitochondrial acetyl-CoA used to form malonyl-CoA is thought to be derived from citrate via the

ATP-citrate lysate reaction (see Figure 1-II). The activity of ACC is under the regulation of AMP-activated protein kinase (AMPK), which will be discussed later in this chapter. Inhibition of fatty acid oxidation, for example associated with malonyl-CoA accumulation, is known to increase glucose and lactate uptake and oxidation. This is achieved by decreasing citrate levels and inhibition of PFK-1 and by lowering acetyl-CoA and/or NADH levels in the mitochondrial matrix, thereby reversing inhibition of PDH (Figure 1-II).

The ATP yield and oxygen requirement for mitochondrial oxidative phosphorylation differs for the different metabolic substrates. Fatty acid oxidation yields more ATP, but requires more oxygen per ATP production, and do not yield ATP under oxygen-independent conditions. Therefore, during ischaemic conditions, promotion to glucose oxidation would be more "energy efficient", as the process produces more ATP per oxygen consumption [9].

Figure 1-II: Regulation of mitochondrial nutrient oxidation: a summary of the "Randle cycle"



Abbreviations: GLUT = glucose transporter; HK = hexokinase; Gluc-6-P = glucose-6-phosphate; Fruc-6-P = fructose-6-phosphate; PFK-1 = phosphofructokinase-1; OMM = outer mitochondrial membrane; IMM = innter mitochondrial membrane; PDH = pyruvate dehydrogenase; LCFA = long chain fatty acid; CPT-1(/2) = carnitine palmitoyl transferase-1(/2); CAT = carnitine acyltranslocase; ACC = acetyl-coA carboxylase; MCD = malonyl-CoA decarboxylase; p-AMPK = phosphorylated AMP-activated protein kinase; ETC = electron transport chain; ADP = adenosine diphosphate; ATP = adenosine triphosphate; PCr = phosphocreatine; Cr = creatine.

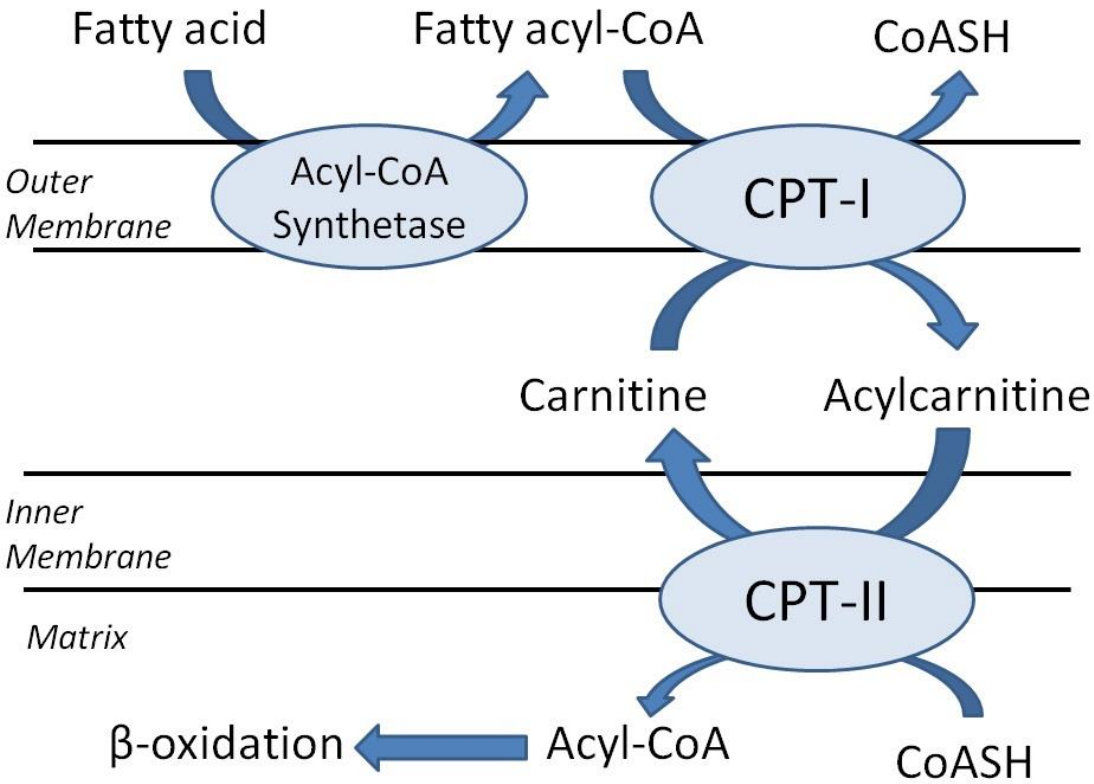
1.1.2.1 Physiology of carnitine palmitoyl transferase-1

The conceptual framework of how carnitine controls the oxidation of long-chain fatty acids by mammalian tissues first arose from studies in the 1950s to 1960s [10-12]. These studies provided preliminary evidence showing that carnitine enables long-chain fatty acids to be esterified in the extramitochondrial compartment, in order to overcome the permeability barrier and gain access to the inner membrane (see Figure 1-III). Despite the elegance of this proposed mechanism, little attention was received until in the mid-1970s. At that stage, it was apparent that CPT-1 activity regulation plays a central role in cellular metabolic homeostasis. Furthermore, it was found that genetic defects at the level of carnitine palmitoyl transferase (CPT) system form the basis of serious human disease which lead to recurrent myoglobulinuria [13] and that CPT also plays an important role in the liver regulating fatty acid oxidation [14].

During their investigations into how liver accelerates production of ketone bodies in ketotic conditions such as starvation or uncontrolled diabetes, McGarry and Foster [15] hypothesised that a metabolite from glucose oxidation is responsible for regulating fatty acid biosynthesis and degradation. After screening multiple intermediaries of the glycolytic pathway, the pentose phosphate pathway and the tricarboxylic acid cycle, of the ability of such intermediate to inhibit oxidation of oleate in rat liver homogenates, the authors found that malonyl-CoA (to be discussed in detail in Chapter 1.1.2.3) inhibited oxidation of oleate powerfully and this effect was reversible [15,16].

Although the authors also observed an inhibitory effect of malonyl-CoA on fatty acid oxidation in rat heart and kidney, it was thought at that time that malonyl-CoA could not be present at inhibitory concentration as neither tissues had a cytosolic fatty acid synthesis pathway [15,16]. However, it was later found that non-hepatic tissues, such as heart and skeletal muscle, also contain acetyl-CoA carboxylase (ACC: to be discussed in more detailed later) which is responsible for the synthesis of malonyl-CoA from acetyl-CoA [17-19]. This implied that the malonyl-CoA/CPT-1 pathway constituted a potentially important element of the fuel cross-talk in other non-hepatic tissues.

Figure 1-III: The mitochondrial CPT system.



Abbreviations: CPT-I/II = Carnitine palmitoyl transferase-I or II.

1.1.2.2 CPT-1 isoforms

Considerations that skeletal muscle and hepatic CPT-1 are different from each other arise from the observation that the inhibitory effects of malonyl-CoA on rat CPT-1 varies with the tissue source [20]. Specifically, the IC_{50} of malonyl-CoA in liver was ~3 uM as compared to ~ 0.03 uM in skeletal muscle, and the Km values for carnitine were ~ 30 uM and 500 uM, respectively [20]. Additionally, it was found that when labelled with radioactive triglyceride-CoA [21] or etomoxir-CoA [22], there were two proteins with sizes of approximately 88kDa (L: liver) and 82 kDa (M: muscle) that migrated onto SDS/polyacrylamide gels. These two isoforms of CPT-1 were later confirmed to exist in humans using genomic cloning methods [23,24]; and that both isoforms also exist in various other tissues. In particular, in rat heart mitochondria, CPT-1 displayed IC_{50} of malonyl-CoA and Km for carnitine to be intermediate between that of liver and skeletal muscle [20]. Rat heart tissue expresses M-CPT-1 predominantly, but it also contains sufficient L-CPT-1 to account for its overall responsiveness to malonyl-CoA [22,25]. Importantly, both isoforms are found in the human heart [26].

1.1.2.3 Malonyl-CoA: the endogenous inhibitor of CPT-1

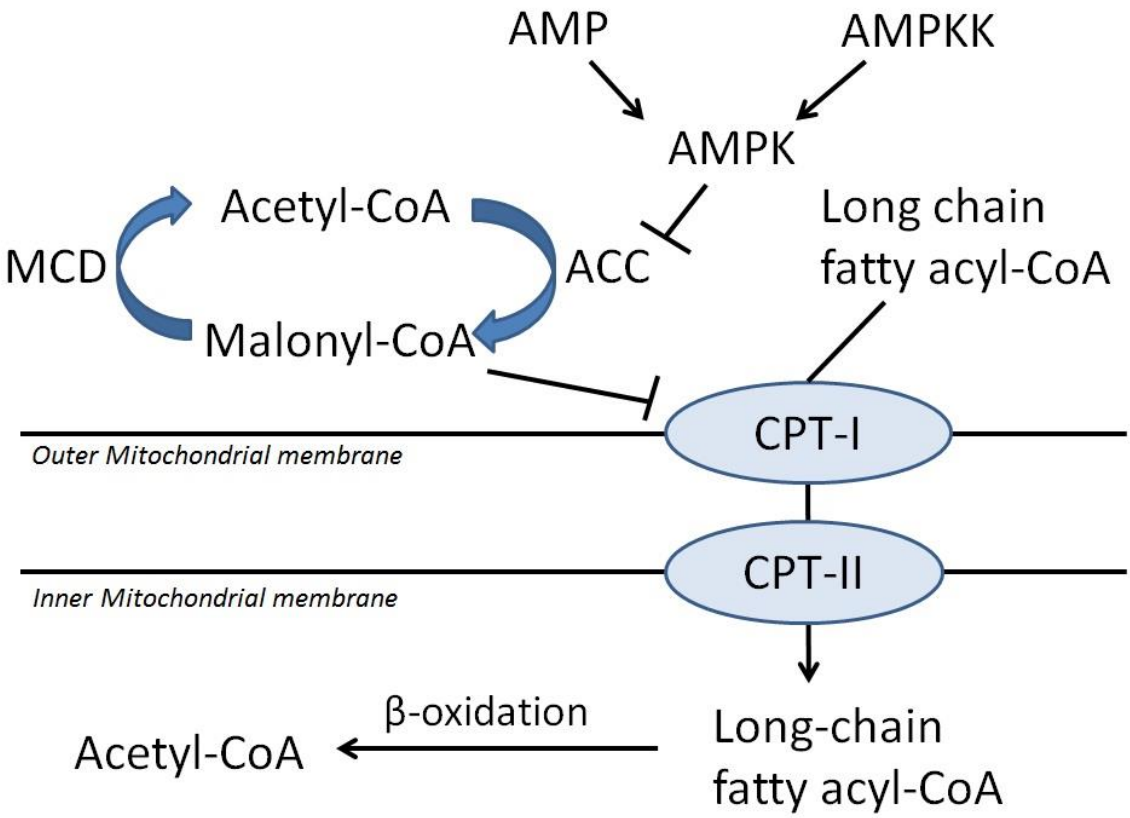
Malonyl-CoA is an endogenous inhibitor of mitrochondrial CPT-I, thus providing a potential limit to the rates of oxidation of fatty acid [27] irrespective of synthetic rates. It has a rapid turnover, with half-life of approximately 1.25 minutes [27]. Therefore, both the production and degradation of malonyl-CoA are important determinants of malonyl-CoA levels and thus of subsequent fatty acid oxidation. Consistent with this, a reduction in malonyl-CoA level increased the uptake and oxidation of fatty acids [28-30]; whereas an increase in malonyl-CoA suppressed fatty acid oxidation [31,32].

Malonyl-CoA is synthesized via carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC) (Figure 1-IV). There are two isoforms of ACC in the myocardium: the α and the β-isoforms, with the latter being the predominant isoform in the heart [17]. It has been suggested that malonyl-CoA produced by the β-isoform of ACC is more involved in the regulation of fatty acid oxidation [31]. This role contrasts to that of the high abundance of ACC-α in the liver, which produces malonyl-CoA primarily for the regulation of fatty acid biosynthesis [33]. The activity of ACC is also postulated by phosphorylation or dephosphorylation of AMPK (described in Chapter 1 section 1.2.3.1), which has a key role in the cardiac energy metabolism (see Figure 1-IV).

Malonyl-CoA is degraded via decarboxylation by malonyl-CoA decarboxylase (MCD). Previous studies showed that inhibition of MCD led to an increase in malonyl-CoA levels, which then limited rates of fatty acid

oxidation and caused a secondary increase in glucose oxidation [34,35]. This activity of MCD represents a potential therapeutic target. Additionally, the expression of MCD has also been shown to be regulated by peroxisome proliferator activated receptor alpha (PPARα), a transcription factor involved in fatty acid oxidation. Activation of PPARα increased MCD expression, accompanied by increase in fatty acid oxidation [36,37].

Figure 1-IV: Regulation of malonyl-CoA in the aerobic heart.



Malonyl-CoA is synthesized via carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC), and degraded via decarboxylation by malonyl-CoA decarboxylase (MCD). ACC is negatively regulated via phosphorylation by AMPK. Increased malonyl-CoA production inhibits fatty acid oxidation. During stressful conditions when ATP is low (e.g. ischaemia), adenosine monophosphate (AMP) and AMPK kinase (AMPKK) are increased and activated, leading to a very rapid activation of AMPK. This then inhibits ACC, which caused a rapid fall in malonyl-CoA levels and increased fatty acid oxidation [38].

1.1.3 ATP transport between nucleus and cytoplasm

Despite a huge fluctuation in the demand for ATP, the human heart maintains a relatively constant cytosolic ATP concentration at 10 mM [39]. This is achieved by matching the rate of ATP synthesis with that of ATP utilization (by myosin, ion pumps, synthesis and degradation of large or small molecules). The reversible interconversion of creatine into phosphocreatine is controlled by creatine kinase (CK), a central controller of cellular energy homeostasis.

In principle, the phosphocreatine-CK shuttle serves as an “energy shuttle”, providing bridges between the sites of ATP generation and ATP consumption. This is largely because the velocity of the CK reaction is approximately ten times faster than the velocity of oxidative phosphorylation (to synthesize ATP) [40]. There are two isoforms of CK, the dimeric cytosolic CK and the mostly octameric mitochondrial CK localized in cristae and intermembrane space. Both isoenzymes contribute to the build-up of a large intracellular pool of phosphocreatine [40]. Phosphocreatine serves as an energy buffer that rapidly donates its phosphorus group to ADP in order to maintain a relatively constant global ATP concentration.

Creatine and phosphocreatine accumulate, in the excitable tissues via the Na^+/Cl^- transporters. The creatine transporter in the sarcolemma then moves creatine against a concentration gradient into the tissue [40]. Creatine is either extracted directly from diet or synthesized from a two-step biosynthesis pathway occurring in liver, kiney or pancreas, that involves arginine:glycine amidinotransferase and guanidineoacetate methyltransferase. Interestingly, in a study of the development of creatine transport in rats, both of these enzymes are not detected in the heart [41].

The other function of CK is based on its subcellular compartmentation. Such compartmentation of CK isoenzymes allows it to supply ATP from PCr during acute increase of energy demand. Such ATP synthesis not only functions to maintain a constant supply of ATP, but also functions to maintain a high ratio of ATP relative to its hydrolysis products, namely ADP and phosphate. This ratio, expressed as the maximal ΔG (change in Gibbs free energy) for the free energy of ATP hydrolysis, is the chemical driving force of all ATP-requiring reactions [40]. A low concentration ratio of ATP to ADP will therefore stimulate mitochondrial oxidative phosphorylation.

1.1.4 Modulation of mitochondrial function and energetics

1.1.4.1 Role of uncoupling proteins

Uncoupling proteins (UCP) were first identified in 1976 in brown adipose tissue (now also found in white adipose tissue and termed UCP-1) [42]. They are members of the mitochondrial anion carrier family located in the mitochondrial inner membrane, and are thought to play a role in dissipitating the proton electrochemical gradient by transferring proton ions generated during oxidative phosphorylation back into the mitochondrial matrix (Figure 1-V). Uncoupling proteins all share the property of dissociating metabolism from high energy phosphate generation, and thus act as “energy” sinks (see Chapter 1.2.2).

During oxidative phosphorylation, protons are being pumped out from the inner mitochondrial matrix into the intermembrane space by complexes I, III and IV of the electron transport chain. This establishes a proton motive force (Δp) across the inner membrane. The re-entry of protons into the mitochondrial matrix through ATP synthase (or complex V) couples the release of Δp to ATP synthesis. All other forms of proton re-entry, including through the uncoupling proteins or adenine nucleotide translocase are considered to be “proton leak” [43].

Activation of proton leak through UCP is thought to be controlled by reactive oxygen species (ROS) (especially superoxide and perhydroxyl radical HO_2). There is also recent evidence that glutathionylation acts as a “switch” for the activation of UCP, especially UCP-2 and UCP-3. In particular,

low dose of ROS leads to deglutathionylation and proton leak; whereas prolonged high level of ROS deactivate UCP by reglutathionylation, potentially to avoid the collapse of the mitochondrial membrane potential and induction of cell death (see Figure 1-V B) [44].

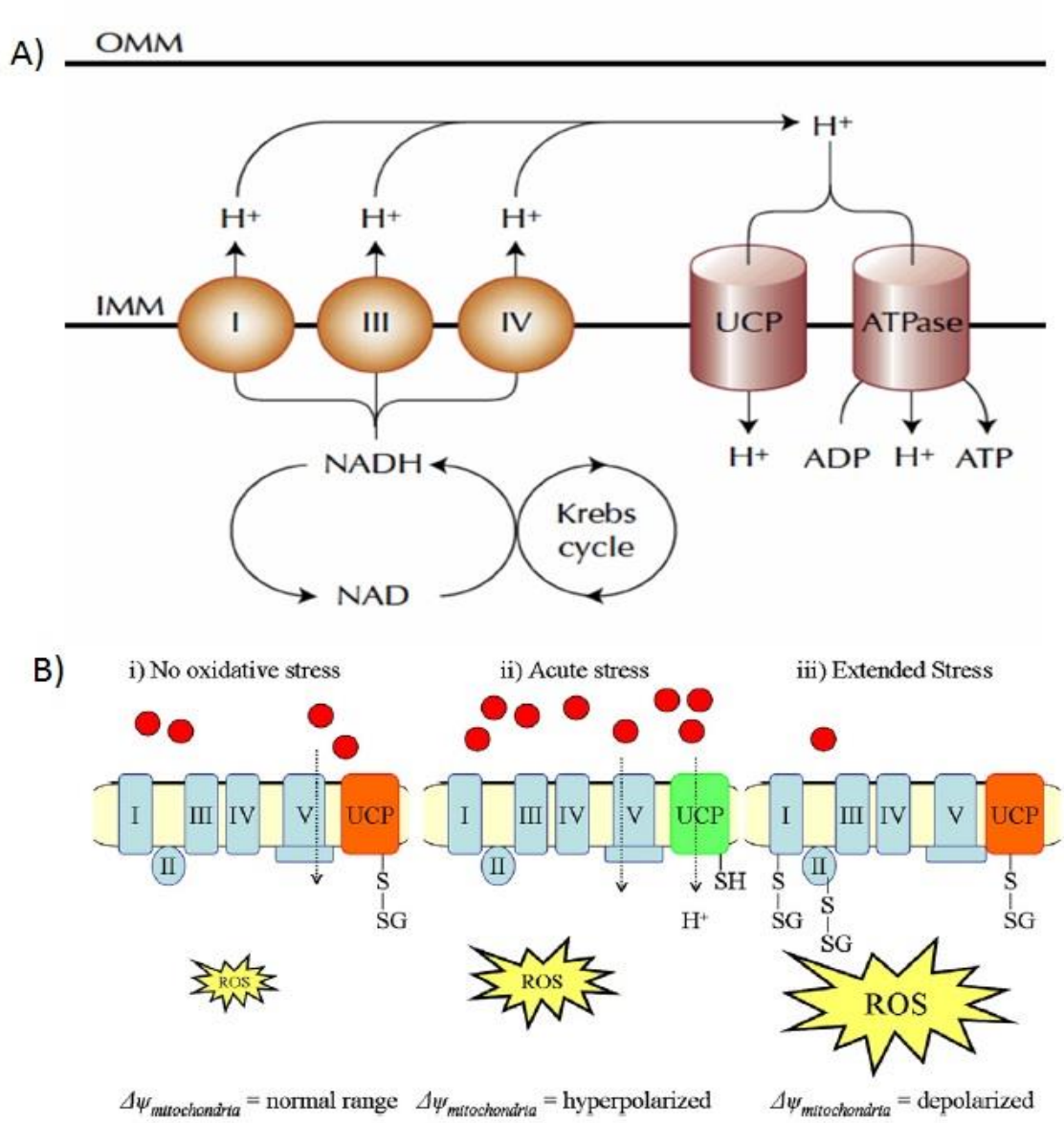
UCP-2 and UCP-3 are structural homologs of UCP-1. While much debate occurs regarding the physiological roles of the UCPs, it is known that UCP-1 is responsible for heat generation; while UCP-2 and UCP-3 are not associated with non-shivering thermogenesis. In regards to location, there is also some controversy of whether or not UCP-2 is expressed in heart (this may be species-specific), but UCP-3 is known to be expressed in the inner mitochondrial membrane of the heart's skeletal muscle.

UCP-3, in particular, is thought to protect the cells from oxidative stress injury. It mitigates reactive oxygen species release from the electron transport chain, and this may involve the glutathionylation of the protein. For example, in UCP-3 knock-out mice, the production of reactive oxygen species from harvested isolated skeletal mitochondria is significantly higher compared to that from wild-type animals [45]. Additionally, there is also evidence that UCP may participate in ischaemic preconditioning: the levels of UCP (both mRNA and protein expressions) are inversely correlated with infarct size [46]; and UCP may also limit atherosclerotic plaque formation by reducing the formation of reactive oxygen species [47].

In 2004, Murray and colleagues found a positive correlation between the concentration of circulating free fatty acids and the content of cardiac mitochondrial UCPs (UCP-2 and UCP-3) from tissue samples of patients undergoing coronary artery bypass graft surgery [48]. Such a relationship

does not explain whether it is beneficial or harmful (as a maladaptive response), nor does it delineate mechanisms of the demonstrated association.

Figure 1-V: Mitochondrial metabolism and the role of uncoupling proteins



Adapted from references [49,50].

A) Protons generated during oxidative phosphorylation and electron transport chain are “leaked” back into the mitochondrial matrix through uncoupling protein. B) Hypothetical scheme of the regulation of ROS emission as controlled by glutathionylation/deglutathionylation state of uncoupling proteins. (Red round dots represent protons). During physiological conditions (i), minimal ROS is generated. Slight increase in ROS leads to uncoupling protein deglutathionylation (ii) and

extended periods of oxidative stress lead to uncoupling protein re-glutathionylation.

Abbreviations: OMM = outer mitochondrial membrane; IMM = inner mitochondrial membrane; I, II, III, IV, V = mitochondrial complex I-V; UCP = uncoupling proteins; ROS = reactive oxygen species.

1.1.4.2 Mitochondrial Permeability Transition Pore and effects of oxidative stress with mitochondria

Apart from energy production, mitochondria also participate in Ca^{2+} signaling, production of reactive oxygen species, cross-talk with other organelles, biogenesis, fusion or fission, and orchestration with other cell death modalities.

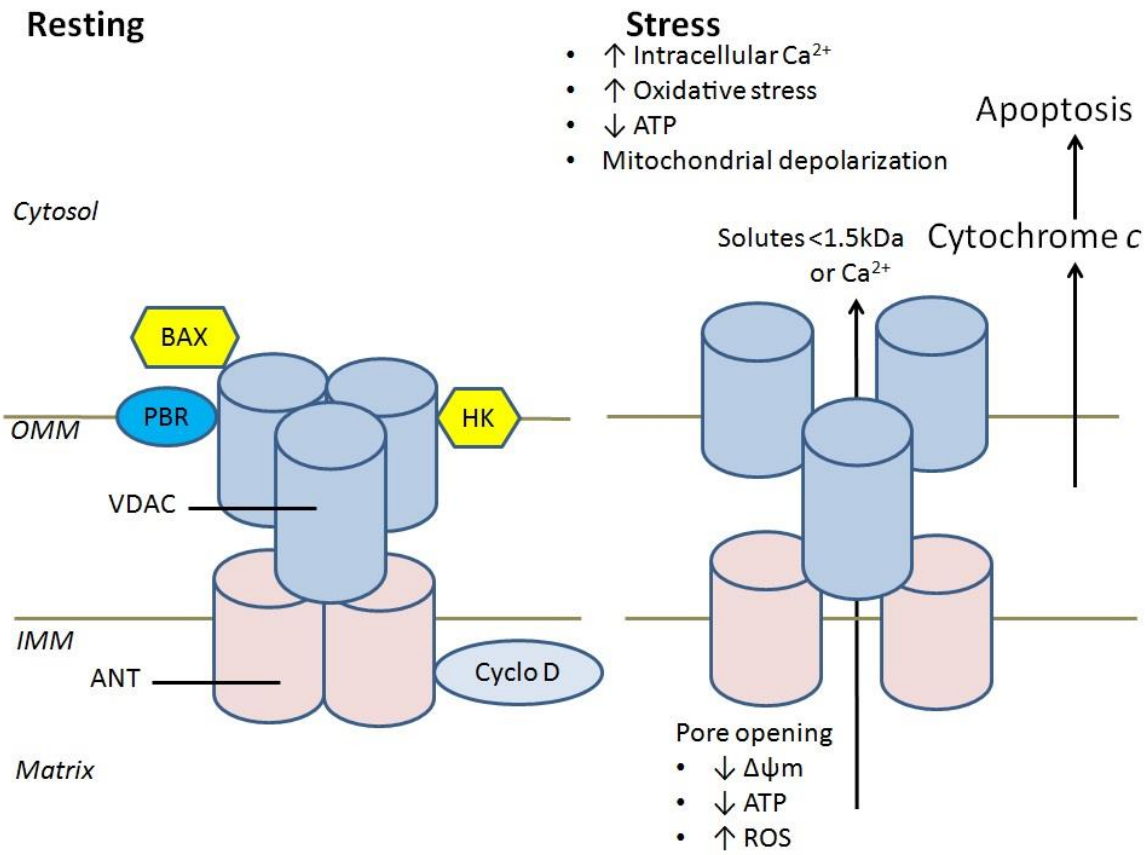
Oxidative stress, together with calcium overload and ATP depletion, lead to the opening of mitochondrial permeability transition pore (mPTP), with high conductance channels (see Figure 1-VI). Three key proteins are generally accepted as the key structural components of the mPTP: the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane, the mitochondrial cyclophilin D (cyclo D) in the mitochondrial matrix, and the voltage-gated anion channel (VDAC) in the outer mitochondrial membrane [51]. Other proteins, such as the peripheral benzodiazepine receptor (PBR), hexokinase (HK) and creatine kinase (CK), might also be associated with the mPTP.

Although the physiological role of mPTP is incompletely understood, it is centrally involved in mitochondrial dysfunction. Under conditions such as increased intracellular calcium, high oxidative stress, ATP depletion, and mitochondrial depolarization, the complex forms an open pore, resulting in influx or increased permeability of solutes with molecular masses up to 1.5 kDa. This eventually leads to mitochondrial matrix swelling, membrane rupture and release of pro-apoptotic or pro-inflammatory proteins such as procaspases and cytochrome c into the cytoplasm [52].

During myocardial ischaemia, the accumulation of lactic acid inducing an acidic environment exerts a strong inhibitory effect on mPTP opening, despite an accumulation of intracellular calcium and oxidative stress mediators. During reperfusion, the rapid washout of lactic acid, restoration of cellular pH in the presence of increased reactive oxygen species release and re-activation of ion-transporters lead to the opening of mPTP, contributing to ischaemia-reperfusion injury [53].

While investigating the mechanism of nephrotoxicity caused by cyclosporine on mitochondria, Crompton and colleagues found that cyclosporine inhibited the opening of mPTP and reduced calcium efflux [54]. Since then, many studies used cyclosporine as an investigative tool, and continued to investigate the pharmacological modulation of mPTP on various areas. This include the reduction of ischaemic-reperfusion injury [53], restoration of spontaneous circulation post cardiac arrest [55,56] or reduction of peri-operative myocardial injury [57].

Figure 1-VI The mitochondrial permeability transition pore: modulators of opening.



Modified and adapted from reference [58]. The opening of the mPTP ultimately results in mitochondrial swelling, mitochondrial Ca^{2+} efflux and the release of apoptotic proteins, such as cytochrome c and procaspases.

Abbreviations: $\Delta\psi m$ = mitochondrial membrane potential; BAX = BCL2-associated X protein; ROS = reactive oxygen species; PBR = peripheral benzodiazepine receptor; VDAC = voltage-dependent anion channel; HK = hexokinase; ANT= adenine nucleotide translocator; Cyclo D = cyclophilin D.

1.2 Disordered myocardial energetics and cardiovascular disease states

1.2.1 Redox stress and the mitochondria

Cytoplasmic generation of reactive oxygen species (ROS) reflects activity of NADPH oxidase, xanthine oxidase, uncoupled (endothelial) nitric oxide synthase, lipoxygenase and cytochrome P450 monoxygenase. However, mitochondrial oxidative phosphorylation accounts for substantial ROS generation [59,60]. Monocytes and neutrophils also use myeloperoxidases to produce ROS, which affect neighbouring cells upon their release. The range of ROS includes the hydroxyl radical ($OH^{\bullet}$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl^-$) [60]. Furthermore, ROS frequently generate reactive nitrogen species (RNS): nitric oxide ($NO^{\bullet}$) reacts particularly with O_2^- to generate peroxynitrite ($ONOO^-$), which is relatively cell-permeable, and causes lipid peroxidation and protein modification [59].

Under physiological conditions, ROS/RNS production is generally counterbalanced by clearance via the cellular antioxidant defense systems. The usually resultant low steady-state level of ROS/RNS serves to exert physiological functions, including acting as signaling molecules involving in cell growth, host defence, inflammation or oxygen sensing [59,61]. In particular, low levels of H_2O_2 are known to activate elements of protective signaling pathways, such as phosphatidylinositol-3-kinase, protein kinase C, or mitogen activated protein kinase [59]. However, under many pathological conditions (such as aging, inflammation or cellular death) a shift in equilibrium occurs favoring the

accumulation of ROS/RNS and causes nonspecific oxidative/nitrosative damage to DNA, proteins, lipids or other macromolecules [59].

There are many studies suggesting a potential association between the level of ROS/RNS and the integrity of mitochondrial function. Under physiological settings, mitochondria undergo continuous changes in morphology and size, including fusion and fission [62]. A healthy balance between mitochondrial fusion (joining ≥ 2 mitochondria to form one mitochondrion) and mitochondrial fission (splitting one mitochondrion into ≥ 2 mitochondria) is required to maintain overall cellular homeostasis [63]. Although this may be cell or tissue-dependent, several of the core proteins (for example, dynamin related protein-1, or Drp1) and participating proteins that are involved in the mitochondrial fusion/fission process contain redox-sensitive motifs, therefore subjecting themselves to potential post-translational S-nitrosylation or nitration [63], with consequent changes in functional status.

1.2.2 Role of energetic "sinks"

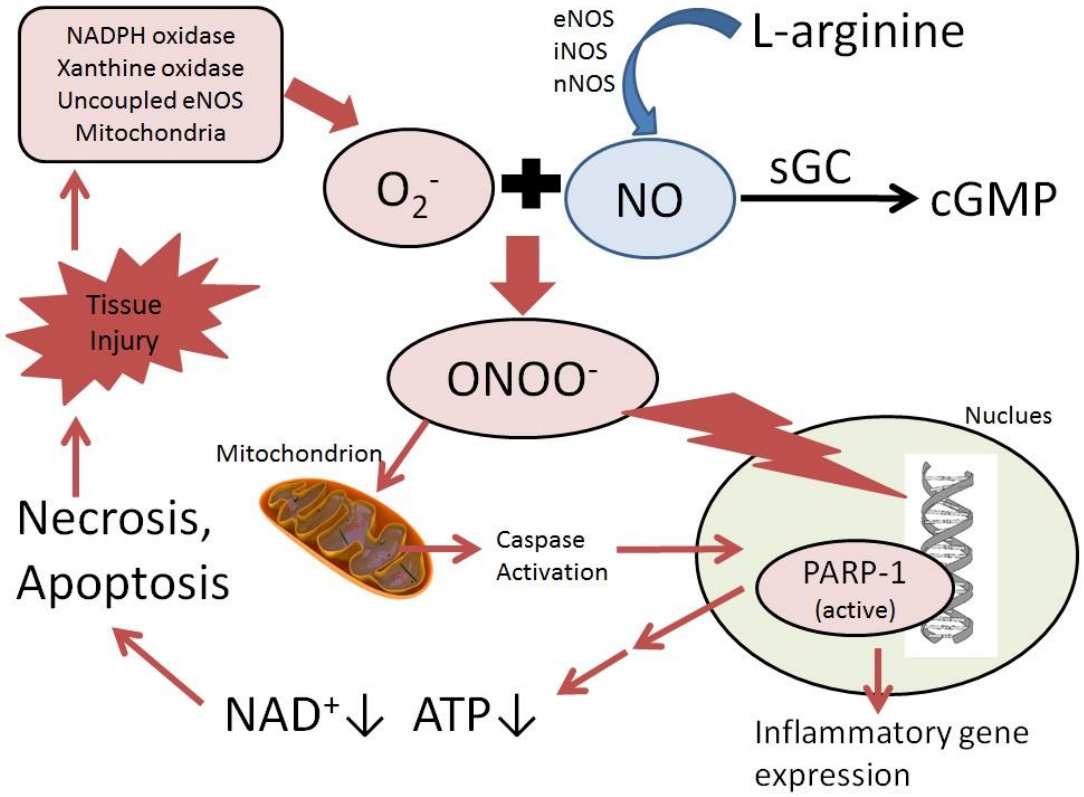
The physiological roles of the various uncoupling proteins have been described in Chapter 1.1.4.1: these represent significant mechanisms for control of high energy phosphate generation. Recently attention has been directed to the role of poly(ADP-ribose) polymerase (PARP).

Peroxynitritre (generated from the reaction between nitric oxide and superoxide) is a key trigger for DNA single strand breakage and the subsequent poly(ADP-ribose) polymerase (PARP) activation. Overactivation of PARP during stressful condition will lead to the depletion of its substrate NAD^+, slowing the rate of glycolysis, ATP formation and eventual cell death (see Figure 1-VII) [61].

PARPs are predominantly nuclear enzymes that are involved in cellular stress responses, both as sensors of cellular damage and as participants in stress response. Human PARPs comprise of a family of 17 enzymes that share common catalytic domain, but the majority (85-90%) of these is PARP-1, and the remainder is predominantly PARP-2. Activated PARPs cleave NAD^+ into nicotinamide and ADP-ribose [64]. This process depletes NAD^+ to 10-20% of its normal levels within minutes upon DNA damage, forces the cell to synthesize NAD^+ using salvage pathways in attempt to maintain viability. These salvage pathways, however, are ATP-consuming. Therefore, persistent PARP activation will ultimately result in ATP depletion, creating a "vicious" feedback loop that jeopardizes cell survival [64]. Additionally, reduced NAD^+ will also impair glycolysis, where NAD^+ is a major cofactor. This leads to reduced glycolysis-derived metabolite that feeds into the mitochondrial tricarboxylic acid cycle, further compromising overall cellular energetic balance [65].

In animal models, PARP-1$^{-/-}$ and PARP-2$^{-/-}$ animals displayed enhanced energy expenditure, increased hypoglycaemic response to insulin, altered mitochondrial mRNA expression of genes involved in respiration, uncoupling and fatty acid oxidation, but also protection against age- and high fat diet-induced body weight gain [66-68]. Perhaps more excitingly, PARP-1$^{-/-}$ mice were resistant to the development of streptozocin-induced diabetes while maintaining normal pancreatic insulin content and islet cell morphology [69].

Figure 1-VII: The nitric oxide-peroxynitrite-PARP pathway in health and in disease states.



Under physiological conditions, nitric oxide activates soluble guanylate cyclase and produces cyclic GMP, which exerts a range of cardioprotective effects. However, during pathological states, over-activation of PARP leads to depletion of NAD^+ and ATP.

Abbreviations: sGC = soluble guanylate cyclase; cGMP = cyclic GMP.

1.2.3 Role of energy sensors

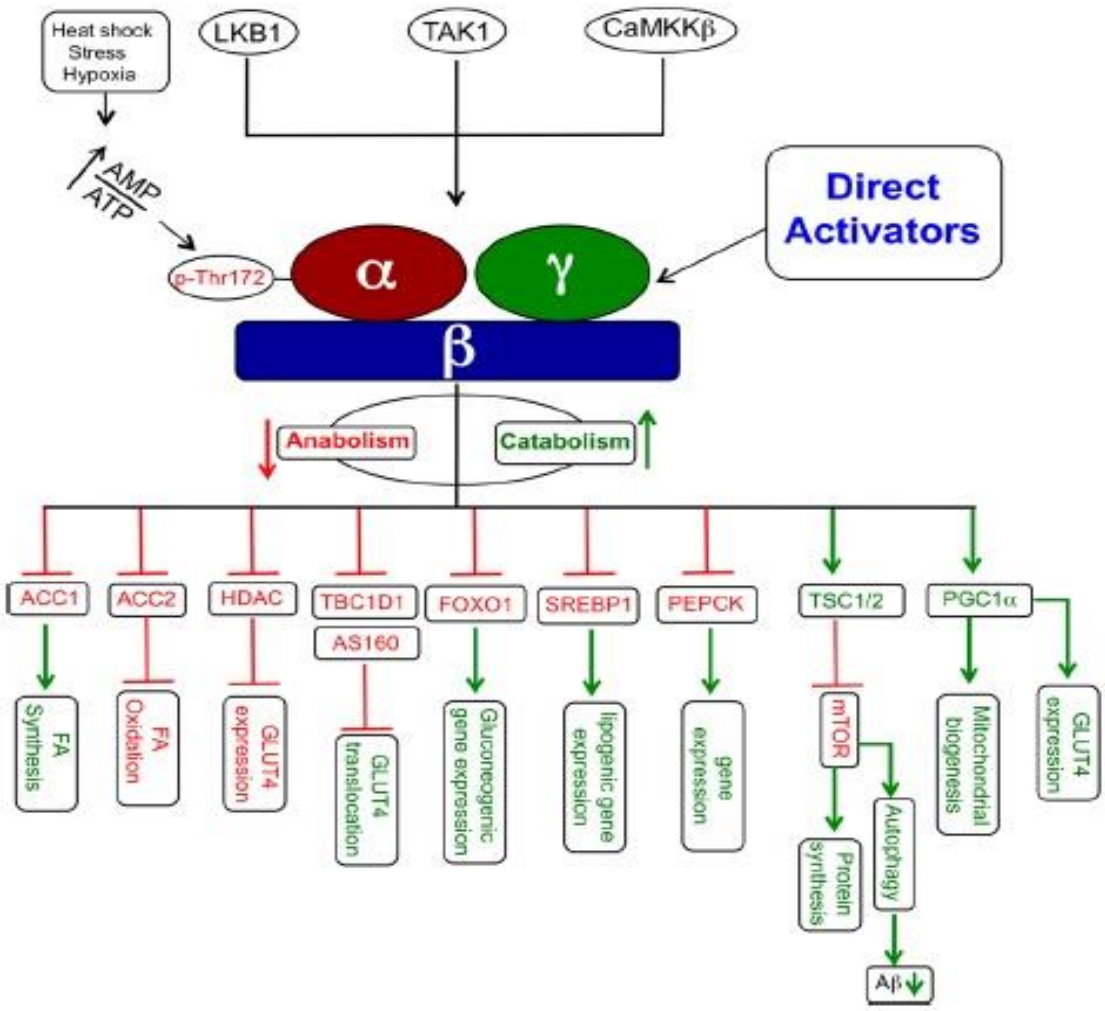
1.2.3.1 AMP-activated protein kinase

The adenosine 5'-monophosphate activated protein kinase (AMPK) is a heterodimeric complex containing catalytic α subunits and regulatory β and γ subunits [70]. It is activated by an increase in the AMP: ATP or ADP: ATP ratios, which occur during metabolically stressful conditions. Its main role, upon activation, is to restore metabolic homeostasis by inhibiting ATP-consuming processes and activating alternate catabolic processes to generate ATP [70].

Upstream kinases that activate AMPK include liver kinase B1 (LKB1), calcium/calmodulin-dependent kinase kinase β (CaMKKβ) and transforming growth factor-β-activated kinase 1 (Tak1). During metabolic stress, LKB1 is the predominant activator of AMPK [71].

The γ subunit of AMPK contains four nucleotide-binding sites, which allow the competitive binding of AMP, ADP or ATP. During metabolic stressful conditions, the increased AMP or ADP replaces ATP and binds to AMPK. Such binding exchange leads to an activation of AMPK, which phosphorylates downstream targets and regulates a range of catabolic effects [70].

Figure 1-VIII Activation of AMPK and its downstream signaling.



Adapted from reference [72].

Note extensive modulation of “downstream” metabolic regulation via activated AMPK.

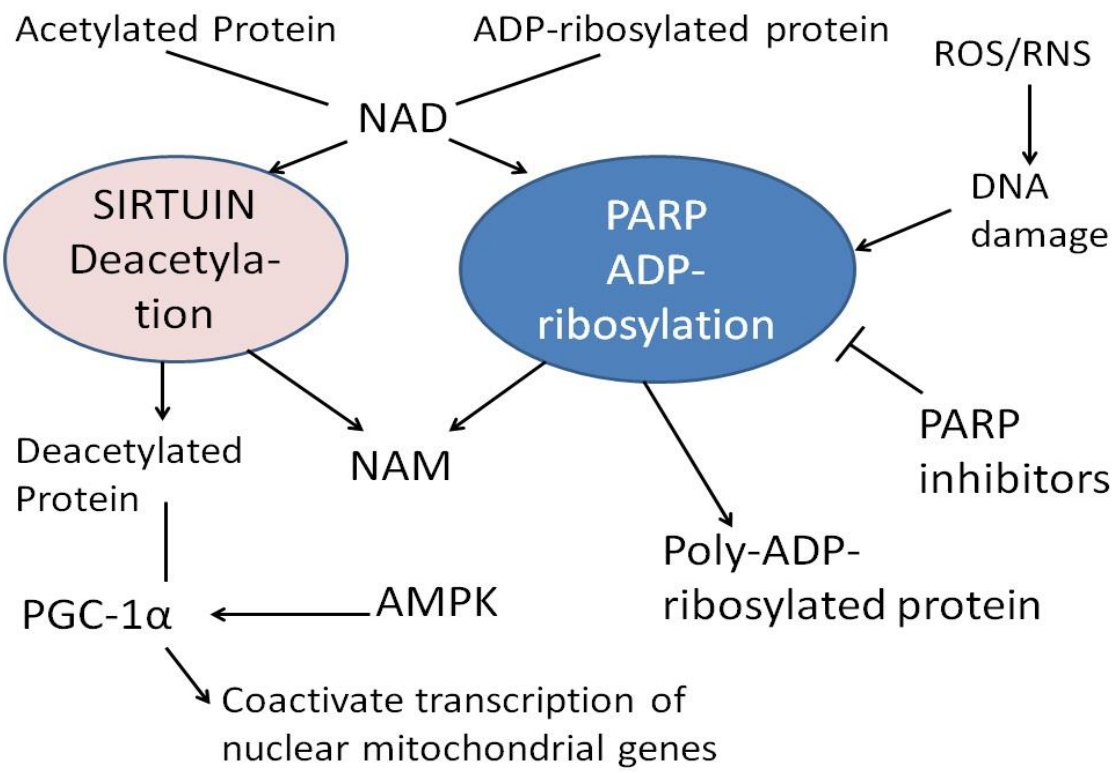
One of these processes is to translocate glucose transporter-4 from intracellular vesicles to the plasma membrane, promoting insulin release (in pancreatic β-cells) and subsequent muscular glucose uptake [73,74]. In cells that express only glucose transporter-1 which are already expressed at the plasma membrane (i.e. most cells except those in muscle, liver and adipose tissue), activated AMPK also promotes glucose uptake to generate ATP. Moreover, AMPK can also promote the translocation of the fatty acid transporter to the plasma membrane to facilitate fatty acid uptake [74].

Other important processes activated by AMPK include mitochondrial biogenesis, which generates increased capacity for the oxidative catabolism of both glucose and fatty acids in the longer term. AMPK directly activates peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), the direct regulator of mitochondrial biogenesis and a co-activator that increases the activity of several transcription factors acting on nuclear-encoded mitochondrial genes [74].

1.2.3.2 Sirtuins

Sirtuins, a family of conserved NAD^+-dependent protein deacetylase, play a crucial role in a variety of cellular process, including energy metabolism, stress response to tumorigenesis and aging [75]. As protein deacetylases, sirtuins have a broad range of substrates, and are able to shuttle form cytosol to nucleus in response to certain environmental stimuli. The expression of SIRT1 (one of the sirtuins) is closely controlled by a number of factors that alter the cellular NAD^+ availability, such as a low-energy environment (e.g. fasting and exercise) that can increase NAD^+ [75]. It is known to control key aspects of fat and glucose metabolism by influencing several transcription factors involved in cardiac energy turnover, for example PGC-1α, a transcriptional cofactor involved in fatty acid/glucose metabolism and mitochondrial biogenesis [76]. Additionally, it has also been previously shown that the deletion or inhibition of PARP-1 elevates NAD^+ levels and promotes the activity of SIRT-1 [67,77]. The major roles of PARP-1, sirtuins and of AMPK in energy homeostasis are depicted schematically in Figure 1-IX.

Figure 1-IX: The interactions between PARP, sirtuins and AMPK in cellular energetic homeostasis.



Sirtuins and PARP are both competitive NAD^+-consuming enzymes. One of the target proteins of SIRT-1 is PGC-1α, which is activated upon deacetylation coactivates a number of mitochondrial genes. AMPK also activates and phosphorylates PGC-1α.

Abbreviations: NAD = nicotinamide adenine dinucleotide; NAM = nicotinamide

1.2.4 Inflammatory activation and disordered energetics

As previously stated, redox signals to and from mitochondria are central to a number of biological processes, including cell proliferation, differentiation, adaptation to hypoxia, autophagy, immune function, hormone signaling, and overall cell survival.

Inflammation, occurring in the vasculature often as a response to injury, lipid peroxidation, and perhaps infection, has recently been found to be associated with a range of cardiovascular risk factors. These include diabetes, hypertension and hypercholesterolaemia. Chronic inflammatory activation not only is linked to increased potential for vulnerable "plaques", being prone to plaque rupture and thereby thrombosis, but also diabetes and metabolic syndrome [78].

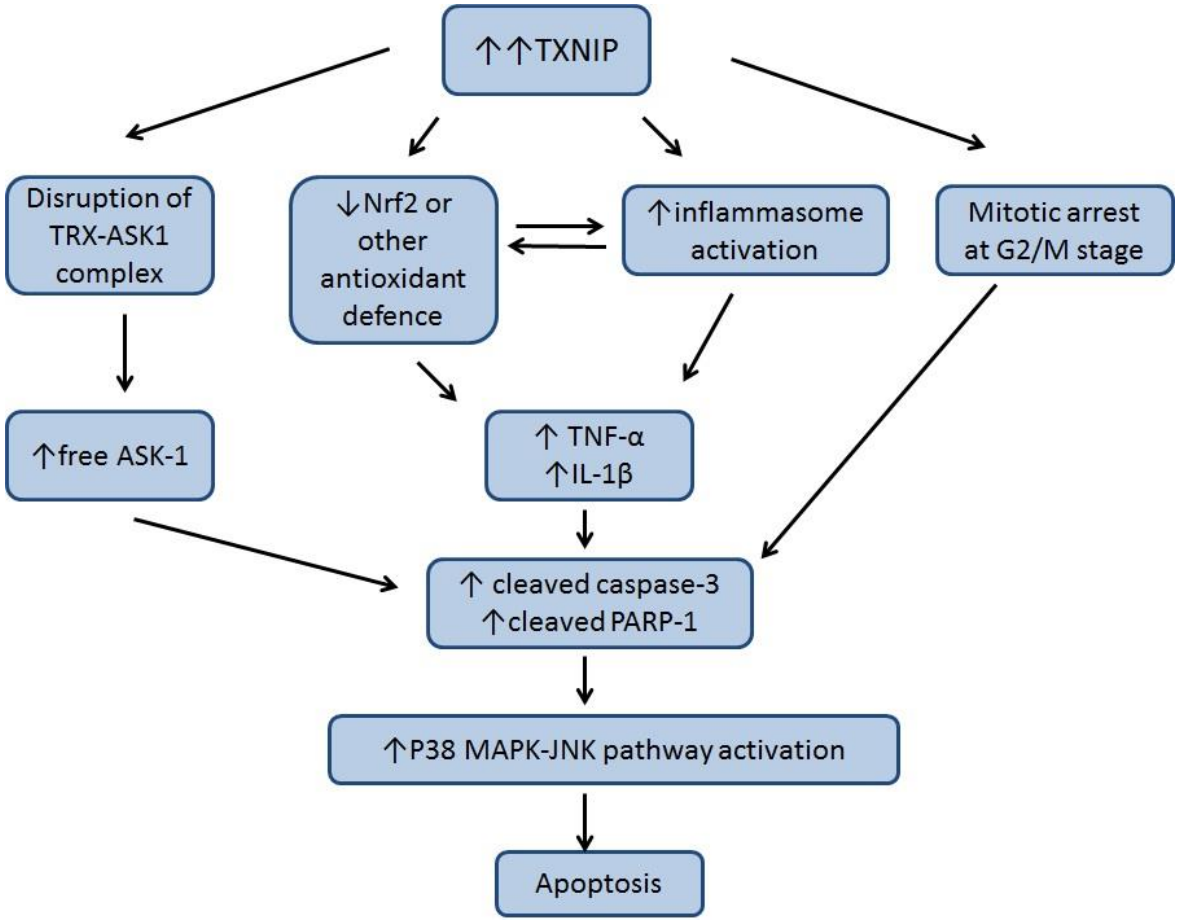
A recent study aimed to explore the possible association of high-sensitive C-reactive protein (hs-CRP) expression in otherwise healthy individuals with metabolic syndrome. Fourteen thousand apparently healthy women, approximately a quarter of whom had metabolic syndrome, were followed up for about an 8-year period for major cardiovascular events (myocardial infarction, stroke, cardiovascular death, and coronary revascularization). It was found that at all levels of metabolic syndrome, CRP improved risk prediction for future cardiovascular events [79]. Additionally, CRP also predicted the onset of type 2 diabetes [80].

Many inflammatory stimuli also are linked to energetic depletion. One such example is thioredoxin-interacting protein (TXNIP), an intracellular protein that exerts its biological effect partially via inhibition of the anti-oxidant thioredoxin (Trx). Pathological suppression of Trx activity by TXNIP has been demonstrated in

a number of clinical conditions such as diabetes mellitus and myocardial infarction. Many of the effects of TXNIP including Trx-independent actions [81] are initiated by its dissociation from intra-nuclear binding with Trx or other SH-containing proteins or PARP-1: these effects include migration of TXNIP to cytoplasm, modulating stress responses and potentially activating apoptotic pathways [82] (see Figure 1-X for further schematic display of increased TXNIP expression).

TXNIP also functions as a general metabolic sensor and modulator of cellular energetics, a stimulus for abnormal calcification, with impact as an immune modulator and vascular mechanosensor (Table 1 provided an overview of major pathophysiological effects of TXNIP relevant to cardiovascular disease). Finally, TXNIP has recently been shown to be a principal activator of the nucleotide-binding oligomerization domain-like receptors pyrin domain-containing 3 (NLRP3) inflammasome [83,84], which modulates innate immune function by regulating maturation and secretion of pro-inflammatory cytokines The pathophysiology of TXNIP is discussed in detail in Chapter 2.1.3.

Figure 1-X: Schematic representation of the impact of increased TXNIP expression on inflammation and on pro-apoptotic pathways.



Published in reference [81].

Abbreviations: ASK1 = Apoptosis signaling kinase; Nrf = Nuclear receptor factor; TNF = Tumour necrotic factor; IL = interleukin; MAPK-JNK = mitogen activated protein kinase c-Jun N-terminal kinases.

Table 1-I: Major pathophysiological effects of TXNIP relevant to cardiovascular diseases.

1. Cardiac Regulation of hypertrophy Activation of apoptosis/ ischaemia-reperfusion injury Pro-inflammatory effect in myocardium and valves Modulation of myocardial metabolism
2. Vascular Suppression of angiogenesis/ interaction with VEGF Induction of inflammation with non-laminar flow ?Pro-atherogenesis ?Suppression of NO effect
3. Platelet ?Suppression of NO effect

Published in reference [81].

Abbreviations: VEGF = Vascular endothelial growth factor; NO = nitric oxide.

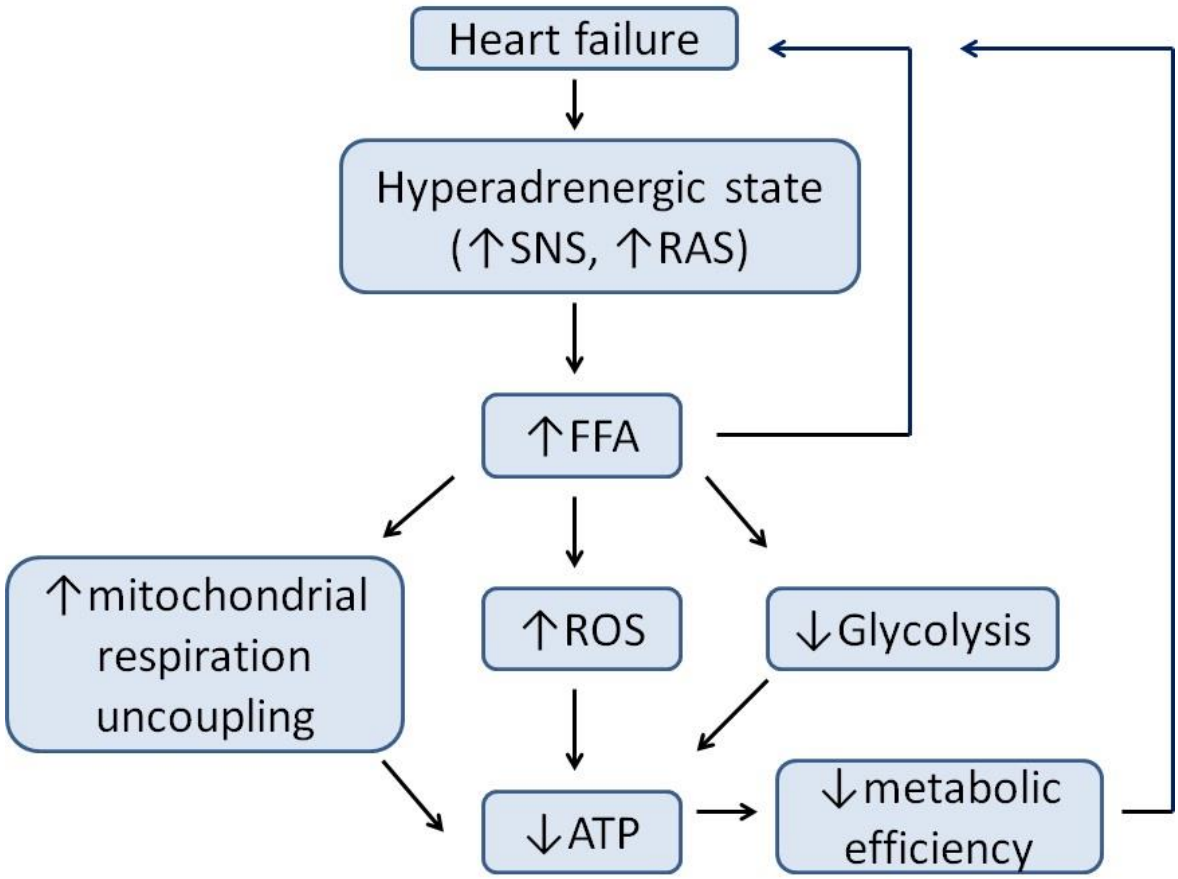
1.2.5 Diabetes mellitus, heart failure and myocardial ischaemia as disorders of cardiac energetics

Pancreatic islet β-cell dysfunction, associated with potential impairment of appropriate insulin secretion and signaling, has the ultimate outcome of diabetes, with exogenous insulin-dependence. In health, following post-prandial hyperglycaemia, β-cells secrete insulin [via generation of mitochondrial adenosine triphosphate (ATP)] for the closure of the ATP-sensitive K_{ATP} channel, leading to the influx of Ca^{2+}, and the ultimate exocytosis of insulin [85]. Any form of dysfunctional mitochondria (for example, defective "fuel-sensing", reduction in mitochondrial mass, or reduction in the ability to generate ATP) contributes to deterioration of not only β-cell function and glucose control, but also increases the adverse effects of hyperglycaemia on other organs [85]. Therefore, interventions to improve mitochondrial energy pathways might also engender protection against organ failure in diabetics. Indeed, it has been shown that exercise [86] and/or metformin [87], which improve efficiency of ATP generation, also offer benefits of reduced morbidity and mortality in T2DM.

In 1996, Nascimben *et al* demonstrated, in biopsies of human hearts obtained during surgery, that there was an approximately 30% reduction of ATP concentrations in patients with systolic heart failure (HF) [39]. Additionally, many human inborn errors of metabolic deficiencies (e.g. Leigh's syndrome, systemic carnitine deficiency) often co-exist with HF. Subsequent studies also demonstrated that, compared to left ventricular ejection fraction and New York Heart Association functional class, cardiac energetic status is a better predictor of both total and cardiovascular mortality in patients with HF [88]. The hyperadrenergic state of HF increases plasma free fatty acid concentrations that, upon entry into mitochondria,

not only uncouple mitochondrial respiration, but also diminish the efficiency of contraction (that is, ATP generation per unit oxygen consumption) of the heart. Additionally, increases in free fatty acid concentrations also decrease glycolysis, which further diminishes efficiency (see Figure 1-XI). Overall, such a metabolic "vicious cycle" argues in favour of a metabolic approach to the treatment of HF. Intriguingly, many of these homeostatic anomalies are also present in diabetics, affecting the efficiency of the heart and predisposing to HF symptoms.

Figure 1-XI: The metabolic vicious cycle of heart failure.



Abbreviations: SNS = sympathetic nervous system; RAS = renin angiotensin system; FFA = free fatty acid; ROS = reactive oxygen species.

A similar “metabolic vicious cycle” has also been described in the early phase of acute coronary syndrome (ACS). High plasma free fatty acid levels have been found in patients with acute myocardial ischaemia (MI) within 30 minutes of the onset of chest pain. Such levels of free fatty acid could be reduced by glucose-insulin-potassium (GIK) [89]. Additionally, in 1972, Braunwald’s group found that in dogs, GIK infusions commenced 30 minutes after experimental coronary occlusion had a protective effect against ischemia and reduced myocardial necrosis [90]. Several other animal studies also confirmed the presence of metabolic dysfunction during the early stage of MI and the benefits of metabolic manipulation by GIK in the reduction of oxygen wastage and the prevention of cardiac remodeling [35,91,92].

However, the translation of GIK (or components thereof affecting metabolic manipulation) during MI from animal to human clinical studies has been largely unsuccessful, with the possible exception when used chronic ischaemic LV dysfunction in the viable myocardium, as assessed by dobutamine echocardiography [93], or of the recent IMMEDIATE [94] trial. Howell *et al* also reported that perioperative GIK improved haemodynamic stability in high-risk patients undergoing aortic valve replacement, with an associated increase in AMPK phosphorylation within the myocardium [95]. It has been proposed that timing and metabolism have been the critical factors contributing to the results [96]: much of the clinical applications of GIK reported commencement at variable time post onset of symptoms, by which time myocardial ischaemia could have progressed to infarcted tissue and beneficial effects on metabolism are much less likely.

1.3 Physiological consequences of energetic impairment

"The failing heart – an engine out of fuel."

S. Neubauer, NEJM, 2007.

1.3.1 Disordered inotropic state and energetic state

The physiological performance of the heart is regulated primarily by variations in the contractile force developed by individual myocardial fibres, based largely on the Frank-Starling principle. In the heart, the majority of ATP formed during mitochondrial oxidative phosphorylation is used for the mechanical contraction, while a minority is used in the maintenance of other aspects of cellular homeostasis, for example modifying substrates, producing glycogen, synthesizing lipids or proteins, and maintaining the integrity of cellular membranes [97]. Any mismatch or "uncoupling" between ATP demand and generation is likely to lead to an impairment of cardiac function. Although the mechanosensory effects occur within single cells, it is known that such effects can also affect neighbouring cells or even the entire organ's function, perhaps via transmission to the cytoskeleton or an alteration in the duration of action potential. ATP is an important immediate energy source for the interaction between actin and myosin (thus promoting myocardial contraction), and is rapidly replenished by the hydrolysis of creatine phosphate [98].

It has previously been shown that inborn errors of metabolism (such as Leigh's syndrome, malonyl carboxylase deficiency) lead to congestive cardiomyopathy [1]. Additionally, as previously stated, any hyperadrenergic state

of the heart (for example, during heart failure) potentially leads to dysregulation of substrate metabolism and an eventual “vicious cycle” of metabolic dysfunction.

Recently, epidemiological studies showed that diabetes mellitus (a disease characterized by metabolic and mitochondrial dysfunction), increases the incidence of heart failure by 2.5-fold, independent of age or concomitant hypertension, obesity, dyslipidaemia or coronary heart disease [99,100]. Previous studies showed that diastolic dysfunction occurs in 50-75% of asymptomatic, normotensive diabetic patients with preserved left ventricular ejection fraction and no known coronary disease [101-103], when ejection fraction was used as the measure of cardiac function. Nowadays, detection of subclinical dysfunction is made available via the use of echocardiographic strain imaging or cardiac magnetic resonance, with reduced longitudinal contractility and impaired systolic circumferential strain in patients with diabetes [104,105]. Additionally, it has become possible to assess the impact of both diabetes and heart failure on myocardial energetics in vivo using phosphorus magnetic resonance spectroscopy (MRS). A study in apparently uncomplicated type 1 diabetes was associated with impaired myocardial phosphocreatine to ATP equilibrium as measured by MRS [106].

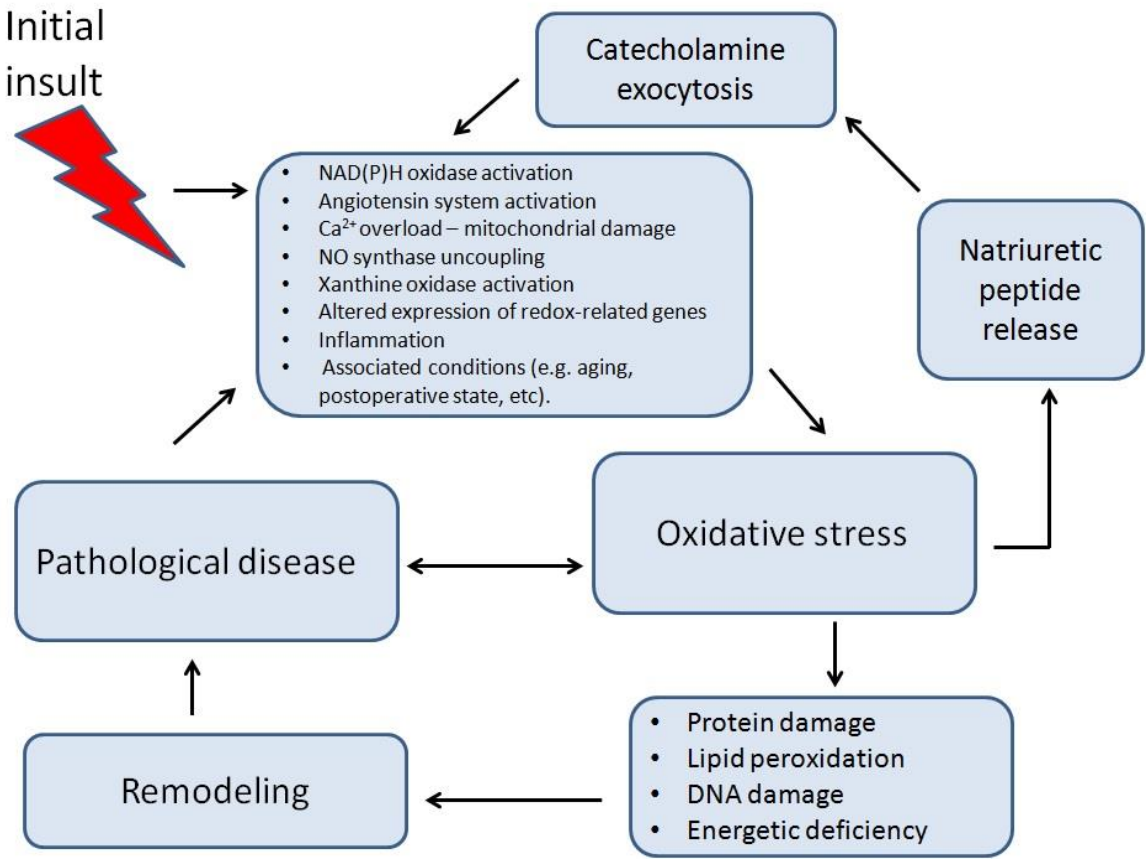
1.3.2 Perpetuation of redox stress

During the initial insult causing redox stress, physiological compensation occurs towards restoring homeostasis. However, such homeostatic response is often inadequate during pathological conditions, leading to a self-perpetuating inflammatory “vicious cycle” (see Figure 1-XII; a process very much similar to the

“metabolic vicious cycle” as mentioned in Chapter 1.2.5). For example, in atrial fibrillation (the most common form of arrhythmia) [107,108], myocardial ischaemia [109], and stress cardiomyopathy (or known as Takotsubo cardiomyopathy) [110,111], inflammatory activation persists beyond the initial insult and appears to contribute to the gravity of the resultant clinical syndromes. For example, in the case of coronary disease, a reduction in coronary perfusion leads to a deprivation of blood flow that first affects the subendocardium. Histological findings of fibrosis and vacuolization in this area together with ATP depletion provide evidence that such culmination of events often leads to the gradual loss of subendocardium [109].

While perpetuation of redox stress may occur as a result of the loss of balance between compensatory and the "insulting" mechanisms, another possibility is a paradoxical effect of the "compensatory" hormone(s) contributing to this perpetuation. For example, natriuretic peptide, a "compensatory" hormone upregulated in the setting of heart failure, which acts by increasing intracellular cyclic GMP, is theoretically "protective" through smooth muscle relaxation and vasodilation. In 2012, Chan and colleagues [112] found that in isolated hearts, natriuretic peptides and other cell-permeable cyclic GMP analogues cause catecholamine exocytosis. Such a proadrenergic action may have counteracted any potential protective effect of natriuretic peptide, providing an explanation for not only the self-perpetuating redox stress process in heart failure but also for the loss of beneficial effect of natriuretic peptide analogues in the treatment of heart failure. This putative effect is schematized in Figure 1-XII.

Figure 1-XII: Proposed scheme for self-perpetuating mechanisms of oxidative stress.



Adapted and modified from reference [113].

1.4 Measurement of energetic impairment and its determinants

1.4.1 Experimental

Myocardial energetic status can be assessed by measuring the various components of energy metabolism in the heart, utilizing standard methods in myocardial specimens obtained by a biopsy or tissue collection from animals. However, due to the instability of the molecules, the measurement of ATP and phosphocreatine is challenging, requiring care to minimize ex vivo degradation.

Under conditions of high energy phosphate depletion, for example associated with increased demand within the myocardium, repletion of ATP can occur approximately 100 times faster than that of phosphocreatine; hence phosphocreatine to ATP ratios tend to fall [1].

1.4.2 Clinical

Clinically, the assessment of cardiac energy status can be performed in a number of ways, for example via determination of the ratio of phosphocreatine to ATP non-invasively utilizing cardiac phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) [1]. ^{31}P-MRS yields peaks for phosphocreatine and the three phosphorus atoms of ATP, all of which are proportional to the cellular concentrations of these metabolites. The system also provides potential assessment of the rates of oxidative phosphorylation and ATP transfer [114].

The ratio of phosphocreatine to ATP has been found to correlate well with New York Heart Association (NYHA) functional class and indices of systolic and diastolic dysfunction [115]. In a study of 39 patients with dilated cardiomyopathy, PCr: ATP ratio was found to be a stronger predictor than functional (NYHA class) or clinical indices of systolic function of both total mortality and mortality rates attributable to cardiovascular diseases [88].

The measurement of intracellular and compartmental ATP, especially the availability of ATP at the sarcoplasmic reticulum or ion channels, instead of average cellular level of ATP, should theoretically provide better insight into the energetic status. However, no currently available method is able to make such subcellular measurements.

A method for the assessment of consequences of cardiac energetic status is to determine the cardiac mechanical efficiency:- the ratio of useful energy produced (or, stroke work) to oxygen consumed. This can be achieved invasively via coronary catheterization or non-invasively via positron emission tomography [116,117]. The former is considered the "gold standard": it measures cardiac work relative to myocardial oxygen consumption (MVO_2) and correlates with high-fidelity catheter-derived pressure-volume loops as indices of changes in LV contractility and stiffness. This method is invasive, unable to assess compartmental (rather than global) oxygen consumption and the needs to correct for LV mass to calculate oxidative metabolism per gram of tissue. It is more suitable for assessing changes in energetic status than for single estimates per patient [116,117].

The latter non-invasive assessment of mechanical efficiency by positron emission tomography is a little less straight-forward. The input energy is assessed by the kinetics of (1) carbon-11-labelled carbon dioxide produced after injection of

carbon-11-labelled acetate (^{11}C-acetate) to assess oxidative phosphorylation via TCA cycle and (2) oxygen-15-labelled-water and oxygen-15-labelled-carbon monoxide produced after inhalation of oxygen-15-labelled molecular oxygen. The combination of all the radio-labelled products allows the quantification of myocardial blood flow and oxygen extraction fraction and therefore the subsequent measurement of MVO_2. The output energy is measured as end-systolic LV pressured derived either by magnetic resonance imaging or echocardiography. Overall, this methodology is not only a semiquantitative index of oxidative metabolism and likely subject to observer variability, but also the correlation of the metabolic fate of ^{11}C-acetate to oxidative metabolism is also highly subject to pathological disease states (such as ischaemia) [116,117].

1.5 Scope of the present study

The current thesis aims at exploring the mechanisms of effect and safety of long-term treatment with perhexiline, its enantiomers, and other aspects of myocardial energetic therapeutics. See Figure 1-XIII for schematic representation of experimental design.

Chapter 2 discusses the current pharmacological aspects of modulating myocardial metabolism; including the modulation of long chain fatty acids, mitohondrial protective agents and the metabolic "sensors" AMPK activators. Other agents discussed include insulin and the impact of its signaling pathway, which affects not only metabolism but also cell signaling, inflammation and platelet aggregability. TXNIP, of which production is mainly stimulated by hyperglycaemia, also has adverse effects on inflammation and apoptosis and its expression is affected by a number of pharmacological agents used either clinically or under investigation. Lastly, the history of use and development of perhexiline, its mechanisms of actions, and the recent development are discussed.

Chapter 3 investigates the current status of chronic use of perhexiline in 170 patients, during the modern era of regular therapeutic drug monitoring. Apart from investigating the incidence of serious adverse effects in patients taking long-term perhexiline, determinants of mortality events in this cohort of ageing population are also reported.

Chapter 4 describes the modulation of pharmacokinetics and its relationship with effects are reported in patients treated with short-term perhexiline (for an average of 9 days) prior to coronary bypass surgery. During bypass, biopsies of left ventricle and atrial appendage were obtained. The relationship between

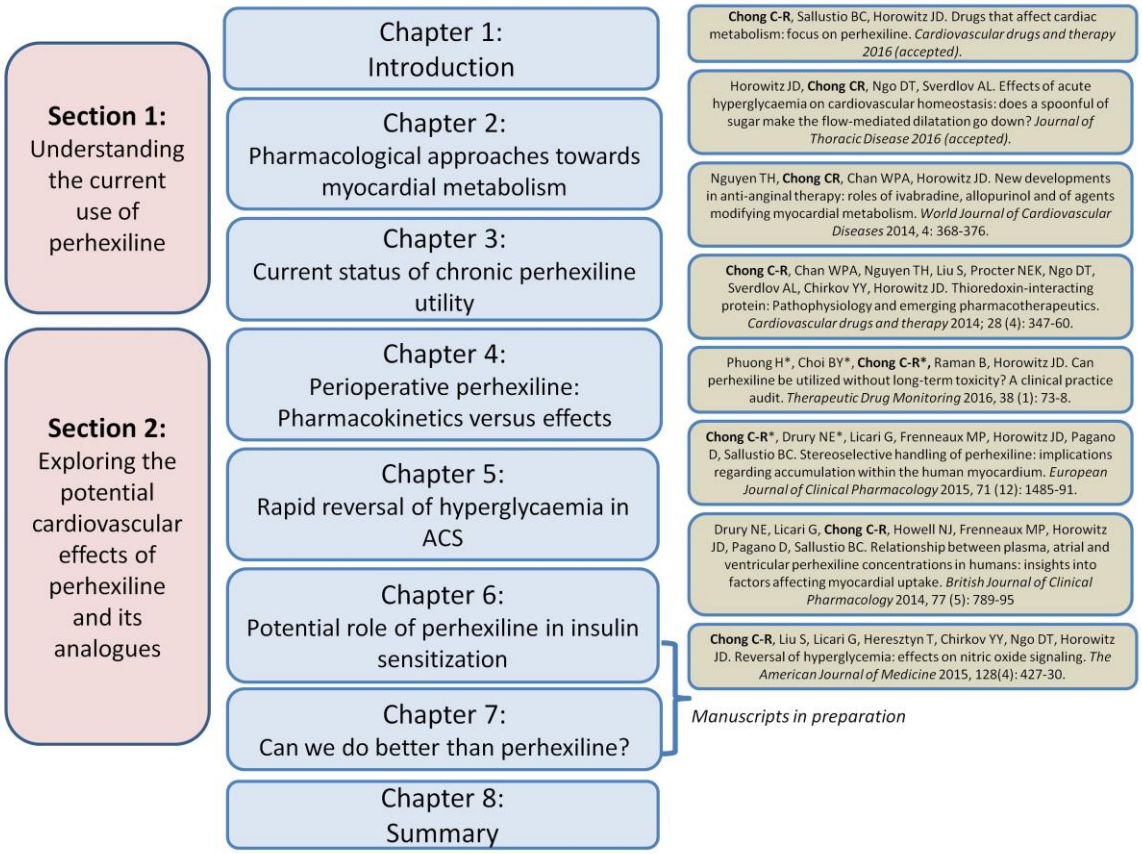
plasma and myocardial perhexiline (enantiomer) concentrations, and the modulation of myocardial uptake are described.

Chapter 5 investigates the relationship between oxidative stress, insulin sensitization, nitric oxide signaling and therapeutic approaches to improved myocardial efficiency. This chapter begins with the investigation of the mechanism(s) of benefits associated with rapidly correct hyperglycaemia in the presence of acute coronary syndrome using insulin infusion, an effect potentially reflecting the benefit of myocardial metabolic intervention.

In a further cohort of patients (Chapter 6), the interaction between perhexiline, TXNIP and insulin sensitivity is explored via an investigation of the potential insulin sensitizing effect of perhexiline in thirty stable diabetic patients. Plasma samples were collected before and after two weeks of perhexiline administration. Association of biochemical correlates are reported.

Chapter 7 compares the effects of various myocardial drugs modifying myocardial energetics on activity of CPT-1 in myocardium, focusing on the enantiomers of perhexiline as well as analogues of perhexiline currently in development, some are pharmaceutical products already clinically available.

Figure 1-XIII: An outline overview of the current thesis.



Chapter 2: Pharmacological approaches towards correction of energetic impairment

2.1 Approaches in diabetes:

2.1.1 Insulin

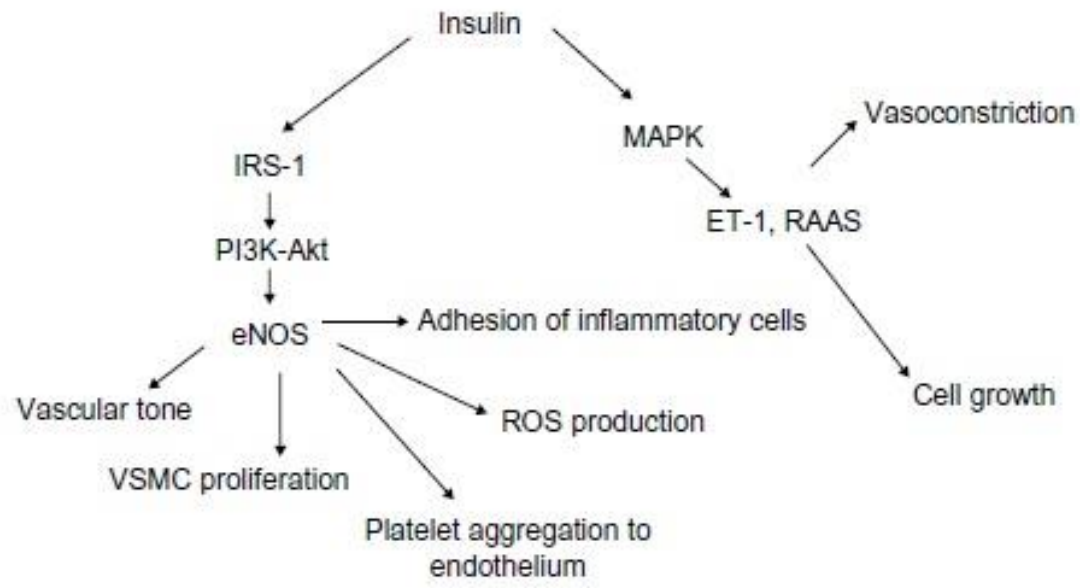
Insulin is synthesized, packaged and secreted by the pancreatic β-cells. The action of insulin is initiated via binding to the heterodimeric insulin receptor on the cell surface of target cells. Insulin receptors are composed of two α-subunits (insulin-binding) and two intracellular β-subunits (signal transduction domains). The binding of insulin to the receptor results in conformational change of the α-subunits, which enables the subsequent binding of ATP to the intracellular β-subunits. ATP binding leads to the phosphorylation of the receptor, which then in turn phosphorylates other protein substrates such as insulin-receptor substrate 1 and 2 [118,119]. The signal is then further propagated through a network of phosphorylation that involves other intracellular substances.

These signaling pathways enable insulin to act as an important regulator of metabolic function. Importantly, insulin-receptor activation of the mitogen-activated protein (MAP) kinase pathway has been implicated in the insulin's effects on vasoconstriction, growth and proliferation [119].

Insulin receptors are widely represented on the surface of cells lining the vascular walls. Apart from the above, the activation of insulin receptor substrate leads to the activation of the phosphatidylinositol 3-kinase-Akt pathway, which eventually activates the endothelial nitric oxide synthase [120]. Additionally, insulin also stimulates the production of prostaglandin PGI_2 from vascular endothelium [121], which, together with nitric oxide produced via eNOS, plays a role in vasodilation [120,122]. It should also be noted that nitric oxide exerts effects other

than vasodilation; it also inhibits platelet aggregation, reduces vascular smooth muscle cell proliferation, and reduces the adhesion of inflammatory cells [120,123] (see Figure 2-I).

Figure 2-I Effects of insulin: molecular mechanisms



Adapted from reference [120].

Abbreviations: IRS = Insulin receptor substrate; PI3K-Akt = Phophatidylinositol-3-kinase and protein kinase B; eNOS = endothelial nitric oxide synthase; VSMC = vascular smooth muscle cell; MAPK = mitogen activated protein kinase; ET-1 = endothelin-1; RAAS = renin-angiotensin-aldosterone system.

2.1.2 AMPK activators

AMPK, a "universal sensor" of energy status, plays a key role in the regulation of whole body metabolic homeostasis. Many classical agonists activate AMPK indirectly by altering the AMP/ADP:ATP balance intracellularly. Such drugs, for example the biguanides metformin and phenformin, act by inhibiting the electron transport chain complex I. Other drugs such as 5-aminoimidazole-4-carboxamide-1-D-ribo-furanoside (AICAR) inhibit adenosine deaminase causing an increase in ATP and adenosine concentrations.

Metformin, as a classical AMPK activator, is one of the most widely prescribed anti-diabetic agents, which reduces cardiovascular mortality in type 2 diabetic patients [87] . It has been in clinical use for more than 50 years and is weight-neutral (hence beneficial in obese diabetic patients) [124]. Metformin has been shown to reduce cardiovascular mortality when compared to non-intensive treatment [87] and more recently, to induce a potential reduction in cancer incidence in most [125-127], but not all [128] studies. Apart from its effect on the reduction of gluconeogenesis [129], metformin also inhibits mitochondrial complex 1 [130], and has been shown to reduce reactive oxygen species [131] and increase nitric oxide synthesis [132].

2.1.3 TXNIP: Impact on energetics and modulation of expression

Thioredoxin interacting protein (TXNIP), also known as vitamin D-upregulated protein, may be regarded partially as a component of the thioredoxin system, which also consists of thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (Trx). TXNIP inhibits Trx, and this effect has been demonstrated in diabetes and cardiovascular diseases. However, TXNIP effects are also partially Trx-independent, and these include direct activation and inhibition of glucose uptake.

The potential involvement of TXNIP in cardiovascular or non-cardiovascular pathological processes is central to its extensive physiological significance. In particular, TXNIP activates inflammation (by antagonizing the antioxidant transcription factor nuclear factor erythroid factor-2 related factor 2 [133], produces superoxide [134], and activates the NLRP3 inflammasome to produce interleukin-1β [135]) and activates apoptotic signaling pathways [136]. In addition, TXNIP also interferes with nitric oxide signaling [137,138], potentially via augmentation of superoxide production. Such effects are potentially critical to survival rates of both β-cell and cardiomyocytes.

In 2002, TXNIP was discovered to be the most upregulated gene in response to elevation of tissue glucose concentrations in human islet oligonucleotide gene expression microarray study [139]. This suggested that TXNIP is potentially implicated in the pathogenesis of diabetes. TXNIP-related activation of inflammasome has been shown to induce inflammation and endothelial dysfunction in intra-ocular vessels of diabetic models [140], implicated

in the pathogenesis of diabetic cardiomyopathy [83,141], and shown to mediate renal fibrosis [142].

TXNIP is known to reside mainly in the nucleus. In 2010, Saxena and colleagues found that TXNIP “shuttles” from nucleus to mitochondria under oxidative stress [143]. It was found that such “shuttling” allowed TXNIP to bind to and oxidize Trx2, therefore allowing ASK1 phosphorylation/activation, leading to induction of mitochondrial apoptosis with release of cytochrome c and caspase-3 cleavage [143]. In another study of TXNIP knock-out mice, TXNIP deletion led to decreased mitochondrial function and morphological changes [144]. In particular, mitochondrial ATP synthesis was decreased by TXNIP deletion, along with a reduction in cellular ATP content and an increase in lactate formation [144]. Recently, a targeted metabolomics study utilizing TXNIP knock-out mice found that TXNIP deficiency is associated with a reduction in enzymes not only required for the catabolism of substrates (amino acids, ketones, and lactate), but also those required for β-oxidation and the tricarboxylic acid cycle [145]. Therefore the available data suggest that TXNIP mobilization may act as an energetic "switch", increasing ATP generation.

The dissociation of TXNIP from its binding protein leads to its accelerated clearance. Therefore, a fundamental mechanism for the rapid clearance of TXNIP involves the ubiquitination-proteasomal pathway. [146] It has been demonstrated that insulin accelerates this process in some tissues [146,147]. Four hours of insulin treatment was associated with reduction in TXNIP mRNA expression, and was dependent on intact insulin receptor signaling rather than simply a reduction in circulating glucose levels. This was supported by the observation that insulin failed to reduce TXNIP levels in insulin-receptor knockout diabetic mice [147].

AMPK activation has been shown to accelerate degradation of TXNIP in some key experiments. It has been shown that AMPK regulates the responsiveness of the carbohydrate-response element binding protein on TXNIP promoter gene [148]. Glucose “starvation” in Hep G2 cells resulted in the AMPK-activation-dependent phosphorylation of TXNIP [149] (a process mimicking the pharmacological activation of AMPK). Such a process results in increased degradation of TXNIP, resulting in prolonged increases of glucose utilization.

Other insulin secretogogues like glucagon-like peptide 1 (GLP-1) agonists also accelerate TXNIP degradation via similar ubiquination/proteasomal pathway. Exenatide, a GLP-1 agonist, reduced levels of pancreatic TXNIP expression in vitro, and was found to be associated with an increase in cAMP signaling, involving not only protein kinase A but also exchange protein activated by cAMP (Epac) singaling [150,151].

There is also substantial evidence that the angiotensin converting enzyme inhibitor ramipril reduces tissue content of TXNIP. First, in a vitamin D-treated rabbit model, aortic valve expression of TXNIP was shown to be increased; conversely, ramipril reduced TXNIP content and retarded valvular fibrosis [152]. A subsequent human study also showed that two weeks of ramipril treatment in high risk acute coronary syndrome patients reduced platelet TXNIP levels, and that this effect was associated with improved platelet responsiveness to nitric oxide [138].

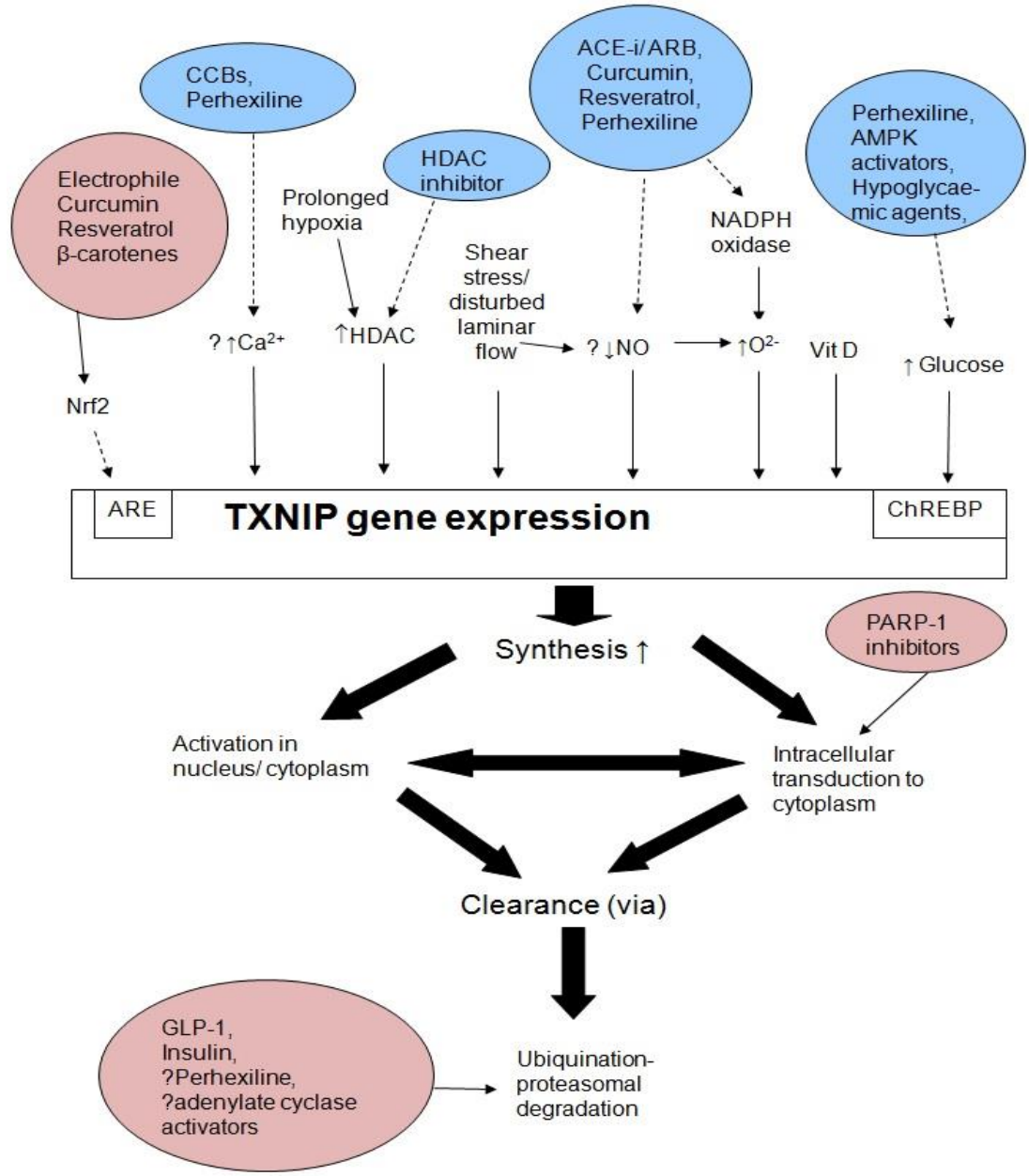
The L-type calcium channel antagonists, verapamil and diltiazem, also suppress TXNIP expression. In cultured cardiomyocytes, Chen *et al* showed that verapamil and diltiazem reduced TXNIP transcription and protein expression [153]. Futhermore, three weeks of verapamil treatment in streptozocin-treated animals also duplicated this results in cardiomyocytes and pancreatic β-cells [141,154].

Perhexiline was also recently found to reduce TXNIP expression in non-diabetic patients awaiting coronary artery bypass surgery [155]. The pathophysiological consequences of this change have not been explored to date.

Figure 2-II provides an overview of the potential site of pharmacological modulation of TXNIP expression, including some experimental agents currently under investigation.

Although hyperglycaemia is associated with incremental oxidative stress and impaired nitric oxide signaling, as well as poor outcomes in patients with concomitant cardiovascular disease, it remains uncertain whether suppression of TXNIP plays a major role in the restoration of cardiovascular homeostasis on reversal of hyperglycaemia. Additionally, it remains a theoretical [156-158] and debatable [159-161] concern that suppression of TXNIP may increase the risk of carcinogenesis.

Figure 2-II Activation/ inactivation pathways of TXNIP: Potential sites for therapeutic manipulations



Published in reference [81]. Blue box or dash line arrows indicate inhibitory effects; pink box or solid line arrows indicate stimulatory effects.

Abbreviations: NO = nitric oxide; Nrf2 = nuclear receptor factor erythroid 2-related factor 2; O_2^- = superoxide; CCBs = calcium channel blockers, ACE-i = angiotensin converting enzyme inhibitors; ARBs = angiotensin-II receptor blockers; ChREBP =

carbohydrate response element binding protein, ARE = Antioxidant response element.

2.2 Mitochondrial protective agents

Given that mitochondria plays a significant role in the development of a number of myocardial and metabolic diseases, this organelle has become a potential site of therapeutic target.

Initial experiments concerned compounds named Szeto-Schiller (SS) peptides that selectively target the mitochondrial electron transport chain. These were discovered while scientists were working on a synthetic opioid peptide with high affinity and selectivity for the μ–opioid receptor [162,163]. The index compound in this group, despite a molecular weight of 640 and being highly polar, was readily taken up by a variety of cells without involvement of transporters or receptors. It was later found out that this compound was selectively partitioned to the inner mitochondrial membrane (thus colocalizing with the electron transport chain) with minimal concentrations in the matrix. A number of peptide analogues were later designed (named SS-31 and SS-20), with negligible affinity for the opioid receptors [163], thus presumptive selectivity for mitochondrial metabolic effects.

The selective binding of SS-31 and SS-20 to cardiolipin has been found to account for their relative concentration on the inner mitochondrial membrane. Cardiolipin, a dimeric phospholipid with small acidic head group and four acyl chains, decreases water permeability of the lipid membrane and decreases the energy required to create folds or cristae in the inner mitochondrial membrane [164]. It is important for cristae formation, and cardiolipin deficiency, such as occurrs in Barth syndrome, leads to loss of cristae and mitochondrial failure [163]. Additionally, cardiolipin content declines with age, and has been found to be

associated with reduced efficiency of electron transport, inhibition of oxidative phosphorylation and increased leakage of reactive oxygen species [165,166]. This attenuation also plays a role in a number of pathological conditions, such as ischaemia-reperfusion injury, heart failure [164], diabetes [167], and cancer cachexia [168].

SS-31, also known as Bendavia or MTP-131, has now entered into clinical trial development for a variety of complex diseases. First, the administration of Bendavia in a number of animal models of ishcaemia-reperfusion injury led to reduced infarct size and reduced cellular levels of reactive oxygen species [169,170]. In a canine model of heart failure induced by repeated intracoronary embolizations, daily administration of Bendavia for three months was associated with contractile improvement (left ventricular ejection fraction, cardiac output, stroke volume, and contractility index) without affecting vasomotor tones, and improvement in mitochondrial potential and increased ATP synthesis [171-173].

A subsequent randomized, double blinded, placebo-controlled trial (EMBRACE STEMI) for patients with first-time anterior STEMI undergoing primary percutaneous coronary intervention has been recently completed (NCT01572909) [174]. Patients were randomized to receive MTP-131 at 0.05mg/kg/hour or placebo as an intravenous infusion. The primary endpoint of the study was infarct size, as measured by both creatine kinase release and cardiac magnetic resonance imaging with gadolinium enhancement [174]. Data from the phase 2a of the study revealed no beneficial effect of MTP-131 on this endpoint [175].

2.3 Agents modulating long-chain fatty acid uptake or utilization

Perhexiline (to be discussed in detail in Chapter 2.4) is (inter alia) an inhibitor of the rate-limiting enzyme of fatty acid oxidation, carnitine palmitoyl transferase (CPT)-1, and to a lesser extent CPT-2 [176].

Etomoxir is another inhibitor of CPT-1 [177], which was initially developed as a potential insulin sensitizer. It was shown to improve left ventricular ejection fraction and exercise haemodynamics in patients with chronic heart failure [178]. In a model of transverse aortic constriction, etomoxir also limited the extent of resultant left ventricular hypertrophy, an effect of potential interest in the context of the current use of perhexiline in hypertrophic cardiomyopathy [179]. However, there was also substantial toxicity. In some models of heart failure, etomoxir increased left ventricular wall thickness, although it is unclear whether this represented hypertrophy, oedema or infiltration [180-184].

Oxfenicine, another irreversible CPT-1 inhibitor, also retards the development of heart failure in a canine rapid pacing model [185]. However, when given in higher doses for prolonged periods of time, it also caused what was labeled as “hypertrophy” of the heart, but actually represented cardiac phospholipidosis [186].

Trimetazidine inhibits the intramitochondrial enzyme long-chain 3 ketoacyl CoA thiolase, causing a reduction in fatty acid oxidation and increasing glucose oxidation [34]. Thus it can be termed a partial fatty acid oxidation inhibitor, but it also is a weak CPT-1 inhibitor [187]. It improved cardiac energetic status (as measured by both magnetic resonance spectroscopy [188] and 18-

fluorodeoxyglucose positron emission tomography [189]) in patients with non-ischaemic systolic dysfunction [188,189] and was shown to be effective as an add-on therapy to heart failure management [190]. This was also confirmed in a recent meta-analysis of randomized controlled trials (with a total of 326 patients) showing that trimetazidine as an add-on therapy reduced all-cause mortality and increased event-free survival time in patients with systolic heart failure [191]. Interestingly, little effect on cardiac fatty acid uptake was observed, despite a significant reduction in fatty acid utilization from intracellular stores [189]. Additionally, in the trial comparing trimetazidine with placebo as an “add-on” therapy for heart failure, there was an increase in the intraventricular septal thickness in patients taking trimetazidine after approximately 6 months of therapy (from 8.5 ± 2.0 to 10 ± 1.4 in the trimetazidine group versus 8.4 ± 1.1 to 8.4 ± 0.5 in the placebo group; $p<0.002$) [189]. This process has not yet been evaluated fully, but resembles the effects of oxfenicine.

Given such similar findings (albeit in animal studies) involving etomoxir and oxfenicine, this was an unexpected, and potentially undesirable consequence of heart failure treatment even though it is uncertain whether this represents phospholipidosis. Currently, the use of trimetazidine is diminishing, largely due to associated reports of Parkinsonism syndrome [192-194].

Ranolazine is an effective prophylactic antianginal. It is also a partial inhibitor of fatty acid β-oxidation [195]. However, this may not be its principal mechanism of action under therapeutic conditions. Recently, ranolazine has also been shown to be an inhibitor of the late inward sodium channel, reducing intracellular calcium (via the sodium calcium exchange channel), and reducing the left ventricular diastolic wall tension [196]. Importantly, although anti-anginal

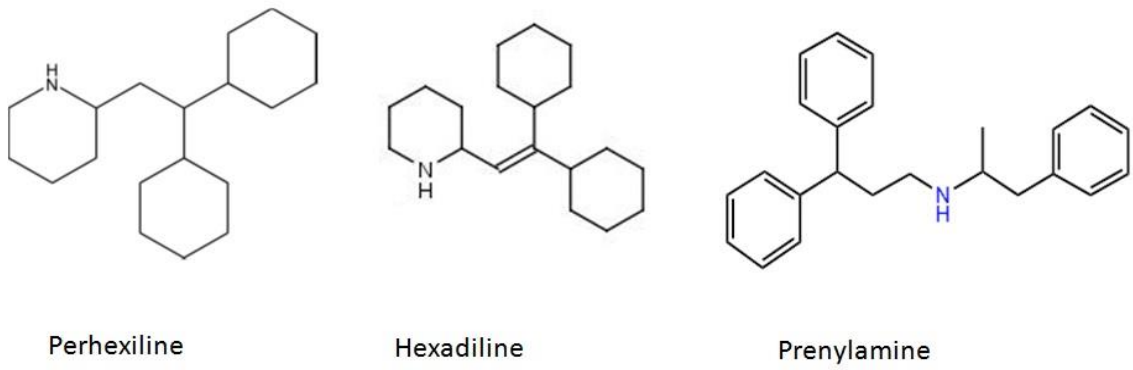
effects have been demonstrated [197], no clinical studies to date have evaluated effects of ranolazine on myocardial energetics in any patient group.

2.4 Focus on perhexiline

2.4.1 Historical

Around 1960, Richardson-Merrell Pharmaceuticals (Cincinnatti, USA) attempted to develop novel vasodilator anti-anginal agents. Hexadiline and prenylamine, both structurally similar to perhexiline, were developed prior to the development of perhexiline (see Figure 2-III for structures). Hexadiline has never been used clinically:- its role is limited to serving as an internal standard to the chromatography assay of perhexiline [198] or other laboratory purposes. Prenylamine, on the other hand, was used once as an anti-anginal [199]. However, it was pro-arrhythmic [200,201] and as such, its indication for angina treatment diminished over time after being replaced by other safer agents. Its use has recently been resurrected as a potential local anaesthetic. It was demonstrated that prenylamine is metabolized to amphetamine in vivo [202].

Figure 2-III: Molecular structures of perhexiline, hexadiline and prenylamine.



Perhexiline Hexadiline Prenylamine

Perhexiline was first marketed in France in the 1970s as a prophylactic antianginal. It was an extremely effective antianginal given as monotherapy, typically in doses of 200 to 400mg per day [203,204]. However, in the late 1970s, several reports of hepatotoxicity and peripheral neurotoxicity appeared: these were poorly understood at that time, but led to the gradual withdrawal of the drug worldwide in the 1980s, except in Australia and New Zealand.

Through continual research to better understanding of the cellular mechanisms of the drug and careful plasma drug concentration monitoring, the drug now is regarded as potentially conferring great benefits to patients with refractory angina. Perhexiline has been re-registered in several European countries, due to favorable clinical trials (to be discussed later), and has also very recently approved by the Food and Drug Administration (United States of America) for development in the treatment of non-obstructive hypertrophic cardiomyopathy (NCT02431221).

2.4.2 Mechanistic insights

In 1980, Vaughan Williams [205] first proposed that perhexiline's mechanisms of action might be based upon changes in myocardial metabolism, but did not identify the specific changes involved. Jeffrey *et al* [206] also provided evidence that perhexiline might increase efficiency of myocardial oxygen utilisation, consistent with the presence of such an effect. However, only in 1996 after perhexiline had been utilized clinically as an anti-anginal for over 20 years, was it found that it was a potent inhibitor of the carnitine "shuttle", which transports long-chain fatty acids across mitochondrial membranes to undergo β-oxidation

[176]. Inhibition of the enzymes CPT-1, and to a lesser extent, CPT-2 [187] would result in secondary activation of glucose utilization via increased activity of the pyruvate dehydrogenase complex, a process which is a component of the so-called "Randle cycle". This metabolic shift results in increased glucose utilization, and ATP production per unit of oxygen consumption.

In 2005, Unger and colleagues found that in isolated non-ischaemic working rat hearts, pretreatment with perhexiline increased cardiac efficiency by about 30%. However, this effect was independent of palmitate oxidation [207]. This was the first evidence to suggest that perhexiline may exert effects beyond CPT-1 inhibition, and it is quite possible that these contribute to both its therapeutic efficacy and potential toxicity.

Indeed, it was subsequently found that perhexiline inhibited the preassembled neutrophil NADPH oxidase [208]. This effect seems to occur selectively: being present in the membranes of pig valve interstitial cells, human umbilical vein endothelial cells and cardiac fibroblasts, but not in the rat aorta. Such an effect in neutrophils and/or platelet may also contribute to effect of potentiation of platelet responsiveness to nitric oxide by perhexiline utilizing whole blood aggregometry [209]. Liberts *et al* (2007) [210] documented that perhexiline potentiated the effects of infused glyceryl trinitrate in suppressing neutrophil superoxide release without accentuating the vasomotor effects of the nitrates.

Although known to cause phospholipidosis (to be explored further in Chapter 2.4.4: Early and late toxicity), the effect of perhexiline on lipid metabolism has never been investigated fully. In a recent study, perhexiline was found to activate Kruppel-like factor 14 (KLF14) and increase high density lipoprotein cholesterol (HDL-C) [211]. In two *in vivo* models of atherosclerosis, administration

of perhexiline retarded the progression of atherosclerosis development [211]. Although it is not known if perhexiline, when administered in therapeutic concentrations, has any effect on the metabolism of HDL-C, this study provides a potential for the development of newer therapeutic agents in this area. It should also be noted that previous genome-wide association studies found genetic variance of KLF14 associated with HDL-C [212,213]; and epidemiological studies and experimental observations revealed decreased levels of HDL-C and apolipoprotein A-I (ApoA-I) to be independent predictors of coronary heart disease [214-217].

Perhexiline was once thought to be a potential antiarrhythmic and a significant L-type calcium antagonist [218,219]. The former mode of action was later disputed as perhexiline's main action, as it was found that perhexiline administration did not markedly affect QT interval, QRS duration, PR interval, intra-atrial conduction time, atrioventricular nodal conduction time, and His-Purkinje conduction velocity significantly [219]. Indeed it has been shown that perhexiline has some inhibitory effects in vitro on a number of potassium channels in myocardium [220], a finding which may account for the single report in the literature of perhexiline-induced *torsades de pointes* [221]. On the other hand, perhexiline was shown to be a calcium channel antagonist in early studies [222,223], as it was found to reduce heart rate significantly, though at doses at least ten times higher than established calcium channel antagonists [222]. In 1985, Barry and colleagues compared the 50% inhibitory concentrations (IC_{50}) of several calcium channel antagonists on spontaneous contractile amplitude in chick embryo ventricular cells (which are L-channel-dependent), and found that perhexiline was a weak calcium channel antagonist [224]. In fact, the IC_{50} values

for diltiazem and verapamil were 13.5 and 10.0 x 10^{-8} M respectively, compared with 83 x 10^{-8} M for perhexiline [224]. Clinical evidence of the minimal potency of perhexiline in the treatment of Prinzmetal angina [225] attests to the concept that its calcium antagonist effects in vascular smooth muscle are minimal.

Some recent studies have suggested additional mechanisms of action of perhexiline. First, in a desperate attempt to search for potential treatments of otherwise untreatable fibrodysplasia ossificans progressiva (FOP), a rare autosomal dominant disorder caused by gain-of function mutation in ACVR1 bone morphogenic protein type II receptor and characterized by progressive heterotopic ossification, Yamamoto and colleagues [226] screened 1040 FDA-approved drugs that suppress Id1, a transcriptional factor upregulated by the mutant receptor. They found that perhexiline (together with another similar hexadiline derivative fendiline) suppressed the Id1 promoter in a dose-dependent manner. Furthermore, perhexiline treatment also downregulated the phosphorylation of Smad1/5/8, driven by bone morphogenetic protein-2. Animal experiments showed promising results, that perhexiline treatment reduced the volume of heterotopic ossification by approximately 40% compared to controls. However, a subsequent open-labelled clinical trial in five patients failed to show prevention of heterotopic ossifications in FOP patients [227].

In another study searching for chemical modulators of autophagy, Balgi and colleagues used an automated cell based assay and screened > 3500 chemicals [228]. They found that perhexiline, together with amiodarone, niclosamide and rottlerin, were capable of increasing autophagosome content. Further assays showed that they inhibit signaling to mammalian target of rapamycin complex 1 (mTORC1) [228]. Autophagy is an adaptive cellular “self-eating” process that

compensates for the lack of extracellular nutrients by engulfing cytoplasmic components and also potentially increases efficiency of metabolism of residual mitochondria. Therefore, an increase in autophagy might contribute to the effects of perhexiline in increasing efficiency of myocardial metabolism. Although the therapeutic application is unclear, such results should facilitate the preclinical and clinical testing of autophagy induction.

In a similar bulk-screening process, it was found that perhexiline possesses some unique anti-cancer properties. It was found to internalize and degrade human epidermal growth factor receptor HER3, which is implicated in promoting the aggressiveness and metastatic potential of breast cancer [229]. A subsequent *in vivo* study showed that perhexiline inhibited breast cancer proliferation and tumour growth [229]. Another study found that perhexiline antagonized Hairy and Enhancer of Split 1 (HES1), a critical mediator required for tumour cell survival in T-cell acute lymphoblastic leukaemia, both *in vivo* and *in vitro* [230].

2.4.3 Spectrum of clinical efficacy: cardiac therapeutics

As originally marketed, perhexiline is useful for the prophylactic treatment of chronic exertional angina. It was highly effective, not only as an adjunct therapy but also as monotherapy, resulting in improved exercise tolerance and reduced consumption of glyceryl trinitrate [204,231]. With careful plasma drug concentration monitoring, Horowitz *et al* first demonstrated improved angina symptoms while avoiding significant adverse effects [232]. Cole *et al* further consolidated the effectiveness of therapeutic drug monitoring of perhexiline, demonstrating that in patients with otherwise refractory angina unsuitable for

coronary revascularization, perhexiline treatment (subject to therapeutic drug monitoring) effectively improved angina symptoms [233]. Similarly, in an open-labelled study, perhexiline was also reported to be associated with symptom improvement in patients with severe aortic stenosis unsuitable for valve replacement surgery [234]. A critical feature of these reports was the safety of perhexiline in the presence of systolic left ventricular dysfunction, in contrast to other calcium antagonists.

Perhexiline has more recently been investigated clinically both in the treatment of heart failure [235] and symptomatic non-obstructive hypertrophic cardiomyopathy [179]. For example, 56 chronic heart failure patients (with or without underlying myocardial ischaemia) with left ventricular ejection fraction of <40% (NYHA class II or III) on optimal drug therapy were randomly assigned to perhexiline or placebo. After 8 weeks of treatment, patients on perhexiline demonstrated significant improvement in oxygen consumption during peak exertion (peak VO_2), quality of life, and left ventricular ejection fraction [235]. When 46 symptomatic patients with non-obstructive hypertrophic cardiomyopathy were randomized to perhexiline or placebo for duration of 3 to 6 months, patients on perhexiline demonstrated improved myocardial ratios of phosphocreatine to ATP, left ventricular diastolic function, quality of life, and peak VO_2 [179].

2.4.4 Early and late toxicity

Early trials of perhexiline reported occasional nausea, epigastric pain and dizziness in patients at the beginning of therapy. In 1996, Stewart *et al* hypothesized that the emergence of nausea/dizziness was related to elevated plasma perhexiline concentrations [236]. It was found that such symptoms occurring during the first three days, following commencement of therapy was indeed strongly correlated with “toxic” plasma perhexiline concentrations. This suggested that in such patients, the development of nausea and/or dizziness is strongly predictive of accumulation of perhexiline beyond the therapeutic range of the drug (to be discussed further in the next section).

Consistent with the previously described effects of other CPT-1 inhibitors [237,238], there are also occasional reports of hypoglycaemia in diabetic patients associated with initiation of perhexiline treatment [239-242]; this change may be sufficient to require reduction in dosage of other hypoglycaemic agents.

Limited previous studies in patients with stable angina also indicated that utilization of a loading dose of 400mg per day of perhexiline maleate for three days is generally associated with therapeutic perhexiline concentrations within two to three days [232,236]. With such a loading regimen, 10 to 28% of patients experienced transient dizziness and/or nausea. Nevertheless, the lack of an intravenous preparation prompted the investigators to also evaluate a more aggressive loading regimen of perhexiline: - 800mg on the first day followed by therapeutic drug monitoring and 200mg daily on the next day in ten consecutive patients who were prescribed perhexiline, in order to attain therapeutic concentrations rapidly in the context of acute coronary syndromes. Given the lack

of reported adverse effects (apart from nausea and vomiting) in the study, such a rapid loading regimen has since been adopted widely [243], especially for high risk patients (see Table 2-I for general guidance on commencement of therapy and subsequent dose adjustment).

Reports of hepatic injury associated with chronic perhexiline began to emerge in the 1970s, after the drug was widely used for the prevention of angina pectoris [244-248]. In severe cases, cirrhosis and death have been reported [248]; while histological findings showed enlarged lysosomes containing myeloid figures, and phospholipidosis consistent with that seen with alcoholic liver disease [248].

Similarly, reports of peripheral neuropathy associated with long term treatment also have been reported [249-253]. An in vivo study found that four weeks of perhexiline treatment induced heavy lipid deposition in the neurons of the dorsal root and sympathetic ganglia in the rats. Furthermore, there was also a significant reduction in neuronal motor latency; and these effects were correlated with increased plasma and tissue concentrations of the drug [254]. When discovered early and treatment withdrawn, most of the adverse effects were reversible [250], but the return of neurological or hepatic function to normal may be slow.

Table 2-I: General algorithm for commencement of perhexiline therapy and interpretation of results.

Day	Dosage	Additional note
1	600-800mg stat	Measure plasma perhexiline and OH-perhexiline concentrations 12-24 hours post loading dose
2 onwards	200mg daily (or adjust as follows)	Continue on 200mg daily until results of day 1 returns.

Interpretation of result post loading of perhexiline:

If [OH-perhexiline] = 0 mg/L or undetectable (indicating poor metabolizer),

then hold dose for one week and change immediately to 100mg WEEKLY.

Repeat TDM after 4-6 weeks.

If [OH-perhexiline] is detectable but

(1) [Perhexiline] > 0.6mg/L or if patient develops nausea or vomiting, then hold dose for about 5-7 days and repeat TDM.
(2) [Perhexiline] falls between 0.15 – 0.60 mg/L, then continue on 200mg daily. Repeat TDM in about 5-7 days.
(3) [Perhexiline] falls between 0.10 – 0.15 mg/L, then increase dose to 250mg daily. Repeat TDM in about 5-7 days.
(4) [Perhexiline] < 0.10 mg/L, then increase dose to 400mg daily. Repeat TDM in about 5-7 days.

Suggested dosage adjustment for subsequent TDM, or during steady-state:

Perhexiline concentration (mg/L)	**Recommended New Daily Dosage**
< 0.15	Double daily dose
0.15 to 0.59	No change
0.6 to 0.89	Reduce by 25%
0.9 to 1.19	Halve daily dose
> 1.2	Cease for 1 week, then reduce daily dose to 25% of previous dose

OH-perhexiline/perhexiline ratio: indicates metabolising ability.

- **Ratio of <0.3:** indicates poor metaboliser status. Poor metabolisers will have a very low metabolite level (or no metabolite) and generally require doses of 50-100mg per week.
- **Ratio of 0.3 to < 2.5:** may indicate intermediate/saturated metabolism or inhibition of CYP2D6 by concurrent drugs. When increasing dose use very small (50mg) increments. These patients may require daily doses of <200mg.
- **Ratio >2.5 to 20:** indicates normal/ extensive metabolizer.
  Normal (intermediate) metabolisers generally require a dose of 100-200mg daily.
- **Ratio of >20:** may indicate ultra-rapid metaboliser.
  These patients may require a dose of >400mg daily.

Adapted from references [97,179,255]. Abbreviations: TDM = therapeutic drug monitoring.

2.4.5 Pharmacokinetics, pharmacogenetics and clinical utility of therapeutic drug monitoring

2.4.5.1 From pharmacokinetics to therapeutic drug monitoring of perhexiline

The (almost) global withdrawal of perhexiline from the market during the mid and late 1980s was not due to its lack of efficacy for the treatment of refractory angina pectoris. As mentioned in the previous sections, perhexiline was found to be very effective. Instead, it was the toxicities of perhexiline, which were poorly understood at that time, that led to its gradual withdrawal. Several observations during the late 1970s and early 1980s paved the way to better understanding the role of pharmacokinetics, pharmacogenetics and the metabolism of perhexiline play in its toxicities.

First, it was observed by Singlas *et al* [256] that plasma concentrations of perhexiline were elevated in patients with long-term therapy and experienced hepatotoxicity and neurotoxicity, when compared to unaffected individuals. They reported that the mean ratio of plasma concentrations of the parent drug to those of its major metabolite, monohydroxy-perhexiline, was up to nine times higher in the patients who experienced neuropathy than the unaffected patients [256], suggesting at that time that the accumulation of perhexiline was likely causative of its toxicities, and raising the possibility that accumulation reflected saturability of perhexiline metabolism.

Perhexiline is a lipophilic drug, well absorbed from the gastrointestinal tract. Its elimination is dependent on its metabolic oxidation to the more polar monohydroxylated and dihydroxylated metabolites which are cleared renally [257]. There is a large interindividual variation in the apparent plasma half-life of perhexiline [258]. It was suggested that perhexiline-induced neuropathy was secondary to some latent inborn metabolic disorder which increased individual's susceptibility [259].

In 1982, Shah *et al* [260] suggested genetic mutation in the metabolism of debrisoquine and perhexiline as a basis of impaired metabolism and potential toxicity. Twenty patients who had developed perhexiline-induced neuropathy had significantly lower oxidation of the antihypertensive drug debrisoquine than fourteen who did not. Eight hour urinary samples were collected after a single oral dose of 10mg debrisoquine, and the urinary ratio of unchanged debrisoquine over 4-hydroxy-debrisoquine (metabolite) was determined. The patients who had neuropathy had a median ratio of 14.4, compared to 0.65 in unaffected patients, suggesting an impairment of metabolic oxidation of both debrisoquine and perhexiline in affected patients. Furthermore, such impaired metabolism was independent of the patients' hepatic function, concurrent drug therapy, and tobacco or alcohol consumption [260]. Hence it was suggested that perhaps monitoring the phenotype of drug oxidation may provide a better prediction of potential risk of long-term toxicity in patients treated with perhexiline. Monitoring of levels might also facilitate appropriate dosing during long-term therapy.

Parallel with these findings, Horowitz *et al* [261] demonstrated that the metabolism of perhexiline in unselected patients with angina was nonlinear. This implied that the risk of disproportionate increase in plasma concentrations with small increase in daily drug dosage. Subsequently, it was observed that by adjusting steady-state doses of perhexiline to achieve plasma concentrations between 0.15 mg/L to 0.6 mg/L, long-term toxicity of perhexiline can be avoided [232]. This therapeutic range was later employed in a double-blinded placebo-controlled crossover trial of 3-month perhexiline in patients who already on maximal conventional antianginal therapy. During this trial, more than 60% of patients on perhexiline had significant symptomatic improvement (compared to none in the placebo group), without any apparent significant adverse effects [233].

2.4.5.2 Pharmacogenetics and clinical practice in relation to the administration of perhexiline

Having recognized the dependence of perhexiline (and debrisoquine) metabolism on the capacity of the individual to oxidatively metabolize the drug concerned in the liver, the mechanisms underlying such polymorphic variation was left to be recognized. During that time, it was known that the metabolic pathways of debrisoquine were also important for clearing many other drugs. Phenotypically, extensive metabolizers may excrete 10-200 times more of the urinary metabolite of debrisoquine than poor metabolizers [262-264]. Family studies also showed that the poor metabolizer phenotype behaved as an autosomal recessive trait with an incidence between 5% to 10% in the Caucasian population of Europe and North America [262-264]. In 1988, a study by Gonzalez *et al* [265] cloned and sequenced the human P450db1 complementary DNA, the molecular basis of this inherited trait. It was found that three variant messenger RNAs are products of mutant genes producing incorrectly spliced db1 pre-mRNA, providing a molecular basis for such defective gene. This member of the P450 family was later called CYP2D6, which is now known to affect the metabolism of more than 30 commonly used medications (see Table 2-II for examples) [266].

Table 2-II Currently used drugs affected by CYP 2D6.

Inhibitors of CYP2D6
Abiraterone, amiodarone Bupropion (strong) Celecoxib, cimetidine, cinacelcet (moderate), cobicistat Duloxetine (moderate) Fluoxetine (strong) Methadone, mirabegron Paroxetine (strong) Terbinafine (moderate)
Substrates of CYP2D6
Amitriptyline, aripiprazole, atomoxetine Bortezomib Carvedilol, chlorpromazine, cinacalcet, clozapine, codeine Dapoxetine, darifenacin, dextromethorphan, donepezil, duloxetine Flecainidine, fluoxetine, fluvoxamine Galantamine, gefitinib Haloperidol Imipramine Lignocaine Metoclopramide, metoprolol Nebivolol, nortriptyline Olanzapine, ondansetron, oxycodone Paroxetine, perhexiline, propranolol Risperidone Tamoxifen, tolterodine, tramadol Venlafaxine, vortioxetine

Adapted from reference [266].

Confirming the central role of CYP2D6 in the hydroxylative metabolism of perhexiline has a number of implications. First, to genotype patients who are likely to be on long-term treatment to identify at-risk patients and individualize therapy would seem ideal. However the benefit of such process might be outweighed by the potential for more than one metabolic pathway involved, need for prospective testing, lack of correlation between pharmacology and genetic testing (for example, via drug-drug interactions), and an unnecessarily high cost associated with genetic testing [267]. Therefore, in the case of perhexiline, phenotypic expression of perhexiline metabolism was usually derived, using the ratio of plasma cis-OH-perhexiline/perhexiline concentrations. In 70 patients who had attained steady-state plasma perhexiline concentrations, it was found that the clearance of plasma perhexiline was correlated not only with steady-state daily dose but also with the ratio of metabolite/parent drug [255]. This phenotypic metabolizer ratio of metabolite/parent drug concentration ratio provided a guide to individualize therapy and drug dosage determination at steady-state. CYP2D6 “poor metabolizers” were identified as those with a concentration ratio of cis-OH-perhexiline to perhexiline of ≤ 0.3; ultra-rapid metabolizers were defined as those with a ratio of > 20; and those with a metabolizer ratio in between were identified as intermediate to extensive metabolizers [255].

Such metabolizer ratio status was later evaluated in prospective studies. Patients with one functional allele of CYP2D6 had significantly higher plasma perhexiline concentration and lower metabolic ratio compared to those with two functional alleles, thus confirming a gene-dose relationship

for the metabolism of perhexiline [268,269]. Furthermore, such a relationship between genotype and metabolic ratio of perhexiline was established in patients who had a standard loading dose regimen consisting of 200mg of perhexiline maleate twice daily for three days [269]. This suggested that assessing the relative CYP2D6 metabolic capacity using concentration ratio of cis-OH-perhexiline and perhexiline during the early phase of therapy is useful for dosage individualization and determining potential long-term maintenance dosage regimens.

Today, CYP2D6 is one of the best characterized polymorphic CYP enzymes, with more than 75 allelic variants identified [270], resulting in a spectrum embracing increase, reduction, or complete loss of enzyme activity. Phenotypically, this variable activity corresponds to classification as either poor, intermediate, extensive and ultrarapid metabolizers. In general, the prevalence of "ultra-rapid metabolizers" among the Ethiopians is probably highest at about 29% [271]. On the other hand, the prevalence of "poor metabolizer" in the Caucasian population ranges from 7 to 10%, the Hispanics and the African-Americans only about 2-6%, and among East Asians are less than 2% affected [270]. The issue of regional heterogeneity of CYP2D6 status among Asians remains incompletely evaluated [272].

While accounting for only 2-5% of all hepatic CYP enzymes, CYP2D6 is known to affect the metabolism of approximately 25% of all clinically used pharmaceutical agents [270]. The investigation of CYP2D6-related metabolism of drugs had originally proven to be difficult in animals. It was not known until later that the expression of the CYP2D6 enzymes, like in humans, are also highly variable between species and strains, hence

affecting the kinetics of drugs depending on the species or strain of animals used in the experiments [273]. In general, female Dark Agouti rats serve as phenotype of "poor metabolizer" [274], and male Sprague Dawley or Wistar rats serve as "extensive metabolizer" phenotype [273].

2.4.5.3 Impact of drug-drug interactions

In general, any drugs with pharmacokinetics that are affected by CYP2D6 have the potential to affect the kinetics of perhexiline. Reported cases have involved antifungals like terbinafine [275] and selective serotonin reuptake inhibitors like paroxetine and fluoxetine [276]. However, given the availability of therapeutic plasma drug monitoring, it is likely that the adverse effects arising from such predictable drug-drug interactions may be minimized via increased frequency of plasma drug concentration monitoring.

There is also theoretical scope for drug interactions based on overlapping biological effects. First, given that perhexiline is a known inhibitor of CPT-1, it could be speculated that concurrent treatment with another CPT-1 inhibitor may increase potential neuro- or hepatotoxicity. Additionally, excessive inhibition of fatty acid metabolism without adequate compensation of increased glucose oxidation could also be potentially detrimental to myocardial energetics. In a retrospective case series of twenty six patients on concomitant treatment with amiodarone (another CPT-1 inhibitor [176]), of whom twenty were on long-term treatment, none of the patients had either medication ceased due to adverse effects. Additionally, onset of hypoglycaemia (acute adverse effect) was also rare (occurring only in five of the patients) and was clinically silent [240].

2.4.5.4 Impact of disease-drug interactions

After the hepatic hydroxylation of perhexiline into mono- or dihydroxylated metabolites, the (predominantly) inert metabolites are cleared renally. To date, there is no evidence that these metabolites cause any clinically significant effect. However, despite a lack of supporting evidence to suggest that renal failure affects the safe use of perhexiline, the product information still cites renal dysfunction as a contraindication. In a retrospective study involving two hundred patients on steady-state long term perhexiline, it was found that a positive correlation was observed between creatinine clearance and maintenance dose on a univariate comparison. However, on a multivariate analysis, this relationship was insignificant as weight and renal function were interdependent. Although statistical association may not suggest causation, and a role for renal dysfunction cannot be completely excluded, the prediction of maintenance dose does not require adjustment for renal function *per se* [277].

In the same study, it is also found that impaired left ventricular systolic function does not influence the maintenance dose of perhexiline required, suggesting that despite perhexiline's narrow therapeutic index and potential toxicity, dosage adjustment is not required in patients with systolic dysfunction. Additionally, the study also found a decrease in the maintenance dose with age, but an inverse relationship with weight. Such a finding may reflect the influence of age-related muscle wasting on the volume of distribution or changes in first-pass metabolism and bioavailability in relation to the liver size [277].

2.4.6 Enantiomer-specific actions

Many drugs are marketed as a racemic mixture of two enantiomers. Given that most drugs often exert their effects on proteins, which are three-dimensional in nature; it is no surprising that pharmacokinetic and pharmacodynamics differences often exist between the enantiomers. Pharmacokinetic processes may be enantioselective at the level of absorption, distribution, metabolism and excretion. Genetic considerations and patient factors such as age, concomitant drugs or disease state can also play a role in determining the relative concentration of enantiomers of a racemic drug. Perhexiline is clinically administered as a 50:50 racemic mixture of (+) and (-)-enantiomers. Some previous studies have suggested that these enantiomers may exert different effects.

This was first investigated by Gould *et al* in 1986 [278]. In this study eight healthy volunteers were given 300mg of individual enantiomers and racemate perhexiline. Blood samples were collected every two hours and urine samples were also collected for measurement of perhexiline metabolites. The study showed that there were higher plasma and urine cis-hydroxyperhexiline concentrations for those who were administered (-)-perhexiline than its antipode. However, none of the volunteers were phenotyped or genotyped with respect to their CYP2D6 metaboliser status and hence the potential contribution of metabolic heterogeneity to the results was uncertain.

Recent (unpublished) work in our laboratory suggested that while the different enantiomers exerted similar effects on superoxide production from patients' or healthy volunteers' neutrophils [as measured by superoxide formation

in neutrophils when stimulated with N-formylmethionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA)], in the Dark Agouti rats which were treated with individual enantiomers, the enantiomers induced differing histological changes in hepatocytes. In the rats that were treated with (+)-perhexiline, histology showed increased fatty deposits throughout hepatocytes, suggesting that (+)-perhexiline is potentially associated with inhibition of fatty acid utilization. In addition, von Frey filament testing (study of neuronal function) and electron microscopy studies on the dorsal root ganglion of the treated animals suggested that (+)-perhexiline is associated with impaired neuronal function and neuropathy. These hepatic and neuronal effects of (+)-perhexiline are similar to the toxicity profile of chronic perhexiline clinically, and potentially resulted from extensive CPT-1 inhibition resulting in reduction of fatty acid oxidation (one of the hypothesis to be explored in this *thesis*: see Chapter 7).

On the other hand, the rats that received (-)-perhexiline seemed to display different metabolic effects. Both histological and biochemical results indicated that there was a significant higher glycogen content and lack of lipid droplets in the hepatic tissue of rats that were treated with (-)-perhexiline, suggesting that a potential independent effect of (-)-perhexiline in association with increased carbohydrate utilization. This indicated that metabolic pathways other than that of CPT-1 inhibition might be involved with the administration than perhexiline. In addition, in a rat model of myocardial necrosis induced by isoprenaline injection, the extent of cellular necrosis (as measured by an elevation of troponin levels) was greater in the group that was previously treated with (-)-perhexiline. This disparity suggested that there is a substantial difference in the pharmacology of the

enantiomers and in their potential clinical effects. However, these results should currently be regarded as preliminary only.

2.5 Unanswered questions regarding perhexiline

"New tricks for an old drug."

M Frenneaux, 2002.

2.5.1 Is perhexiline safe when used long-term in the modern era of therapeutic drug monitoring?

The currently used therapeutic range for perhexiline concentrations in plasma has been utilized in short term trials, ranging from three to twelve months [97,233,235]. As demonstrated in previous sections, significant adverse effects are rarely reported when therapeutic monitoring of perhexiline is in place. However, given the increasing recognition that heart failure and many other myocardial disease states (including hypertrophic cardiomyopathy) share a common association with myocardial energetic impairment, the spectrum of clinical indications for perhexiline long-term therapy will potentially increase and therefore an understanding of its long-term safety is warranted. When used in the long-term, it is also recommended that plasma level monitoring is required every three months. However, in the modern era of regular therapeutic monitoring of perhexiline, it is unknown whether such precautions achieve their safety objectives.

2.5.2 Modulation of kinetics

Although the utility of therapeutic drug monitoring is predicated on the idea that plasma drug concentration is predictive of tissue drug concentration (whether

it represents target tissue for efficacy or toxicity), such a hypothesis has rarely been tested in the case of cardioactive drugs in humans, including perhexiline. Additionally, the uptake of this drug into the myocardium, have not previously been reported, due to the inherent difficulty in obtaining human myocardial tissue samples. On the other hand, animal studies have provided adequate information on the principles modulating myocardial drug uptake, which is not affected by plasma drug concentration but by heart rate and the relative octanol: water partition coefficients [279]. Additionally, active transport mechanisms may also further modulate the uptake kinetics and therefore, in theory, the ratio of plasma to myocardial drug concentrations may vary between individuals. This area of interaction is potentially further complicated by the concept that fibrotic infiltration of myocardium might affect the uptake of perhexiline into this ultimate "central" compartment.

2.5.3 The pleiotropic effects of perhexiline

2.5.3.1 Energetics-based effects (other than CPT-1)

Previous chapter (Chapter 1.5.1.3) discussed the effects of perhexiline on the expression of TXNIP, an inflammatory modulator highly expressed in diabetics. It was found that TXNIP expression in human myocardium was lower in perhexiline-treated patients than in those receiving placebo [280]. The relevance of this to the clinical utility of perhexiline is unknown. First, it is unclear if the occasional hypoglycaemic events observed with commencement of perhexiline in insulin-dependent patient [239] reflected a resultant effect from increased

glycolysis or improved insulin sensitivity. It is possible that suppression of TXNIP secretion contributes to the favourable impact of perhexiline on energetics; with TXNIP changes being driven by activation of AMPK. Potential comparison of perhexiline effects in mice with intact versus knockout [144] TXNIP expression might delineate the potential role of TXNIP in mediating perhexiline effects.

2.5.3.2 Non-energetics based effects

Perhexiline is known to exert anti-inflammatory effects [187,209,281], essentially involving NOX-2 production by leukocytes. While these are desirable in cardiovascular disease, it is again unknown if these biochemical effects contribute to the clinical benefits of perhexiline. The recently described effect of perhexiline to potentially increase HDL is surprising [211] and may serve as basis for future therapeutic development. Finally, within the last 2 years, a number of preclinical studies have suggested that perhexiline may represent an adjunctive treatment for various neoplasms: again it is not yet clear whether such anti-neoplastic effects are entirely CPT-1 dependent [229,282-285].

2.5.4 Enantiomer-specific actions of perhexiline: unanswered questions

Previous research within the Clinical Pharmacology Deparment, the Queen Elizabeth Hospital showed that long-term administration of individual perhexiline enantiomers resulted in differential neuropathic and hepatotoxic effects in Dark Agouti rats [286]; suggesting that the two enantiomers may possess differential

major pharmacological actions. If one of the enantiomers possess favourable-effect-versus-side-effect profile, this may lead to further pharmaceutical development. The ongoing search of the other non-energetic based effects of the enantiomers (as described above about perhexiline) is warranted.

Parallel to this is the development of "new" perhexiline analogues by chemists at the Kosterlitz Institute, Aberdeen, United Kingdom. These analogues would ideally mitigate the necessity a derivative's being a substrate of CYP2D6 (and thence its susceptibility to human genetic variance and necessity for clinical therapeutic drug monitoring). Their mechanism(s) of actions, and therefore clinical utility, warrant further investigation but shed light on the potential future role of cardiac energetic agents.

Chapter 3: An audit of current outcomes in patients treated chronically with perhexiline

3.1 Abstract

3.1.1 Background

Perhexiline, originally utilized as a first-line prophylactic anti-anginal agent, is now regarded primarily as a treatment for otherwise refractory myocardial ischaemia. Recent studies have also demonstrated its short-term utility in heart failure, hypertrophic cardiomyopathy and inoperable aortic stenosis. Its benefits on myocardial energetics state are potentially counter-balanced by risk of hepatotoxicity and peripheral neuropathy during long-term treatment if drug accumulation occurs. Since perhexiline exhibits complex pharmacokinetics with wide inter-individual variabiltiy, its long-term use requires regular plasma level monitoring. In the current study, the risk of neuro- and hepato-toxicity during long-term perhexiline therapy in relation to the intensity of therapeutic drug monitoring was investigated. Furthermore, determinants of mortality during perhexiline treatment were evaluated.

3.1.2 Method

In 170 patients treated with perhexiline for a median of 50 months (IQR: 31 – 94 months), outcomes and relationship to plasma drug concentrations were documented.

3.1.3 Results

Rationale for treatment with perhexiline included myocardial ischaemia (in 88%), and severe systolic heart failure in 38%. Plasma concentrations were within the therapeutic range of 150-600ng/ml on 65% of assay occasions and toxic levels accounted for 8.8% of estimates. No patient developed hepatotoxicity attributable to perhexiline while three developed peripheral neuropathy possibly induced by treatment. Actuarial 5-year survival rate was 83% overall, and 76.3% in patients with associated systolic heart failure.

3.1.4 Conclusion

This first audit of a large population treated long-term perhexiline demonstrates that:-

(1) therapeutic drug monitoring effectively limits occurrence of toxic drug levels and virtually eliminates long-term hepato/neurotoxicity.

(2) mortality rates during long-term therapy, notably for patients with concomitant heart failure, are surprisingly low.

3.2 Introduction

Perhexiline maleate (discussed in detail in *Chapter 2*) was used originally as a first-line, prophylactic anti-anginal agent, both as monotherapy and later as add-on therapy. Despite its efficacy, reports of development of long-term adverse effects (hepatitis and/or peripheral neuropathy) and increased availability of other anti-anginals led to its gradual decline in usage in the 1980s. However, later recognition of the mechanism of perhexiline toxicity and its prevention via therapeutic drug monitoring had kept the agent in clinical usage, usually as a last-line therapy. Subsequent studies had also provided evidence that it might improve symptomatic status in patients with aortic stenosis [234], systolic heart failure (ischaemic or otherwise) [235] and hypertrophic cardiomyopathy [179].

In the current analysis, the long-term safety of perhexiline in a cohort of Australian patients treated, in the main, for otherwise refractory ischaemia and/or heart failure were evaluated. It was sought to determine:-

1. The incidence and determinants of serious long-term perhexiline toxicity (that is, hepatotoxicity and/or peripheral neuropathy).
2. Mortality rates during perhexiline therapy for the entire patient cohort and for those with concomitant systolic heart failure.
3. The relationships between long-term outcomes and
    (a) achievement of therapeutic drug concentrations
    (b) frequency of monitoring of steady-state perhexiline levels, given that 3-monthly determination of levels is currently recommended [266].

The results provide incremental evidence of the safety of perhexiline in long-term treatment. Furthermore, the survival data raise the possibility that this form of treatment may improve prognosis in the populations currently evaluated.

3.3 Methods

3.3.1 Patient selection

Patients were evaluated on the basis of at least 12 months of perhexiline therapy for a currently recommended indication with associated records of follow-up including cardiac clinic review.

The only criteria for non-prescription of perhexiline in this cohort were: -

(1) Severe chronic hepatocellular disease

(2) Previous severe adverse reaction to perhexiline

(3) Anticipated inability to comply with monitoring requirements

Diabetes mellitus (with or without associated peripheral neuropathy), concomitant renal insufficiency, and treatment with potentially interacting drugs (such as amiodarone [240], selective serotonin reuptake inhibitors [276] or antifungal terbinafine [275]) were not contraindications to perhexiline therapy at entry.

3.3.2 Therapeutic drug monitoring

Determinations of plasma perhexiline concentrations (and those of its major mono-hydroxylated metabolite) were performed via high-performance liquid chromatography as previously described [261]. Ideally, monitoring was repeated frequently until levels were stable within the previously described therapeutic

range and then repeated 3-monthly [266]. However, this was a suggested rather than strictly enforced frequency of monitoring. Actual frequency of monitoring was documented throughout the treatment period, together with results of these analyses.

3.3.3 Follow-up

Hepatotoxicity, whether likely due to perhexiline or otherwise, was defined on the basis of at least 3-fold elevation of liver function tests beyond the upper limit of the testing laboratory. Peripheral neuropathy, whether predominantly sensory, motor or autonomic, was diagnosed only in the presence of objective clinical abnormalities. In all cases, appearance of such anomalies was correlated with simultaneous plasma perhexiline concentrations.

Data regarding mortality rates were retrieved from the Registry of Births, Deaths and Marriages of South Australia and presumptive causes of death recorded. Variables selected for multivariate backward logistic regression analyses to identify independent predictors of mortality were age, gender, duration of perhexiline therapy, history of coronary artery disease, presence of aortic stenosis, heart failure, diabetes mellitus, proportion of drug assays within therapeutic range, and proportion of drug assays over-therapeutic range.

3.3.4 Statistics

All data for normally distributed parameters are described as mean ± SEM; for skewed data medians and interquartile ranges are given.

Survival rates (overall and for patients who had concomitant heart failure) were quantitated by Kaplan-Meier analysis, and correlates of survival rate were evaluated by multiple logistic backward regression analysis. All analyses were performed with the SPSS version 20 software (SPSS, Chicago, IL, USA).

3.4 Results

Table 3-I summarizes the clinical demographics of the 170 patients in this study cohort. In general, patients were elderly. Known coronary disease and therefore potential myocardial ischaemia was present in 88% of cases while 37.6% had symptomatic heart failure. Furthermore, aortic stenosis represented an occasional component of the indication for perhexiline therapy (12.9%).

Figure 3-I summarizes metaboliser phenotype. The majority of the patients were intermediate to extensive metabolisers (see Figure 3-I for criteria [255]) and received daily doses of perhexiline maleate between 125 mg and 200 mg; 1.8% were poor metabolisers who received 50 to 100 mg weekly; and 4.12 % were ultra-rapid metabolisers who received 100 to 250 mg daily.

Table 3-I: Baseline characteristics of the 170 patients in this study cohort.

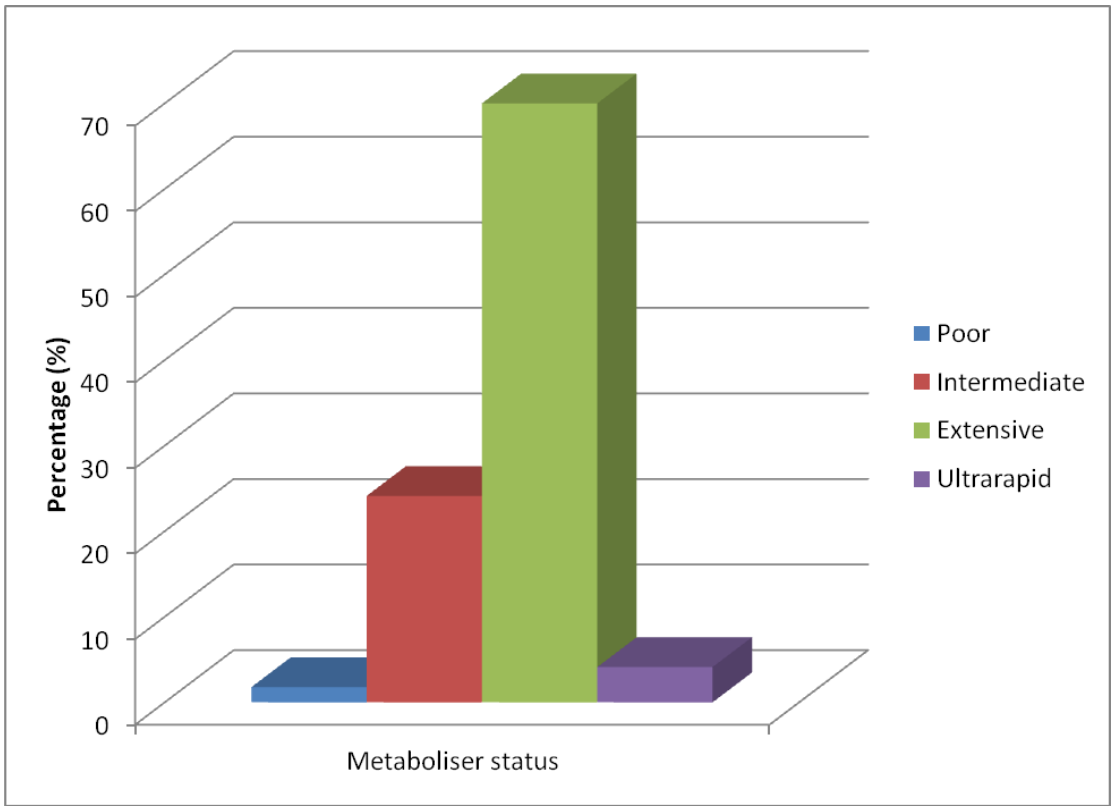
Male (%)	58
Age (years)	74 (65 – 81)*
Duration of treatment (months)	50 (31 – 94)*
Cardiovascular History	
Coronary artery disease (%)	88.2
Previous myocardial infarction (%)	58.8
Previous coronary artery bypass (%)	37.6
Previous percutaneous coronary intervention (%)	33.5
Aortic stenosis (%)	12.9
Systolic heart failure (%)	37.6
Atrial fibrillation (%)	17.1
Non-cardiac disease	
Diabetes mellitus (%)	37.1
┼ Known peripheral neuropathy (pre perhexiline) (%)	7.1
Δ Known hepatic disease (pre perhexiline) (%)	1.2
Significant alcohol intake (%)	2.4

* median (interquartile range)

Δ mild abnormalities of liver function tests at entry

┼ 10 of 12 patients were diabetic

Figure 3-I: Distribution of metabolizers' phenotypes in this cohort of patients.



Metabolizer status was defined by the ratio of plasma concentrations of cis-hydroxyperhexiline to perhexiline at steady-state. Poor metabolizer was defined by a ratio of ≤ 0.3; intermediate metabolizer was defined by a ratio of 0.3 to 2.5; extensive metabolizer was defined by a ratio of 2.5 to 20; and ultra-rapid metabolizer was defined by a ratio of ≥ 20.

Over the period of follow-up, as shown in Table 3-II, 65% of drug concentrations measured were within the therapeutic range of 150 to 600 ng/L [232], the majority of other levels were subtherapeutic (26.2%) rather than potentially toxic (8.8%). The overall frequency of monitoring appeared suboptimal: only 32% of patients had at least 3-monthly drug monitoring, as currently recommended by the Australian guidelines [266].

Table 3-II: The distribution of concentrations with respect to therapeutic range of 150-600 ng/ml and the frequency of monitoring during study period.

Quality of monitoring	
Proportion of concentration within therapeutic range (%)	65
Proportion of concentrations above therapeutic range (%)	8.8
Proportion of concentrations below therapeutic range (%)	26.2
Frequency of monitoring over total study period	
At least three monthly (%)	32
Three to six monthly (%)	48
Less than six monthly (%)	20

Elevation of hepatic enzyme levels beyond population norms, usually transient, occurred in 51 cases. Of these, potential causes included 21 cases of gastrointestinal disease (cholecystitis, pancreatitis and gallstones), 2 of known hepatitis B or C, and 2 of metastatic hepatic infiltration. There were also 8 cases of ischaemic hepatitis associated with acute heart failure. Two of the cases appeared to be related to other drugs such as HMG-CoA inhibitors (statins). In 12 cases of

hepatitis, although of uncertain cause, could not be attributed to perhexiline as the elevation of plasma concentrations of hepatic enzyme was transient and resolved despite continuation of therapy. Overall, none of the cases of abnormal hepatic function appeared likely to be related to perhexiline therapy. In particular, in no case was there "classical" perhexiline hepatotoxicity: that is simultaneous elevation of perhexiline levels and development of abnormal liver function [231,232,287].

There were also a total of four new cases of presumptive peripheral neuropathy in the course of the study, although in one case objective signs were never elicited. In the three other suspected cases, perhexiline could not be excluded as a potential cause of peripheral neuropathy. One of these three patients was a diabetic. In the two non-diabetic patients with define peripheral neuropathy suspected to be perhexiline-induced, one had known peripheral vascular disease. Once again, none of these cases were associated with supra-therapeutic perhexiline levels and none occurred in poor metabolisers (Table 3-III).

Table 3-III: Serious toxicity potentially caused by long-term perhexiline.

Peripheral Neuropathy					
Patient	**Other risk factors**	**Metaboliser status**	**Monitoring frequency**	**Monitoring period (months)**	**Proportion of over-therapeutic values (%)**
1	Peripheral vascular disease	Intermediate	Less than 6-monthly	197	7.15
2	Nil	Rapid	3-6 monthly	58	0
3	(Diet-controlled) Diabetes mellitus	Extensive	Less than 6-monthly	124	10

No cases of hepatotoxicity were deemed likely to be induced by perhexiline.

As regards to mortality, actuarial survival of the entire cohort and that of patients with concomitant systolic heart failure is shown in Figure 3-II. Five and ten-year survival rates were 83% and 72.6% % respectively for the entire cohort, and 76.3% and 63.4% for patients with heart failure. On multivariate analyses (Table 3-IV), deaths on perhexiline tended to occur significantly more commonly during initial years of therapy (p=0.021), and there were trends towards increased mortality with advanced age (p=0.064) and for patients with associated coronary artery disease (p=0.068).

Figure 3-II: Kaplain-Meier curves over the first ten years of therapy for all patients (n=170), and for those who had concomitant systolic heart failure (n=82).

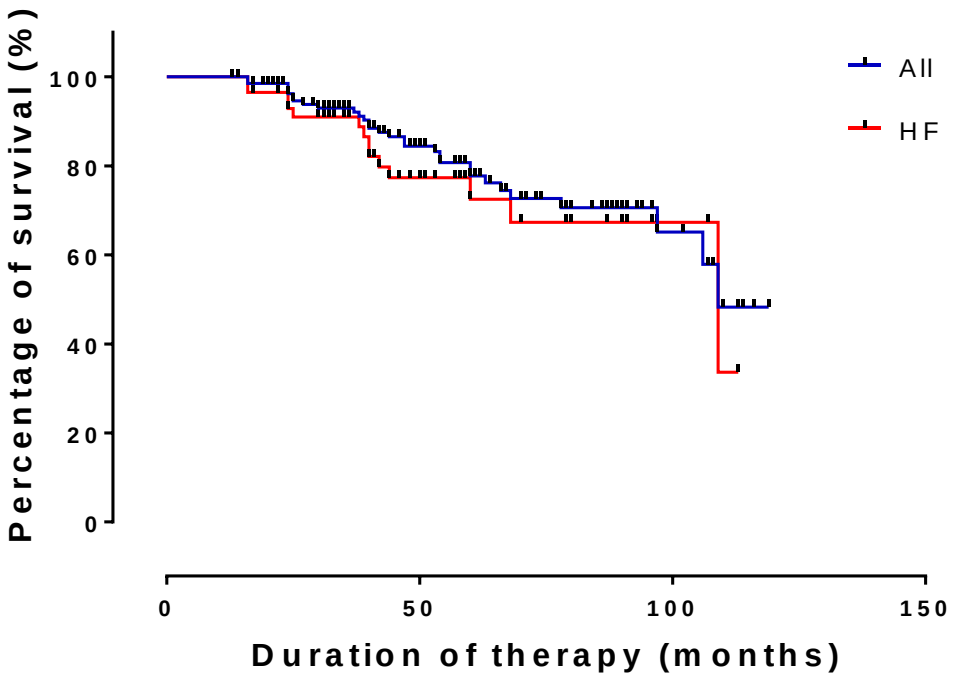


Table 3-IV: Multivariate (backward stepwise multiple logistic regression analysis) correlates of mortality in the current study cohort (n=170).

Parameter	**β-coefficient**	***p*-value**
Age	0.026	0.064
Duration of perhexiline treatment	-1.447	0.021
History of coronary artery disease	1.258	0.068

Cox and Snell R^2 = 0.078

3.5 Discussion

The current data represent the most extensive documentation of long-term safety of perhexiline in the modern era of routine therapeutic drug monitoring. Initial experience with perhexiline, confined to its role as a prophylactic anti-anginal agent, suggested remarkable clinical efficacy, but the serious long-term complications of hepatitis and peripheral neuropathy were documented in as many as 40% of cases [204]. In the current series, presumptive long-term toxicity (limited to three cases of peripheral neuropathy) occurred with an incidence of only approximately one case per 236 patient-years' utilization.

The basis for this vastly improved safety profile was superficially routine monitoring of plasma perhexiline concentrations. There is ample evidence that most cases of previously reported long-term toxicity occur with prolonged (>3 months) elevation of plasma perhexiline concentrations, beyond 600 ng/L [231,232]. The strategy of maintaining levels below that point has been successful in limiting toxicity, although this has been tested previously mainly in short-term exposure [179,235]. Plasma level monitoring at frequencies of at least 3 monthly has been recommended, and the lower limit of the therapeutic range has been set, largely on an arbitrary basis at 150 ng/L [232].

The current data confirm the considerable inter-individual variability in perhexiline pharmacokinetics. As expected, daily dosage to achieve therapeutic levels ranged widely (from less than 100mg per week to 400mg per day). Fewer patients than expected were poor metabolizers of the drug. Furthermore, very few monitored levels (approximately 8.8% of total readings, occurring in 57% of patients) were above the therapeutic range, and levels of more than twice the

therapeutic range were virtually never seen. Hence the avoidance of toxicity was less than surprising.

However, this occurred despite apparently suboptimal frequency of monitoring in two thirds of cases. In some cases, the associated plasma perhexiline levels were below the therapeutic range (i.e. <150 ng/L). The issue of extent of therapeutic effect associated with such low levels should therefore be evaluated in placebo-controlled studies.

The other important change, which has occurred in the general utilization of perhexiline in the last 20 years, has been the transition from use only as a prophylactic anti-anginal agent to that of a second-line treatment for systolic heart failure [235,288], particularly in patients with contra-indications to use of other agents. A substantial minority of the current patient series had heart failure, often with associated renal insufficiency. The 5-year survival rate of the entire cohort was 83%, while in the presence of heart failure that fell slightly. While these data are not placebo-controlled, the very extensive survival of a group of elderly individuals with serious and multiple morbidities seems remarkable, as other heart failure studies (without perhexiline) demonstrated a lower 5-year survival rate at 26-55% [289]. On multivariate analyses, ischaemic heart disease, rather than heart failure, tended to predict increased death rates: these results are superficially surprising.

Furthermore, it is even possible that perhexiline may improve outcomes in patients with myocardial ischaemia secondary to coronary atherosclerosis: a recent study in mice demonstrated that perhexiline functions as an activator of Kruppel-like factor 14, thus elevating concentrations of Apo-A [211]. Therefore,

perhexiline might exert additional anti-atherogenic effects with consequent cardioprotection.

The study has several limitations. In the three cases of hepato- and neuro-toxicity of uncertain cause: perhexiline was potentially, but not definitely, implicated. Indeed, none of these cases had associated “toxic” drug levels. Secondly, measurement of hepatic and neurological functions may have only occurred in patients with clinical symptoms, which may itself present as a source of bias for the study. Thirdly, although most patients would have had moderate to severe impairment of exercise capacity at entry, this was not recorded in all cases, hence the omission. Lastly, given the current study is not placebo-controlled, the therapeutic and mortality efficacy of perhexiline cannot adequately be determined. However, the current data demonstrate considerable safety, and provide preliminary evidence that therapy with perhexiline should be considered as an appropriate option for difficult cases of myocardial ischaemia and/or heart failure. Future prospective randomized controlled placebo trials evaluating the mortality benefits of perhexiline studies will be needed to confirm this.

3.6 Acknowledgement

This chapter forms the basis of publication in *Therapeutic Drug Monitoring* 2015 (see Appendix). Helen Phuong, Bo Y Choi and Betty Raman had provided assistance in data collection and confirmation of adverse events related to perhexiline.

Chapter 4: Utility of perhexiline for perioperative redox stress – pharmacokinetics versus effect considerations

4.1 Abstract

4.1.1 Purpose

Perhexiline is a prophylactic anti-ischaemic agent with weak L-type calcium antagonist effects which has been increasingly utilized in the management of refractory angina. The metabolic clearance of perhexiline is modulated by CYP2D6 metaboliser status and stereoselectivity. The current study sought to determine (1) whether the acute accumulation of perhexiline in the human myocardium is stereoselective, and (2) to investigate the relationship between duration of short-term therapy and the potential stereoselective effects of perhexiline within myocardium.

4.1.2 Method

Patients (n=129) from the active arm of a randomised controlled trial of preoperative perhexiline in cardiac surgery, were treated with oral perhexiline for a median of 9 days. Correlates of atrial and ventricular concentrations of enantiomers were sought via univariate followed by multivariate analyses.

4.1.3 Results

Myocardial uptake of both (+) and (-)-perhexiline was greater in ventricles than atria, and there was more rapid clearance of (-) than (+)-perhexiline. The main determinants of atrial uptake of both (+) and (-)-perhexiline were the plasma concentrations [(+)-perhexiline: β=-0.256, p=0.015; (-)-perhexiline: β=-0.347, p=0.001] and patients' age [(+)-perhexiline: β=0.3, p=0.004; (-)-perhexiline: β=0.288, p=0.005]. Atrial uptake of (+)-enantiomer also varied directly with duration of therapy (r=0.29, p=0.004), while atrial uptake of (-)-perhexiline varied inversely with simultaneous heart rate (r=-0.22, p=0.03).

4.1.4 Conclusion

(1) Uptake of both perhexiline enantiomers into human atrium is greater with advanced age, and displays evidence of both saturability and minor stereoselectivity.

(2) Atrial uptake of (-)-perhexiline may selectively modulate heart rate reduction.

4.2 Introduction

Perhexiline is a metabolic modulating agent that was first introduced into clinical practice as a prophylactic anti-anginal drug in the 1970s. It exerts multiple effects which increase efficiency of myocardial metabolism: amongst these it is known to inhibit mitochondrial carnitine palmitoyl transferase-1 [176], potentially resulting in a shift from fatty acid to glucose utilisation with more adenosine triphosphate production per unit oxygen consumption.

Despite the considerable clinical efficacy of perhexiline in prophylaxis of exertional angina [203,204], its use declined because of the substantial risk of hepato- and neuro-toxicity during chronic therapy [290-292]. However, it emerged that this toxicity reflected drug accumulation in plasma [256] which in turn resulted from inter-individual variability in CYP2D6-mediated metabolism [293,294]. With widespread availability of therapeutic drug monitoring [232,233] and greater understanding of its potential widespread utility for disorders of cardiac energetics [2], the clinical use of perhexiline is now increasing.

A number of studies have specifically evaluated short-term utility of perhexiline in the management of potential cardiac crises, such as the management of high risk patients with unstable ischaemia and for cardioprotection during coronary revascularisation [210,236,243]. The recently reported CASPER trial evaluated its use as an adjunct to myocardial protection in patients undergoing coronary artery surgery but found no clear-cut beneficial effect of prophylactic perhexiline therapy [295]. During this trial, atrial and ventricular myocardial biopsies were taken at the time of surgery. We have previously

reported on analyses of these samples to evaluate the relationship between plasma and myocardial drug concentrations [296]. The objective of the current analysis stems from our recent observation that the effects of racemic perhexiline may result from unequal steady-state concentrations of its two enantiomers [269].

In human liver microsomes, the intrinsic clearance of (-)-perhexiline is greater than that of the (+)-enantiomer [297], which explains the greater clearance rate of (-)-perhexiline at steady state in patients receiving racemic perhexiline [278]. Interestingly, it appears from studies in a rat model, that the safety of the two enantiomers may vary, with greater hepatotoxicity associated with the (+)-enantiomer [286].

Therefore, the CASPER data was re-evaluated in order to determine:

(1) Whether atrial and ventricular myocardial accumulation of perhexiline during short-term treatment are stereoselective, and whether this reflects similar trends in plasma enantiomer concentrations.

(2) The relationship between duration of (short-term) perhexiline therapy and the potential for stereoselective effects of perhexiline within the myocardium.

4.3 Methods

Data were mainly derived from patients in the active treatment arm of the CASPER (Coronary Artery Surgery with PERhexiline therapy) trial (NCT00845364). In brief, this was a double-blind, randomized, placebo-controlled clinical trial evaluating whether preoperative oral perhexiline to improve myocardial protection in patients undergoing cardiac surgery. Non-diabetic patients, who were not taking CYP2D6 inhibitors, undergoing first time coronary artery bypass graft surgery, were randomized to either perhexiline maleate or placebo for at least five days prior to surgery. All patients received the following medication regimen: 200mg twice daily for three days then 100mg twice daily until the morning of the surgery [295].

Following induction of anaesthesia but prior to commencing surgery, the haemodynamic status of each patient, including heart rate, arterial pressures and cardiac index were determined. Plasma collected at this time point was centrifuged and stored at -80°C; this has previously been used to phenotype patients for CYP2D6 metaboliser status [295] according to the plasma concentration ratios of perhexiline monohydroxylated metabolite to parent drug [255]. During preparation for cardiopulmonary bypass but prior to aortic cross-clamping, right atrial and left ventricular myocardial biopsies were obtained, as previously described [295]. These biopsies were initially snap-frozen in liquid nitrogen and stored at -80°C, then later digested in 0.15 M of phosphate buffer solution (pH 6.0) using a homogenizer and tissue grinder to form a suspension (approximately 100mg tissue in 5ml buffer). Plasma and myocardial perhexiline enantiomer

concentrations were determined utilizing a modification of a previously described HPLC assay [298]. Thresholds for detection of myocardial (+) and (-) perhexiline were 0.01mg/L.

4.4 Analysis of results

4.4.1 Determination of relative uptake of enantiomers

Concentrations of each enantiomer in atrial and ventricular myocardium were correlated with plasma enantiomer concentrations to derive tissue to plasma concentration ratios.

Potential stereo-selectivity of uptake was evaluated via determination of:

I. Differential (+) to (-)-enantiomer ratio in myocardium versus plasma (in order to determine whether uptake, rather than clearance, might engender stereoselective myocardial effects).

II. Percentage of (+)-enantiomer concentrations in plasma and myocardium, in relation to time (in order to determine the net effects of stereoselective kinetics on the myocardial uptake of the drug).

4.4.2 Identification of determinants or correlates of myocardial (+) and (-)-perhexiline uptake

Univariate followed by multivariate analyses were utilized for assessment of the atrial uptake of enantiomers. Parameters evaluated were plasma concentration, metaboliser status, age, weight, duration of therapy, heart rate,

creatinine clearance, and cardiac index. Multivariate backward stepwise analyses were performed using statistical software SPSS (version 20, Chicago).

I. Data from poor metabolisers (n=7) were excluded from analysis (unless specified otherwise) because of potential for differential clearance mechanisms and non-attainment of near steady-state kinetics.

II. Data are expressed throughout as mean ± SD for normally distributed parameters and median (interquartile range) for skewed data.

4.5 Results

4.5.1 Patient demographics: metaboliser status and absence of dose titration

129 patients from the active arm of CASPER trial were included and their clinical characteristics are summarised in Table 4-I. While patients had well-preserved renal function, their pre-treatment cardiac function is not known, with formal estimation of cardiac indices and heart rates performed following the induction of anaesthesia for surgery; these data therefore reflect the potential interaction of perhexiline and pre-treatment status. However, the generally low cardiac indices in these patients imply some degree of systolic left ventricular dysfunction at least at the time of measurement. The median plasma concentrations of perhexiline at the time of blood sampling were 0.27mg/L (IQR: 0.13 - 0.47), with approximately one third of patients having subtherapeutic levels (i.e. <0.15mg/L).

Table 4-I: Clinical characteristics of all patients (n=129).

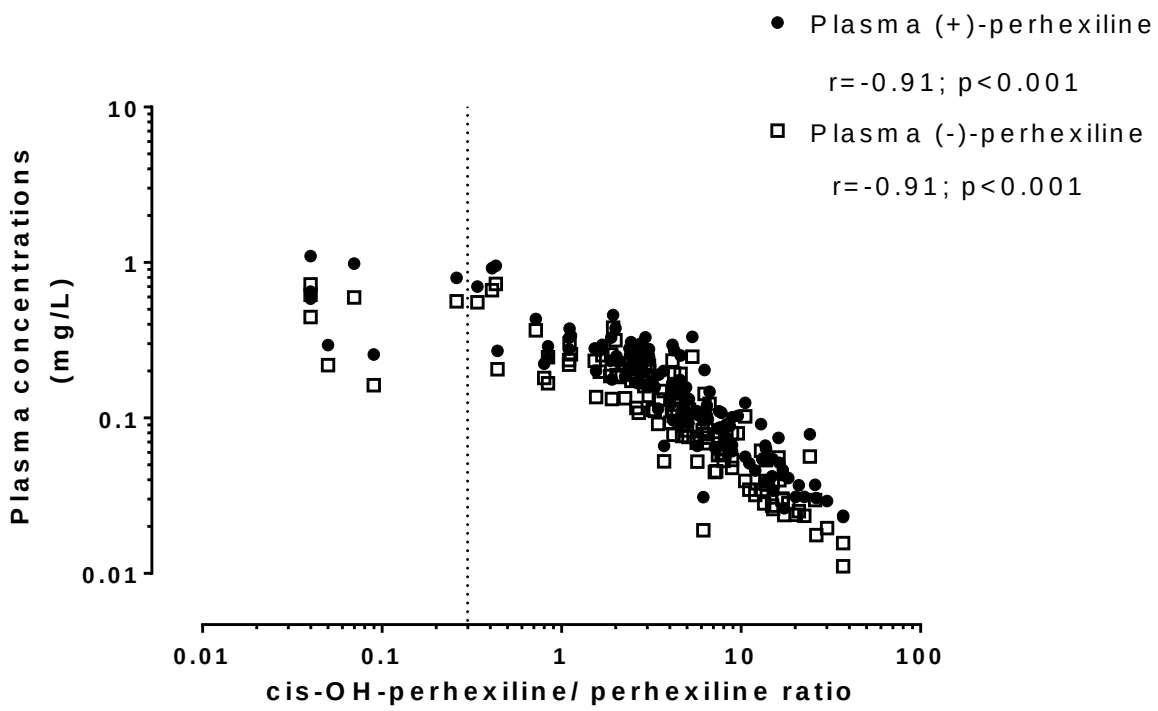
Parameters	
Age (years) [a]	66 (58-73) [a]
Weight (kg)	83 ± 14
Creatinine clearance (ml/min) [a]	70 (60 - 85) [a]
Baseline cardiac index	2.04 (1.78 - 2.34) [a]
Heart rate (beats per minute) ┼	59 ± 10
Duration of therapy (days)[a]	9 (6 - 12) [a]

[a]. Expressed as median (interquartile range)

┼ Measured immediately prior to coronary surgery, that is, on treatment.

Figure 4-I examines the relationship between plasma cis-OH-perhexiline/ perhexiline ratio (used to categorise metaboliser status) and plasma perhexiline concentration at the time of surgery. It is apparent that there was substantial variability in racemic perhexiline concentrations, such that therapeutic concentrations were not generally attained in rapid metabolisers. There were a total of 7 poor metabolisers, all of whom attained therapeutic or potentially toxic perhexiline concentrations.

Figure 4-I: The relationship between rate of perhexiline's metabolism and plasma perhexiline enantiomer concentrations at the time of surgery.



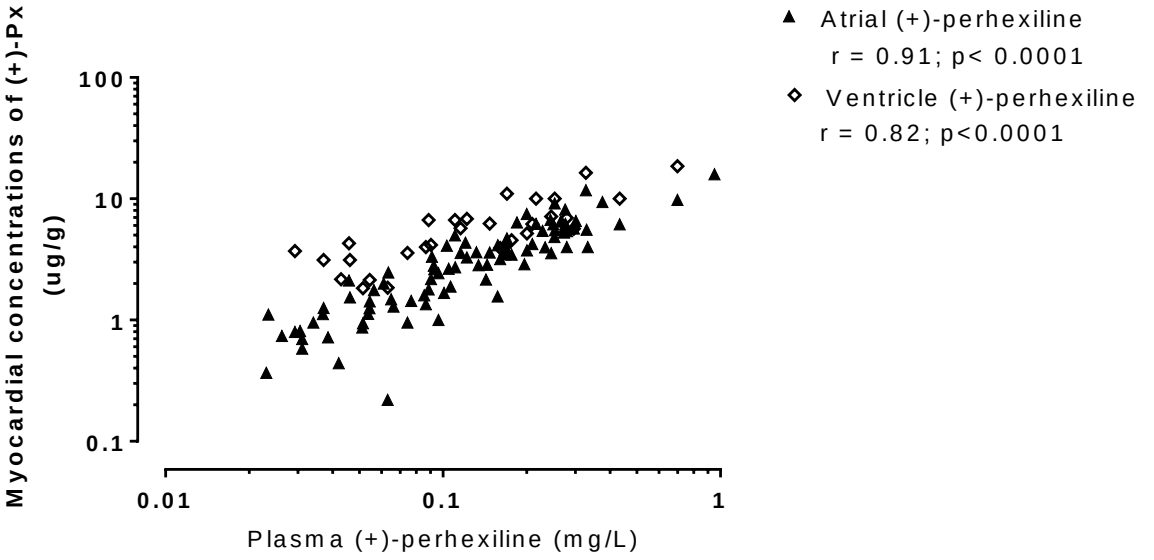
Perhexiline's metabolism is expressed as cis-OH-perhexiline to perhexiline ratio. Data to the left of the dashed line (ratio <0.3) correspond to "poor metaboliser" status.

4.5.2 Myocardial concentrations of enantiomers

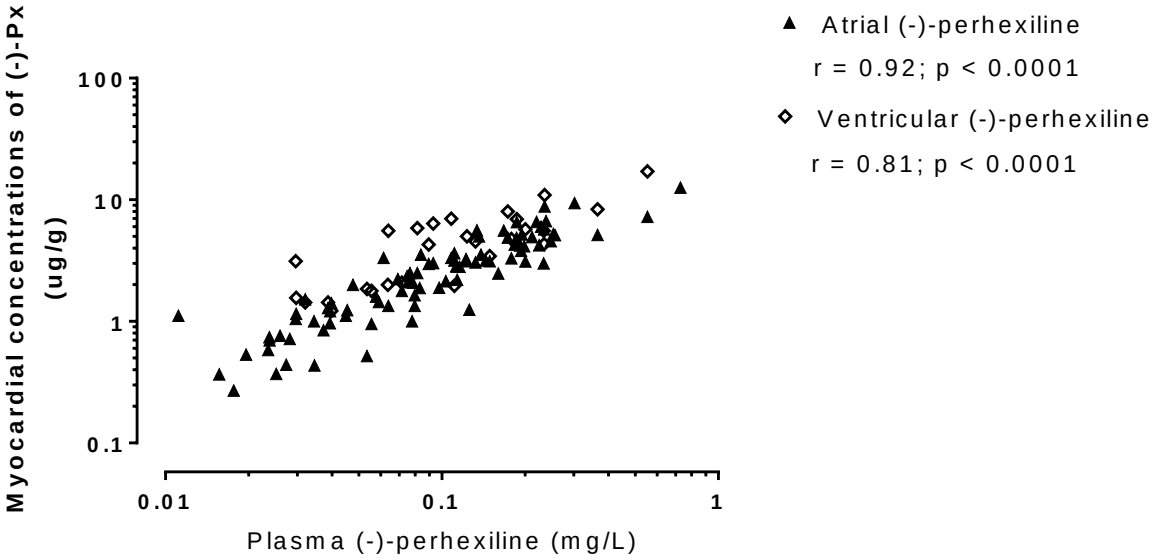
The relationships between plasma and atrial or ventricular concentrations of (+) and (-)-perhexiline are depicted in Figures 4-II A and B respectively. There were strong linear correlations for both enantiomers, with slightly greater concentrations of both enantiomers in ventricle than atrium ($p<0.001$ for both, Spearman's test).

Figure 4-II: Correlations between plasma and myocardial concentrations for (A) (+)-perhexiline, and (B) (-)-perhexiline.

A. (+)-Perhexiline



B. (-)-Perhexiline



Spearman's correlations are shown.

The impact of metaboliser status on these plasma: myocardial concentration relationships is summarised in Table 4-II. In summary, plasma concentrations of (+)-perhexiline tended to be greater than those of (-)-perhexiline for both plasma and myocardium, irrespective of metaboliser status.

Table 4-II: Perhexiline enantiomer concentrations in plasma, atrium and ventricles across all metabolic phenotypes.

Metaboliser status	Plasma concentrations (mg/L)		Atrial concentrations (mg/kg)		Ventricular concentrations (mg/kg)	
	(+)	(-)	(+)	(-)	(+)	(-)
Poor metabolizer	n=7		n=3		n=1	
	0.67 ± 0.32 *	0.48 ± 0.21	16.57 ± 6.57	13.22 ± 4.89	17.5	12.5
Intermediate metaboliser	n =28		n =19		n =6	
	0.28 (0.24-0.37) *	0.23 (0.18-0.29)	6.17 (5.45-8.16) *	5.11 (4.28-6.58)	11.07 ± 5.41$^{\Delta}$	9.08 ± 4.54 $^{\Delta}$
Extensive metabolisers	n =85		n =64		n =21	
	0.11 (0.07-0.17) *	0.08 (0.05-0.13)	2.77 (1.50-3.99) *	2.17 (1.26-3.24)	5.16 ± 2.48 $^{\Delta}$	3.55 ± 2.17 $^{\Delta}$
Ultrarapid metabolisers	n =9		n =8		n =2	
	0.03 (0.026-0.037) *	0.023 (0.017-0.027)	0.84 ± 0.31	0.623 ± 0.314	3.41 ± 0.41	1.56 ± 2.21

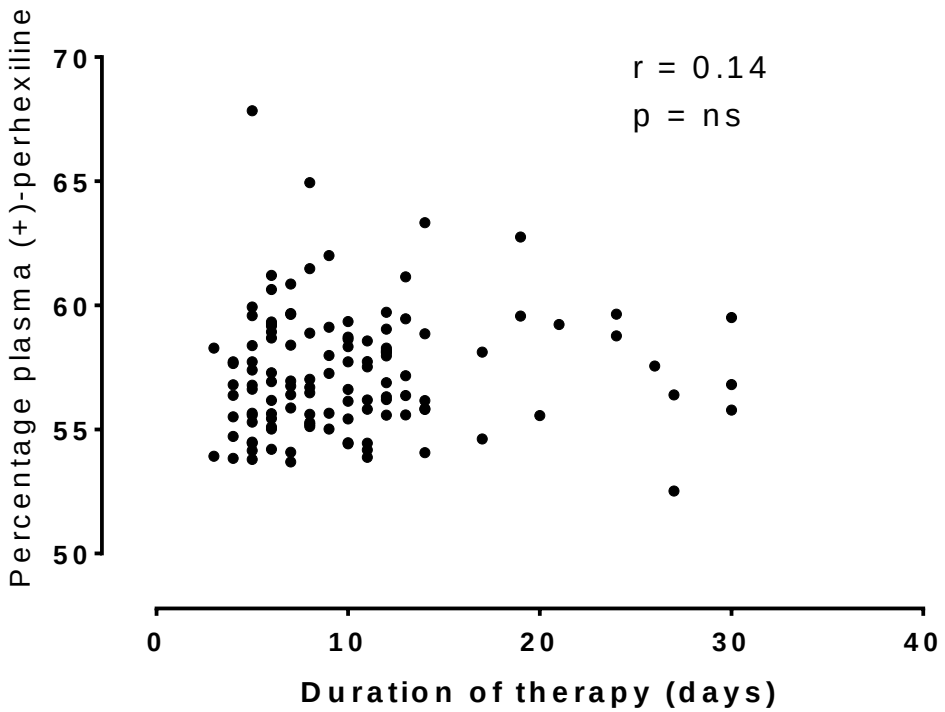
Metabolizer status was defined by the ratio of plasma concentrations of cis-hydroxyperhexiline to perhexiline as previously stated [255].

* $p<0.05$ vs antipode (Wilcoxon); $^{\Delta}$ $p<0.005$ vs atrial:plasma ratio

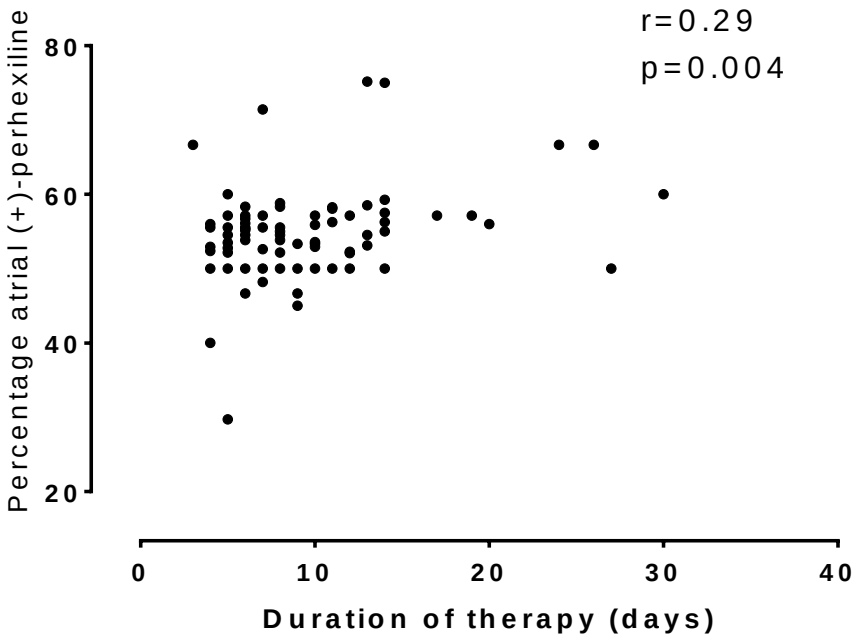
It was next sought to determine whether stereoselective clearance of perhexiline and/or of its uptake into myocardium might vary with duration of therapy. The proportion of (+)-perhexiline in plasma did not vary significantly with duration of therapy (Figure 4-III A), while that in atria increased significantly ($p=0.004$) with time (Figure 4-III B). Thus the ratio of (+)-perhexiline in the atrium to that in plasma tended to increase progressively ($r=0.19$, $p=0.07$).

Figure 4-III: Variations in the percentage of (+)-perhexiline in (A) plasma, and (B) atrium relative to duration of therapy.

A.



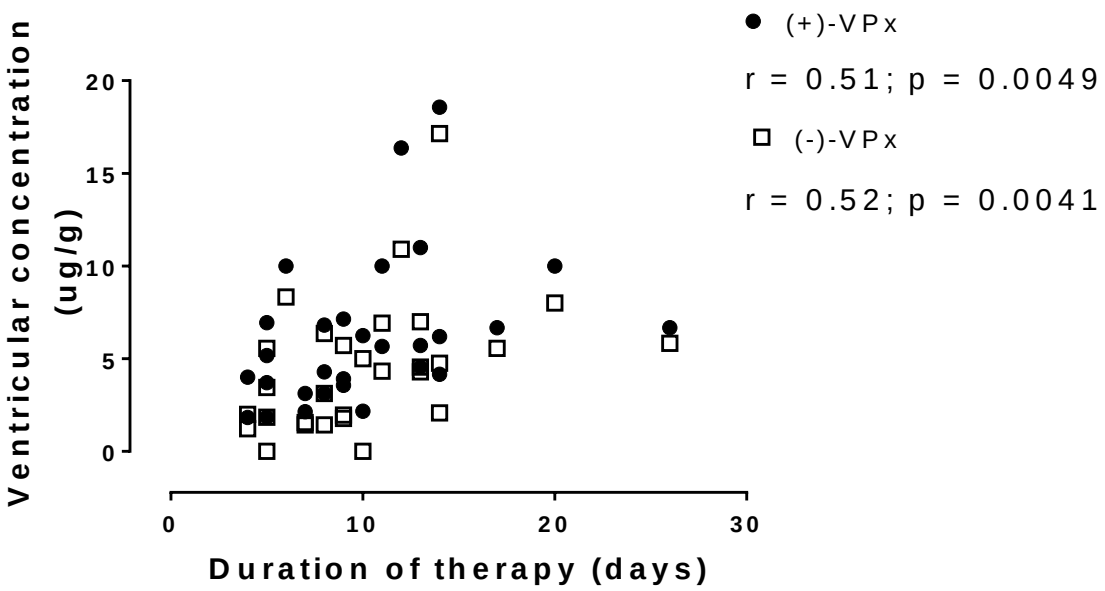
B.



Spearman's correlations are shown.

Concentrations of both (+)- and (-)-perhexiline into the ventricle increased significantly with time (p=0.005 and 0.004 respectively, Figure 4-IV), with no significant difference between the enantiomers.

Figure 4-IV: Impact of duration of treatment on concentrations of perhexiline enantiomers in ventricular myocardium.



Spearman's correlations are shown.

4.5.3 Multivariate correlates of uptake of enantiomer

Table 4-III summarizes atrial: plasma concentration ratios, with all patients (excluding poor metabolisers) evaluated. Plasma perhexiline concentration was a strong negative correlate and age was a positive correlate of this ratio for both enantiomers.

Table 4-III: Correlates of the atrial: plasma ratio (net uptake) of each enantiomer on multivariate analyses.

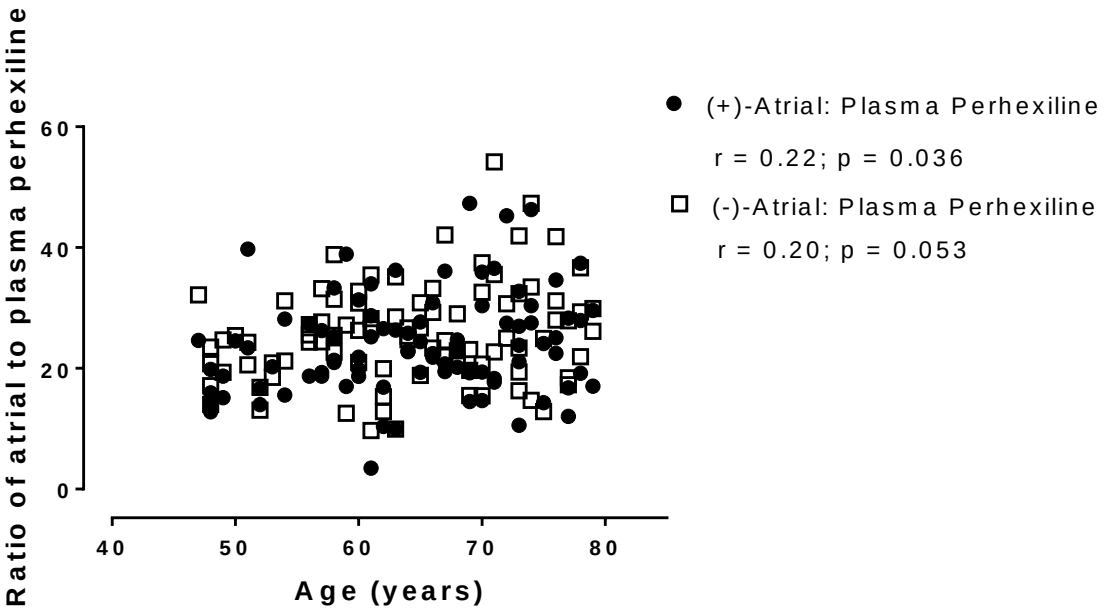
Determinant	Correlates	β coefficient	p-value
Atrial:plasma ratio of (+)-perhexiline	Plasma (+)-perhexiline concentration	-0.256	0.015
	Duration of therapy	0.228	0.025
	Age	0.300	0.004
Atrial: plasma ratio of (-)-perhexiline	Plasma (-)-perhexiline concentration	-0.347	0.001
	Age	0.288	0.005
	On-treatment heart rate	-0.240	0.015

Poor metabolisers were excluded.

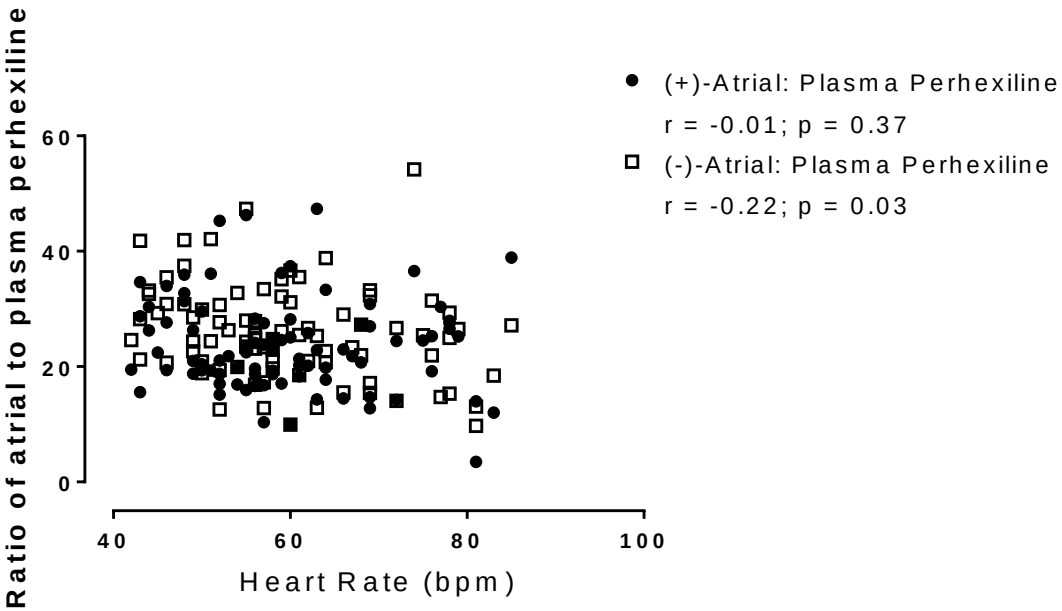
Uptake of (-)-perhexiline also varied inversely with simultaneous heart rate. Similar trends were also apparent with univariate correlations (Figure 4-V).

Figure 4-V: Univariate comparisons (Spearman's correlation) between (A) patients' age and (B) heart rate and uptake ratio of each enantiomer into atrium.

A.



B.



4.6 Discussion

The current analyses complement previously published evaluation of the uptake of racemic perhexiline into the human myocardium [296]. It is apparent from the current studies that the perhexiline dosing regimen utilized, with inadequate time available for adjustment of dosage on the basis of metaboliser status, led to wide variability in plasma and myocardial drug concentrations, but nonetheless, allowed exploration of determinants of myocardial uptake of perhexiline enantiomers.

The main findings of the current analysis are that:

1. The main determinant of concentrations of both (+)- and (-)-perhexiline into atrial and ventricular myocardium is plasma concentration of that enantiomer.

2. Just as plasma (+)-enantiomer concentration exceeds that of (-)-enantiomer, similar trends are present in myocardium, especially atrial muscle.

3. Myocardial uptake of each enantiomer also depends on plasma drug concentrations and patients' age, where a strong inverse relationship is present.

4. Atrial uptake of (+)-perhexiline varies positively with duration of therapy.

5. Atrial uptake of (-)-perhexiline varies inversely with simultaneous heart rate.

These findings carry a number of important implications regarding the myocardial handling and effects of perhexiline enantiomers.

The higher concentrations of (+)- than (-)-enantiomer in plasma are consistent with more rapid clearance of the latter. These data are consistent with previous publications regarding stereoselective clearance of perhexiline [278,297]. On the other hand, myocardial concentration ratios of the enantiomers generally parallel those in plasma, suggesting absence of major stereoselectivity in myocardial uptake. The exception to this is the small but statistically significant increase in the proportion of (+)-enantiomer in the atrial myocardium with time (Figure 4-V A). These data suggest that myocardial uptake and efflux of (-)-perhexiline may be slightly more rapid than that of the (+)-enantiomer.

The relatively prolonged time required to reach steady-state perhexiline concentrations in the ventricular myocardium may reflect greater distribution into mitochondria [299], known to represent a site of intracellular drug accumulation for perhexiline in hepatocytes [300].

As regards the interactions between advanced age and cardiac uptake of the enantiomers, the current data suggest that uptake of both perhexiline enantiomers is greater in older patients, perhaps related to decreased skeletal muscle mass. Indeed we have previously also shown that steady-state dosage requirements for perhexiline tend to fall with age [277], and in the current study plasma perhexiline concentrations increased with patient age ($r=0.24$, $p=0.029$). Together, these data suggest that elderly individuals may benefit from perhexiline therapy despite apparently borderline subtherapeutic plasma drug levels.

Finally, atrial uptake of (-)-perhexiline varied inversely with concurrent heart rate. Perhexiline is a weak L-type calcium antagonist [224,301], although its effects on the myocardium have undergone only limited study. The IC_{50} for inhibition of calcium fluxes in chick embryo ventricular myocardium by racemic perhexiline was 8.3×10^{-7} M [224]. In the current study, atrial concentrations of (-)-perhexiline approximated to these values. It is therefore possible that the calcium antagonist effect of racemic perhexiline is mediated primarily by the (-)-enantiomer.

The current study has several limitations. Most importantly, correlation with cardiac effects of perhexiline is limited by the absence of true pre-treatment data. Second, myocardial drug content after a median of 9 days of therapy reflects both uptake into and efflux from the myocardium, and there is no way to determine the precise component of each, given that steady state has not been reached. Finally, the full implications of the widely variable plasma perhexiline concentrations on variability in drug uptake cannot be fully understood without a strategy of multiple drug dosing per patient.

The current results also need to be related to the clinical context. Recently the effects of acute loading of perhexiline in patients with severe ischaemia are reported [236,243]. The current data are consistent with the idea that there may be early onset of cardioprotective effects, especially in the elderly, at a time when plasma drug concentrations are notionally subtherapeutic. Furthermore, the data regarding heart rate correlations should stimulate evaluation of whether the calcium antagonist effects of perhexiline can be dissociated from its “metabolic” cardioprotective effects [179,235], by selective administration of the (+)-enantiomer.

4.7 Where do we stand regarding the uptake of perhexiline overall?

Generally, it was shown that, in non-poor metabolizers, steady state concentrations of perhexiline were not reached within the ventricles after a week of treatment, even though this has been reached within the atrium [72,296]. Additionally, while the plasma concentrations of perhexiline were well correlated with its myocardial concentrations, the concentrations of perhexiline were almost twice as high in the ventricles than in the atrium. This raised the possibility of not only potential compartmental accumulation of perhexiline, but also possible differential uptake or clearance of perhexiline in the myocardium. Local factors such as heart rate may also have played a role in the slow uptake of perhexiline, especially the (-)-enantiomer [72]. Additionally, patient's age also correlated with increased myocardial uptake of perhexiline, which may be a reflection of decreased skeletal muscle mass; suggesting that older patients may benefit from subtherapeutic levels or the lower threshold of the plasma therapeutic range of perhexiline [72,296].

Although it has been appreciated for many years that myocardial drug concentrations may not be readily predictable on the basis of plasma drug concentrations and methodology for assessment of effects has remained limited, the current studies suggested that assessment of the effect of such metabolic agent may be more difficult than expected. It was possible that without adequate myocardial accumulation of perhexiline in ischaemic patients (plus a lack of adequate dose individualization), cardioprotective effects of perhexiline have failed to be demonstrated in a recent trial [295].

4.8 Acknowledgement

Recruitment of patient subjects, treatment, sample collection and analysis of the CASPER trial were performed in Birmingham, UK and had previously been published [295]. The original authors of the CASPER trial had generously shared the samples and information collected for the completion of current study. The current study involves remeasurement of plasma concentrations using an enantiospecifc assay, and performing analysis to investigate the enantioselective uptake of perhexiline into myocardium, which constituted the majority of work described in this chapter.

Chapter 5: Rapid reversal of hyperglycaemia in acute coronary syndrome

5.1 Benefits of restoring insulin signaling in acute coronary syndrome

It is known that patients with diabetes mellitus are at increased risk for the occurrence of acute coronary syndromes [302], heart failure [303] and atrial fibrillation [304]. However, the contribution of acute elevation of blood glucose level to the overall cardiovascular risk is incompletely understood. For example, while it is known that acute elevation of blood glucose level increases oxidative stress [305], the impact of such oxidative stress on the physiology of vasculature, myocardium and platelets is unknown.

The potential role of impaired NO signaling in the setting of hyperglycaemia and acute coronary syndromes (ACS) was investigated in this chapter: it was shown reduced platelet responsiveness to the anti-aggregatory effects of NO is negatively correlated to admission blood glucose levels (BGL) during ACS [306]. In the study, rapid correction of BGL by intravenous insulin therapy not only rapidly improved platelet responsiveness to NO, resulting in a potential reduction in thrombogenecity, but this was also associated with a reduction of plasma superoxide content and plasma ADMA levels. Given that aggressive correction of hyperglycaemia with intravenous insulin improves NO response, we hypothesised that this effect was also associated with a reduction in TXNIP expression by vurtue of the reciprocal relationship between TXNIP expression and platelet responses to NO at steady-state [138]. In addition, we also sought to assess changes in other markers which are associated with NO signaling, namely plasma ADMA levels, superoxide content, endothelial progenitor cells' function and thrombospondin-1.

5.2 Background of current study

Hyperglycaemia during acute coronary syndromes is associated with substantial mortality [307,308]. Theoretically, this might reflect a number of processes including impaired glycolysis, increased fatty acid levels, increased cytokine activation, platelet hyperaggregability, and impaired microcirculatory function [309].

Hyperglycaemia is also accompanied by rapid up-regulation of thioredoxin-interacting protein (TXNIP). TXNIP is a physiological inhibitor of thioredoxin (Trx), and suppression of Trx by TXNIP has been demonstrated in diabetes and many cardiovascular diseases (see Chong *et al* [81] for review). TXNIP also exerts Trx-independent effects, which include direct activation of inflammation and inhibition of glucose uptake [82]. The production of TXNIP is stimulated by increased glucose levels, hypoxia, and several inflammatory activators (e.g. increased shear stress, transforming growth factor β-1 and interleukin 1-β [81]). Insulin therapy may reduce TXNIP levels in tissues both via reduced synthesis and increased clearance [146], although each of these effects may be organ-specific. For example, in human skeletal muscle and adipocytes in cell culture, it was also demonstrated that four-hour treatment with insulin significantly reduced mRNA expression of TXNIP [147]. Furthermore, insulin accelerates degradation of TXNIP via activation of the ubiquitin-proteasome pathway in some, but not all, tissues [146].

Hyperglycaemia is also linked to reduced availability of nitric oxide (NO), perhaps by virtue of increased scavenging by superoxide (O_2^-) or by increased generation of the endogenous NO synthase inhibitor, asymmetric dimethylarginine

(ADMA). There is increasing evidence that suppression of NO generation may be in part TXNIP-mediated and indeed that TXNIP may function as a physiological antagonist to NO signaling, both in animals [137,150,310] and in humans [138]. For example, in aging normal subjects, platelet TXNIP content varies inversely with platelet NO responsiveness, with a highly significant negative correlation between these two parameters.[138]

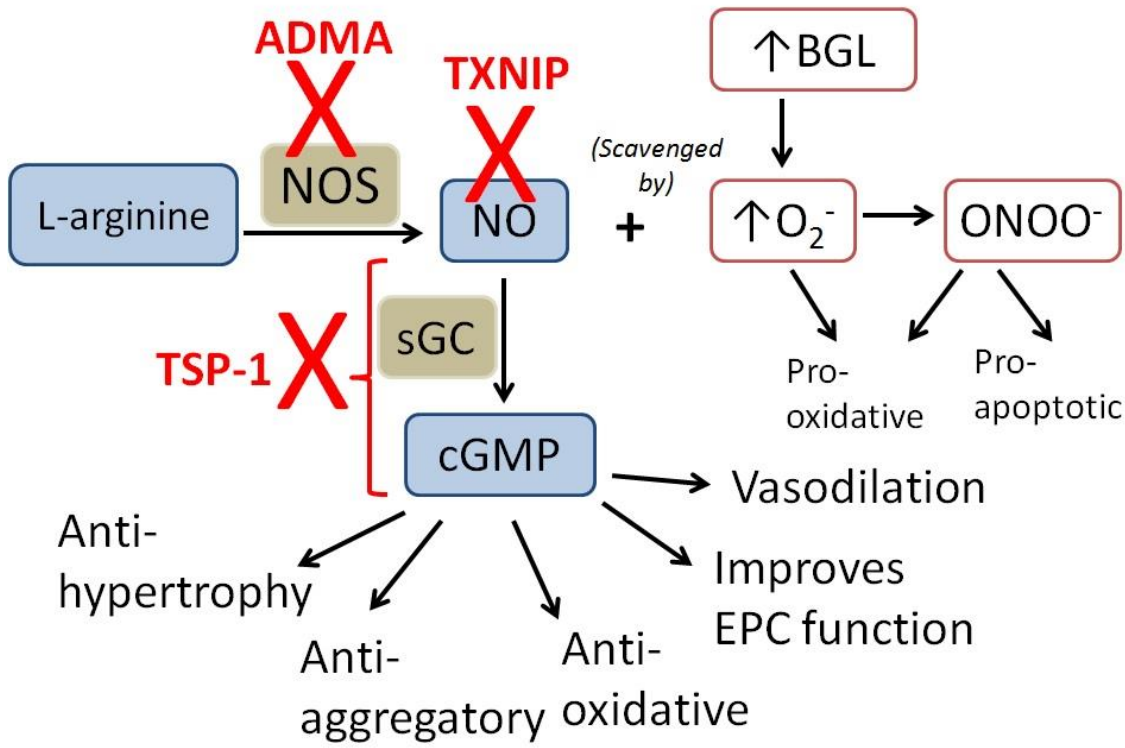
The potential role of impaired NO signaling in the setting of hyperglycemia and acute coronary syndrome (ACS) has previously been investigated, showing that reduced platelet responsiveness to NO varies inversely to admission blood glucose levels (BGL) [306]. The precise mechanism(s) by which correction of hyperglycaemia normalises NO signaling remain uncertain. In the initial investigation, it was demonstrated that baseline blood O_2^- content varied inversely with NO responsiveness, and rapid reversal of hyperglycaemia reduced O_2^- content and potentiated platelet NO responsiveness [306]:- this would suggest diminished NO "scavenging" [123]. In this regard, the potential role of thrombospondin-1 (TSP-1), a matrix glycoprotein derived from platelet α-granules [311], was not initially evaluated. However, TSP-1 has recently emerged as a potent inhibitor of the NO-soluble guanylate cyclase (sGC) signaling cascade [311].

Furthermore, formation of NO, rather than just integrity of its signaling, may also be important. Correction of hyperglycaemia was associated with significant fall in plasma ADMA concentrations [306], which have been shown to correlate with improved NO generation and with improved function of endothelial progenitor cells (EPCs), and thus vascular stabilization [312] (see Figure 5-I for schematic view). So far, this has not been explored in the context of ACS or hyperglycaemia.

Endothelial progenitor cells are increasingly recognised as important biomarkers of vascular function, particularly during the process of "vascular repair". Originating from bone marrow, EPCs have proliferative capacity [313]. Not only do they repair ongoing vascular damage, promote vascular growth, but they also improve endothelialization [313,314]. Additionally, NO and eNOS also play a role in EPC-mediated angiogenesis [315]. Thus changes in EPC function may potentiate vascular damage associated with hyperglycaemia.

Given that aggressive correction of hyperglycaemia with intravenous insulin infusion improves platelet NO responsiveness [306], it was hypothesised that this effect is associated with a reduction in platelet TXNIP content. In addition, the changes in other biomarkers associated with NO signaling, including TSP-1 concentrations, ADMA concentrations, O_2^- content, and EPCs' function were also assessed .

Figure 5-I: A schematic view of the signaling pathways of nitric oxide.



Interacting mechanisms impeding nitric oxide generation / signaling and those inducing nitrosative stress are indicated. The generation of nitric oxide from L-arginine is controlled by the nitric oxide synthase (NOS), of which is inhibited by asymmetric dimethylarginine (ADMA). Upon release, nitric oxide exerts its effects [which includes improvement of the function of endothelial progenitor cells (EPC)], via soluble guanylate cyclase (sGC) - cyclic GMP (cGMP) pathway. Although the protein affected is unclear, this pathway was previously shown to be inhibited by thrombospondin-1 (TSP-1) [311]. It is also known that the pro-inflammatory protein thioredoxin interacting protein (TXNIP) inhibits the effect of nitric oxide [138]. During acute hyperglycaemia, the superoxide generated scavenges nitric oxide and contributes to "nitric oxide resistance" [306], by virtue of increased

inflammatory and apoptotic responses. Therefore, restoration of insulin signaling pathway and reversing hyperglycaemia rapidly may restore nitric oxide signaling.

5.3 Methods

5.3.1 Patients and study protocol

This study was approved by the Ethics of Human Research Committee of the Queen Elizabeth Hospital (protocol number: 2010163). Informed consent was obtained prior to study entry in all cases. Patients presenting with ACS (angina pectoris or non-Q-wave myocardial infarction) excluding S-T elevation infarction and an admission BGL > 11.1 mmol/L to correspond to the entry criteria for the DIGAMI trial [307] were recruited.

Criteria for exclusion were:

1) Concurrent treatment with ADP-receptor blockers (e.g. clopidogrel), glycoprotein IIb/IIIa inhibitors or perhexiline, which is known to potentiate NO response and lowers BGL [209];

2) moderate to severe renal insufficiency (serum creatinine > 0.3 mmol/L).

After baseline investigations, all of the identified patients were subjected to intravenous Actrapid® insulin infusion according to the DIGAMI protocol [307], with the aim of restoring normoglycaemia within 12 hours. Peripheral venous blood samples were collected prior to and 13 ± 0.8 (SEM) hours after the commencement of insulin infusion.

5.3.2 Investigations performed were as follows:

(1) Platelet responsiveness to NO

This was determined as previously described [316]. Briefly, 10ml of blood was collected in tubes containing acid citrate anticoagulant. Platelet aggregation was studied in dual-chamber impedance aggregometer (Model 560, Chrono-Log, Havertown, Pennsylvania, USA). Aggregation was induced by 2.5 μmol/L of ADP. The NO donor sodium nitroprusside (SNP, 10 μmol/L) was used to quantitate platelet responsivess to NO, with results expressed as percentage inhibition of ADP-induced aggregation.

(2) Total ROS content in whole blood and isolated neutrophils

a. Blood sampling and preparation of neutrophils

Blood samples were drawn by venesection from an antecubital vein. Blood was collected into heparinized vacutainer tubes for whole blood total ROS assessment, or into 24 mmol/L EDTA tube for neutrophil preparation.

For neutrophil preparation, blood samples were centrifuged at 150 *g* for 10 minutes, and plasma was replaced by an equal volume of Hanks' balanced salt solution (HBSS) pH 7.4. Neutrophils were isolated using a Ficoll-Hypaque gradient as previously described [209]. The viability of neutrophils was shown to be over 95% by Trypan blue exclusion.

b. Electron Paramagnetic Resonance Spectroscopy measurement of Reactive Oxygen Species

Whole blood:

Quantitation of total ROS in whole blood was performed utilizing electron paramagnetic resonance (EPR) spectroscopy, as previously described [317]. All samples were prepared for EPR using the spin probe CM-H (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine, 400μmol/L) in Krebs-HEPES buffer (pH 7.35) in the presence of 25μmol/L deferoxamine and 5μmol/L diethyldithiocarbamate, and placed in 50μL glass capillaries (NOXYGEN, Elzach, Germany). Reaction of ROS with CM-H generates a stable nitroxide radical, the formation of which was measured by monitoring the amplitude of the low-field component of the EPR spectrum as previously described [318], and calculated from the accumulation of nitroxide, obtained from a calibration curve for intensity of the EPR signal of 3-carboxyproxyl. EPR spectra were recorded using an e-scan M EPR spectrometer (Bruker, Rheinstetten, Germany) and super-high Q microwave cavity with the following settings: field sweep, 10 G; microwave frequency, 9.75 GHz; microwave power, 19 mW; modulation amplitude, 2 G; conversion time, 10.24 ms; time constant, 40.96 ms; receiver gain, 3.2×10^2. Data were expressed as μM/mL/min of nitroxide accumulation.

Isolated neutrophils:

For O_2^- determination by EPR, neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA) (100nmol/L, for 20min) at room temperature, and then samples were scanned immediately after supplementation of the spin probe CM-H

(200μmol/L). EPR settings were identical to those utilized for whole blood ROS determination. All EPR experiments were performed in triplicates.

(3) Platelet TXNIP content

TXNIP content within platelets was analysed by immunohistochemistry [138]. Briefly, peripheral blood collected in EDTA-containing tubes were used to prepare platelet rich plasma, which was then smeared onto a microscope slide and stored at -80°C until further analysis. On the day of analysis, defrosted slides were washed with phosphate buffered saline and incubated with 5% goat serum for an hour, then with 2% of anti-rabbit VDUP (Invitrogen Corporation, CA, USA) in 1% of bovine-serum albumin for overnight. On the next day, after the slides were washed, they were incubated with 2% of phycoerythrin (PE)-labelled CD41 antibody (Becton, Dickinson and Company, NJ, USA) as platelet marker, and 1% of secondary fluorescein isothiocyanate (FITC)-labelled anti-rabbit antibody (Becton, Dickinson and Company, NJ, USA). The slides were then studied using inverted fluorescence microscope (Carl Zeiss Microscopy, Oberkochen, Germany). Analyses were performed using image analysis software (AxioVision 40 verion 4.8.2, Carl Zeiss Microscopy, Oberkochen, Germany), and an average value was obtained per slide.

(4) Plasma ADMA concentration

Plasma ADMA concentrations were determined utilizing derivatization with AccQ-Flor to generate stable fluorescent derivatives followed by high performance liquid chromatography [319].

(5) Plasma TSP-1

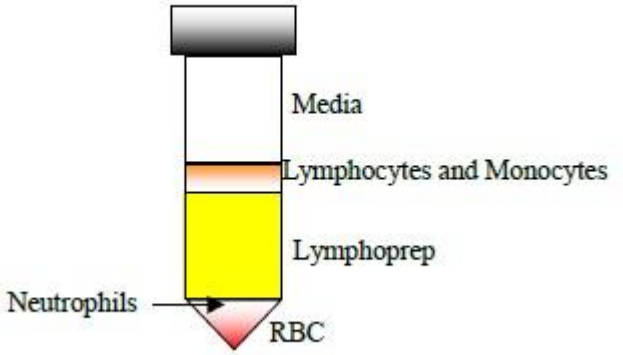
Plasma TSP-1 concentrations were determined by ELISA according to manufacturer's instructions (R&D Systems, MN, USA).

(6) Endothelial Progenitor Cell count

10mls of blood were collected into an EDTA tube, and then transferred into a 50ml tube where 10ml of phosphate buffered saline (PBS) was added and underlaid with 15mls of Lymphoprep® or Ficoll-Hypaque® (Axis-Shield, Oslo, Norway). The sample was then centrifuged at 550 *g* for 30 minutes. After centrifugation, the "buffy" coat containing mononuclear cells was collected using a pipette (see Figure 5-II).

Circulating endothelial progenitor cells (EPC) were quantified using flow cytometric analysis (FACScan, BD Bioscience, NJ, USA) of cells positive for both cell surface antigens, CD34 and CD133 [320].

Figure 5-II: Schematic representation of the various phases of blood following centrifugation of blood via Lymphoprep system.



(7) Endothelial Progenitor Cell function: formation of early colony-forming units

EPC were cultured using the method of Shintani et al [314]. Briefly, mononuclear cells were isolated using a Ficoll-Hypaque® gradient density centrifugation similar to how mononuclear cells are collected in (6). After collection of mononulear cells, the cell suspension was cultured in fibronectin coated 6-well plates in medium supplemented with 20% foetal bovine serum and penicillin/streptomycin (Gibco®). Following 48 hours of incubation, any detached cells were washed away with PBS and the remaining attached cells were left to incubate in complete medium for 7 days to obtain early EPC. Culture medium was changed every two days. EPC function was expressed as colony-forming units.

5.3.3 Statistical analyses

Effects, analysed before and after insulin infusion, were compared utilizing Student's paired t-test or paired Wilcoxon test for non-Gaussian data (GraphPad Prism 6®). The limit of statistical significance was set at $p<0.05$. Data were expressed as mean ± SEM or median (interquartile range), unless otherwise indicated.

5.4 Results

Table 5-I summarizes the clinical demographics of the twelve patients in this study. The mean age of these patients was 66 ± 3 years. Apart from the index elevation of blood glucose levels, the glycosylated haemoglobin concentrations suggested longstanding poor diabetic control. Only 6 of the 12 patients had elevation of troponin-T concentrations beyond the normal range, and none of the patients had peak creatine kinase > 1000U/L, consistent with minimal levels of myocardial necrosis.

Table 5-I: Clinical characteristics of the twelve patients

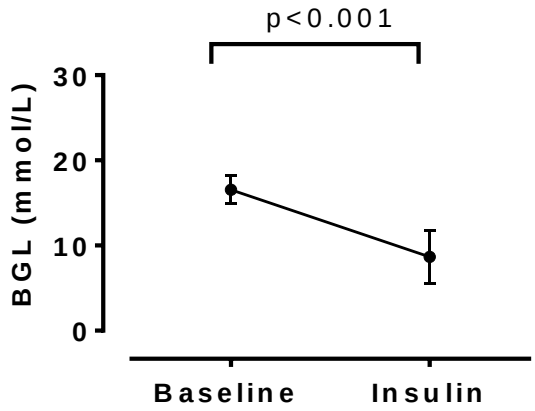
Age (years)	66 ± 3.2
Men/ women (%)	58/ 42
HbA1c (%)	9.8 ± 0.5
Baseline BGL (mmol/L)	16..6 ± 1.6
Peak troponin-T (ng/L)	38 (29-285)
Peak creatine kinase (U/L)	108 (91-438)

Abbreviations: HbA1c = glycosylated haemoglobin; BSL = blood sugar level

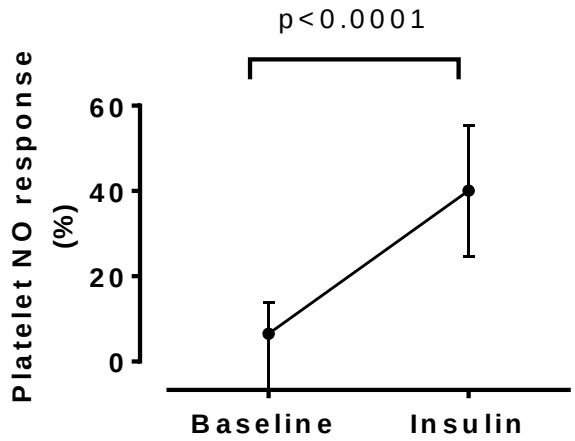
After insulin infusion, the blood glucose level fell by 7.9 ± 1.4 mmol/L ($p < 0.01$; 95% confidence interval: -10.9, -4.8; Figure 5-III A) over 13 ± 0.8 hours of the study. This was associated with a marked improvement in platelet responsiveness to the anti-aggregatory effect of sodium nitroprusside (Figure 5-III B). This was independent of the pro-aggregatory effect of ADP, which did not change significantly (mean difference +1.05 Ω; $p=0.20$).

Figure 5-III: Effects of insulin infusion on (A) (Mean) blood glucose level (BGL) (B) Platelet responsiveness to NO, measured via inhibition of ADP-induced aggregation with SNP.

A.



B.

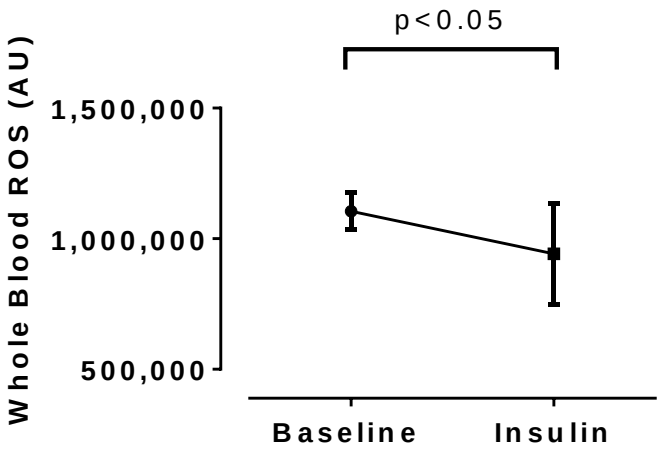


Mean data are shown.

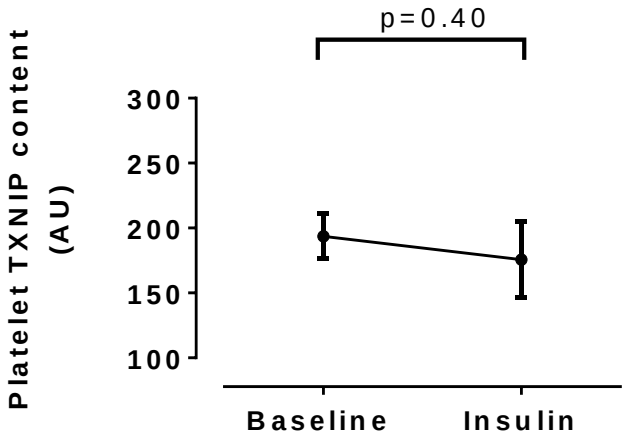
Correction of hyperglycaemia was associated with a significant reduction in whole blood ROS (Figure 5-IV A) but no significant change in PMA-stimulated O_2^- release in isolated neutrophil (1.47 x 10^6 vs 1.31 x10^6; p=ns).

Figure 5-IV: Effects of insulin infusion on (A) whole blood ROS, and (B) Platelet TXNIP content.

A.



B.

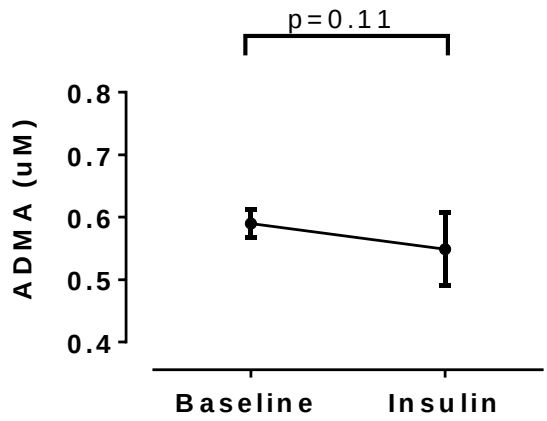


As regards potential modulators of changes in NO signaling and ROS production, plasma ADMA concentrations (Figure 5-V A) tended to fall (p=0.12; mean change -0.04 µmol/L; 95% CI-0.10, 0.01). Neither change in platelet TXNIP

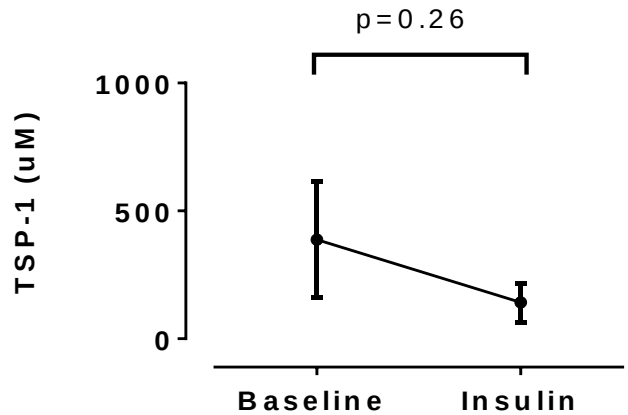
content (mean change -18 AU; 95% CI -63, +27) or plasma TSP-1 concentration (mean change -246 µmol/L; 95% CI -715, +223) reached statistical significance.

Figure 5-V: Effect of insulin on (A) asymmetric dimethylarginine (ADMA), and (B) thrombospondin-1 (TSP-1) concentrations in plasma.

A.



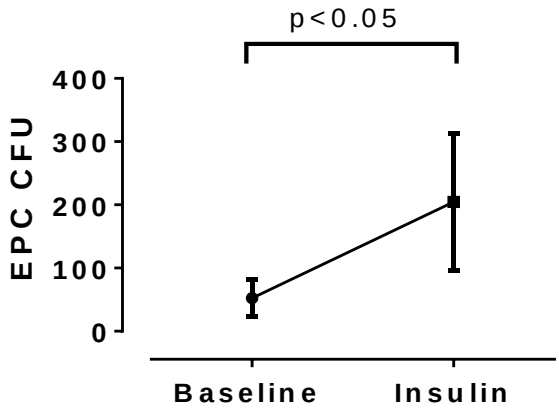
B.



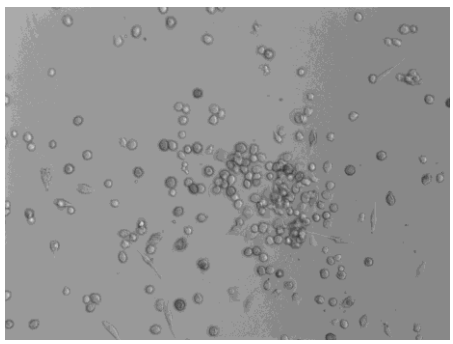
While numbers of EPCs did not change significantly (p=ns; mean change -9.90; 95% CI -52, 33), there was a substantial and significant increase in CFU count (Figure 5-VI).

Figure 5-VI: Effects of insulin infusion on (A) endothelial progenitor cell (EPC) function [colony-forming unit (CFU) counts at 7 days]. Examples of CFU formation at baseline, and post insulin are shown in (B) and (C) respectively.

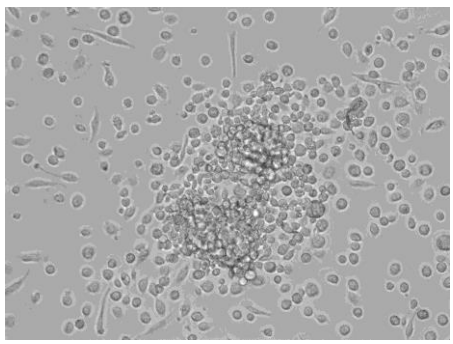
A.



B.



C.



5.5 Discussion

The current study aimed to delineate the mechanism(s) underlying the restoration of tissue NO responsiveness associated with correction of hyperglycaemia in ACS patients. It was hypothesised that the previously observed reciprocal relationship between glucose control and platelet NO signaling [138] is primarily due to a change in platelet TXNIP content, and such improvement in NO effectiveness would also lead to improved EPC functionality. Whilst it was confirmed that correction of hyperglycaemia is associated with markedly augmented platelet NO signaling and a reduction of ROS content, there was not any statistically significant reduction of TXNIP content in platelets. Additionally, it was considered that a reduction in plasma TSP-1 concentration might occur, thus sensitizing sGC to NO [311]: again, this was not observed in practice. EPC function (as measured by CFU formation) was increased significantly after correction of hyperglycaemia, consistent with increased NO effect [315], despite the fact that ADMA concentrations did not decrease significantly.

Previous studies suggest that insulin exerts its effects via (i) activation of the phosphatidylinositol-3-kinase-AKT-eNOS signaling pathway [321] and (ii) suppression of both interleukins 6 and 8 and C-reactive protein [322]. It has also been shown that while glucose upregulates TXNIP expression [139], insulin suppresses TXNIP synthesis at the transcriptional level [146]. However, the mechanisms underlying TXNIP suppression vary between tissues and include both reduced synthesis and increased clearance by the ubiquitin-proteasome pathway [146]. In the current study, platelet TXNIP content did not fall significantly. One possible explanation for this lack of change (and the associated loss of

previously described nexus between platelet NO signaling and TXNIP content) is potential slow turnover of TXNIP in platelets, perhaps related to the absence of a nuclear-bound pool of TXNIP, which seems to play a pivotal role in rapid changes in TXNIP signaling in nucleated cells [143,323]. The implication, however, is that the suppression of ROS generation and associated sensitization of NO signaling is largely or entirely TXNIP-independent.

While the primary hypothesis in the current study related to the potential role of insulin-induced reversal of hyperglycaemia in suppressing TXNIP expression, a number of other potential biochemical modulators of NO signaling were also evaluated serially. Both TSP-1 and ADMA concentrations tended to fall, but this change did not reach statistical significance (see Figure 5-V). In a previous larger study [306], ADMA concentrations fell to a similar extent following 12 hours of insulin therapy, but the change was statistically significant. It is likely that this change reflects the impact of diminishing redox stress on metabolic clearance of ADMA [324]. However, as ADMA may contribute to O_2^- generation via NOS "uncoupling" [325,326], the diminution of ADMA effect may have supported the restoration of NO responsiveness. As regards TSP-1, there was an approximately 60% fall in concentrations over the study period, but this did not reach significance largely due to extreme variability of baseline concentrations. TSP-1 is released into plasma via platelet degranulation: the baseline variability suggests considerable platelet activation in the presence of hyperglycaemia, in accordance with previous data [327]. If indeed a decrease in TSP-1 plasma concentration occurs with insulin therapy, this might contribute to increase NO effect, largely via improved functionality of sGC [311].

While TXNIP suppression may not be the major mechanism for acute sensitization of platelets to NO with correction of hyperglycaemia, there are other potentially important contributing pathways. Apart from the issue of NOS "uncoupling", increased O_2^- production in hyperglycaemic diabetics might reflect activation of a number of redox pathways. For example, in vascular smooth muscle of hyperglycaemic rats, insulin limited O_2^- production not only by NOS, but also by NAD(P)H oxidase and xanthine oxidase [328]. Indeed the specific contribution of TXNIP to the overall increases in O_2^- production during hyperglycaemia has not been formally evaluated in any tissue to date. However, it certainly seems likely that the kinetics of TXNIP responses to variable tissue glucose and insulin levels may be far slower in platelets than in nucleated cells.

Correction of hyperglycaemia during ACS is an important aspect of management and impaired platelet NO signaling may predispose to increased acute thrombogenesis [329]. Additionally, platelet hyporesponsiveness to NO is an independent predictor of all-cause mortality and cardiovascular readmissions in high risk ACS patients [330]. Furthermore, improved NO signaling could potentially be a mediator of the improvement in EPC functionality currently observed and perhaps also of angiogenesis [313,331]. Importantly, these mechanistic considerations would apply only to regimens involving actual reversal of hyperglycaemia in ACS patients, and thus exclude the glucose-insulin-potassium regimen employed in the recently reported CREATE-ECLA trial [332].

The results of the current study do not totally exclude involvement of changes in TXNIP and TSP-1 effect in modulating NO re-sensitization: they merely exclude substantial roles by virtue of the 95% confidence limits on the experimental data. It remains possible that diminution in TXNIP effect actually

occurred via either a (small) fall in overall platelet content or by changes in intracellular TXNIP distribution [143,333]. As regards uncertainty as to whether TSP-1 was suppressed, apart from the issue of Type II error, it is possible that TSP-1 concentrations *within platelets* may have changed in a relevant manner, as might the binding of TSP-1 to its receptors [334].

5.6 Clinical Perspectives

Rapid correction of severe hyperglycaemia with insulin infusion has been shown to improve clinical outcomes in acute coronary syndrome but the mechanisms underlying this benefit remain unclear. The current investigation confirms that in such patients, treatment of hyperglycaemia reverses impairment of platelet nitric oxide signaling, and demonstrates improvement of endothelial progenitor cell function, without significant reduction in platelet thioredoxin-interacting protein content. These data emphasize the benefit of urgent reversal of hyperglycaemia in all patients with acute myocardial ischaemia.

5.7 Acknowledgements

This chapter forms the basis of publication in *American Journal of Medicine* 2015 (see Appendix). Saifei Liu performed neutrophil isolation, quantification of ROS using EPR spectroscopy, and the cell culture studies in this chapter. Giovanni Licari assisted with cell culture, and Tamila Heresztyn performed HPLC quantification of plasma ADMA.

Chapter 6: Evolving therapeutics - potential role of perhexiline in insulin sensitization

6.1 Efficacy of perhexiline in restoring insulin signaling

6.1.1 Diabetes mellitus: a "precursor" of cardiovascular disease

Although great progress has been made in recent years regarding the management of both type 2 diabetes mellitus (DM) and cardiovascular disease, in many patients the eventual outcomes are poor. DM is a chronic disease with potentially serious complications, estimated to affect over 2.5 million Australians within 20 years [335]. With the increasing age and longevity of the general population, DM not only increasingly imposes a substantial impact on the healthcare economy, but it is also an independent risk factor for acute coronary disease and cardiomyopathy [335], with a concomitant two to four fold increase in risks of developing cardiovascular disease [336,337]. Modifications of traditional risk factors in patients with diabetes and insulin resistance have been effective, but these patients still have an increased morbidity and mortality compared to the non-diabetics. On the other hand, in the western world, heart failure (HF) often coexist with DM, and remains the most common cause of hospitalization [338]. Refractory symptoms, contraindications to surgery, and/or intolerability to the currently available pharmacological agents lead to many “sad survivors”.

Epidemiological study showed that DM increases the risk of HF by 2.5-fold, even when adjusted for coronary artery disease and other comorbidities [99,100]. On the other hand, HF presents earlier in the diabetic than non-diabetic patients [99]. Therefore, it is likely that left ventricular (LV) dysfunction seen in diabetic patients is a reflection of both increased coronary disease (secondary to atherosclerosis), as well as a specific "diabetic cardiomyopathy" (DCM) [339].

In the absence of ischaemia or other concurrent cardiovascular disease, diastolic abnormalities are an early functional defect in the diabetic heart, with prevalence rates range from 15-75% in the asymptomatic normotensive patients [339,340]. There is often mild systolic dysfunction, often depending on the severity or duration of disease. However, despite the absence of systolic dysfunction and the lack of known coronary disease or ECG detectable ischaemic changes, patients with diabetes have a lower myocardial PCr to ATP ratio than the matched healthy controls, suggesting that patients with diabetes are “cardiac energy-deficient” [341]. Furthermore, as previously described in Chapter 5, diabetes, especially with concomitant hyperglycaemia, represents the basis for mitochondrial dysfunction and consequent energetic impairment. Therefore, restoring systemic metabolic homeostasis may present a potential pharmacological alternative to prevent the progression of heart disease in patients with diabetes.

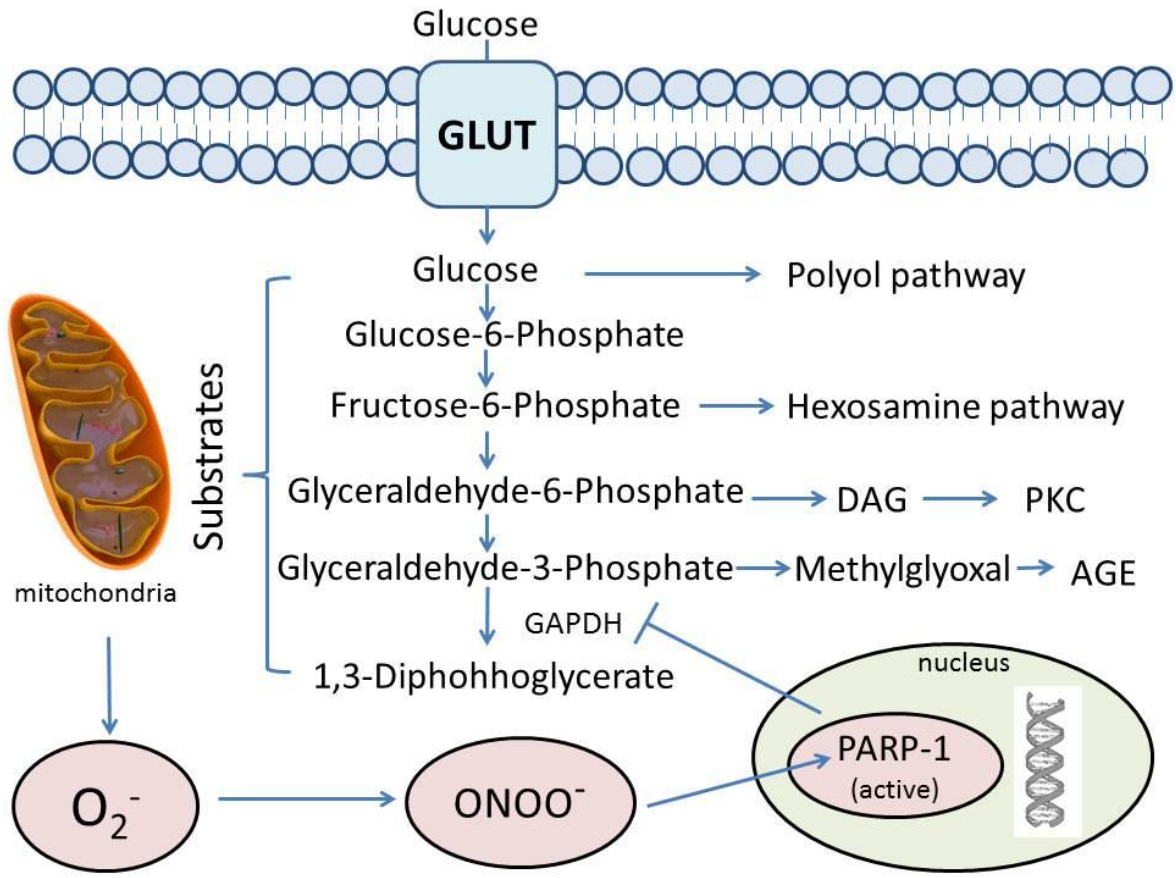
6.1.2 Complications of long-term diabetes: mechanisms of toxicity

Glucose is the driving force of macro- and microvascular complications of diabetes. However, the actions of glucose alone may be inadequate to account for all the excess atherosclerosis and vascular disease observed in diabetics. It has been found that, in diabetic patients there is an increase in plasma free fatty acid concentrations leading to an increased in fatty acid uptake and oxidation [342]. Not only is this process pro-inflammatory [342], it would lead to reduced utilization of glucose as a source of energy during ischaemia and stress, and as suggested previously, would potentially increase the vulnerability of diabetic heart under anoxic conditions.

Additionally, upregulation of TXNIP during hyperglycaemia (as discussed in Chapters 1.2.4 and 2.1.3) is not only pro-inflammatory, pro-aggregatory but also pro-apoptotic; thus representing a potential therapeutic target.

In 2000, Nishikawa *et al* characterised four major signaling pathways activated by hyperglycaemia in endothelial cells: activation of the protein kinase C pathway via diacylglycerol, increased hexosamine pathway flux, increased advanced glycation end product, and increased polyol pathway flux [343] (see Figure 6-I). All these four pathways lead to an integrated eventual increased production of reactive oxygen species by the mitochondrial electron transport chain. Recently, Du *et al* showed that the downstream target of such oxidant stress is PARP (see Chapter 1.2.2 for discussion of effects): its activation leads to ribosylation and inactivation of glyceryaldehyde-3-phosphate dehydrogenase (GAPDH). Inhibition of GAPDH leads to increased intermediaries, which will eventually lead to increased mitochondrial oxidative stress [344].

Figure 6-I: Mechanisms of cellular injury caused by high glucose flux.



During hyperglycaemia, excess glucose flux leads to excessive generation of superoxide by mitochondria when fluxed through the electron transport chain. This leads to the eventual DNA strand breakage and activation of PARP. Activated PARP inhibits GAPDH, which will then shunt glucose oxidation into the polyol pathway and lead to the activation of protein kinase C or advanced glycation end product.

Abbreviations: GLUT = glucose transporter; DAG = diacylglycerol; PKC = protein kinase C; AGE = advanced glycation end product; GAPDH = glyceryaldehyde-3-phosphate dehydrogenase

Parallel to this, there is also recent evidence that chronic supplementation with the antioxidant coenzyme Q_{10}, commenced in the presence of hyperglycaemia (in both type 1 and type 2 diabetic mice models), induced attenuation of cardiac complications [345,346]. Such benefits include reduction in cardiomyocyte hypertrophy, fibrosis and apoptosis (all by approximately 50%), as well as reduced production of superoxide and expression of NADPH oxidase (a major cellular source of superoxide). Furthermore, it has also been shown that targeting ROS *in vivo* prevents diabetes-induced suppression of LV SERCA2a (sarco(endo)plasmic reticulum Ca^{2+}-ATPase) [345], a key mechanism of diabetes-induced LV dysfunction. Therefore, targeting elevated ROS in the diabetic myocardium represents an attractive therapeutic target for the prevention and/or reversal of LV dysfunction in DM.

6.1.3 Therapeutic manipulation of insulin sensitivity: current perspectives

While it is known that insulin therapy augments glucose oxidation, it has been shown that such effect (especially during high oxidative stress, e.g. myocardial ischaemia) is also accompanied with increased platelet nitric oxide responsiveness [306] (a prognostic marker in acute coronary syndrome [330]), reduced celullar oxidative stress, and possibly improved survival and long-term prognosis [307,347].

Analogously with their mechanism(s) of actions and clinical utility in the treatment of diabetes, it is of no surprise that a number of oral hypoglycaemic agents have been shown to prevent type 2 diabetes in high risk individuals. First, in the United State's Diabetes Prevention Program (n=3234), metformin 850mg twice daily reduced incidence of type 2 diabetes by approximately 30% (as compared to placebo) in a group of overweight patients over 3 years [348]. In the STOP-NIDDM trial (Study to Prevent Non-Insulin Dependent Diabetes), treatment with acarbose resulted in reduced rates of abnormal oral glucose tolerance test (relative risk reduction of approximately 30%) after two years of treatment [349]. Rosiglitazone, an agonist of the peroxisome proliferator activated receptor-gamma, has also been shown to reduce incidence of type 2 diabetes and increase the likelihood of regression to normoglycaemia in adults with impaired glucose tolerance [350]. Interestingly, orlistat, an anti-obesity drug, also reduced incidence of diabetes by approximately 37% (compared to placebo) after a median of four year follow up [351].

Importantly, angiotensin converting enzyme inhibitor (ACE-i) or angiotensin II receptor blocker (ARB), two classes of drugs that are commonly used in patients

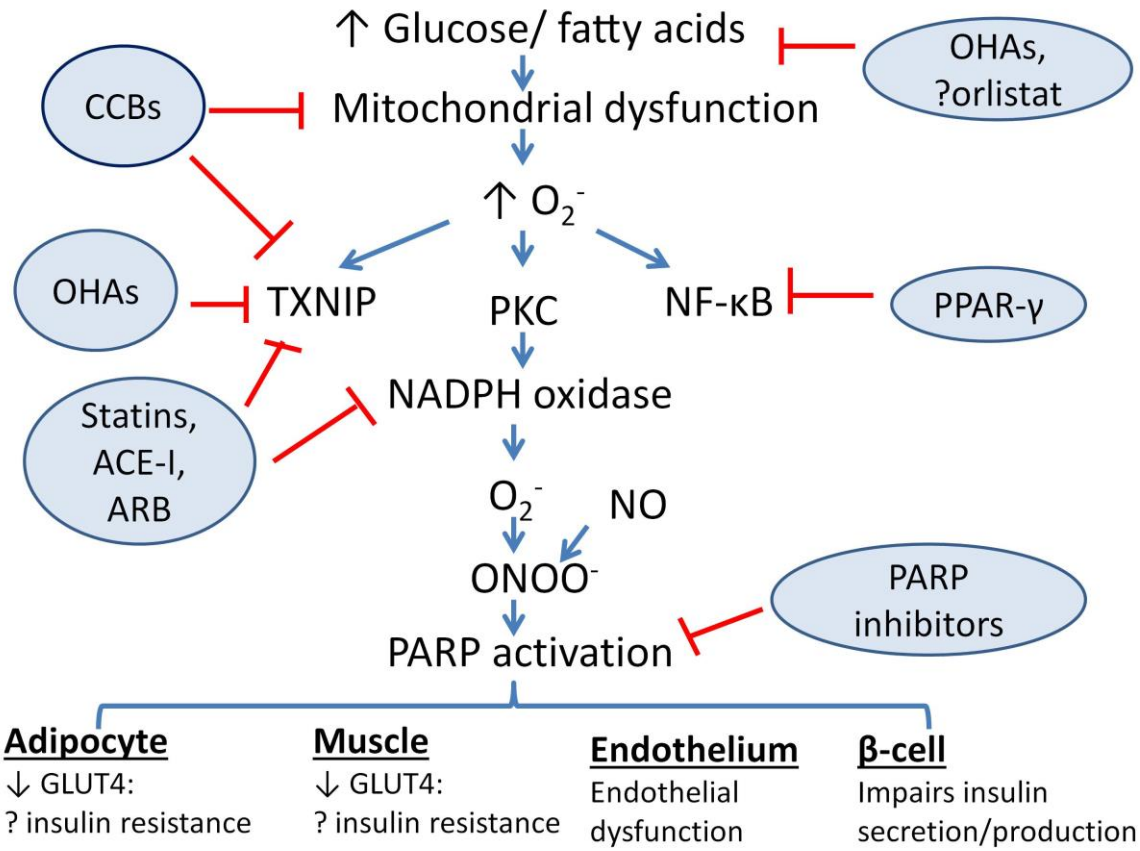
with cardiovascular disease, have also been shown to improve insulin sensitivity. A recent meta-analysis showed that in patients without diabetes, both types of agents improved insulin sensitivity, with ACE-i being more effective than ARB in ameliorating insulin resistance in hypertensive patients without diabetes [352].

Epidemiological evidence that ACE-i or ARB limited development of diabetes is ample, though conflicting. The Captopril Prevention Project (CAPPP) showing that patients treated with captopril had 14% lower rates of newly diagnosed type 2 diabetes compared to controls [353]. This result was then confirmed in the Heart Outcomes Prevention Evaluation (HOPE) trial which showed that during the 4 to 5 year period of the trial, only 3.6% of those treated with ramipril developed diabetes as compared to 5.4% of those in the placebo group [354]. A similar trend was also observed in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE), where there was a 25% reduction in the development of type 2 diabetes in patients randomised to losartan therapy [355]. However, a recent prospective trial investigating the effect of ramipril in pre-diabetic patients showed that ramipril did not reduce the incidence of diabetes after a median follow up of 3 years [356]; nor did it improve β-cell functions, as measured by insulinogenic index and proinsulin concentrations [357].

The mechanism(s) of improvement of insulin sensitivity by ACE-i or ARB are not completely understood but several have been postulated. First, it has long been postulated that mitochondrial dysfunction and subsequent oxidative stress is a common ground for insulin resistance, diabetes and cardiovascular disease [358]. By virtue of reducing cellular oxidative activity, several agents, including calcium channel antagonist and statins may potentially improve insulin sensitivity (see Figure 6-II).

It should also be recognised that angiotensin II reduced the activation of insulin signaling [359,360]. Thus ACE-i or ARB therapy potentially may prevent this inactivation and ameliorate resistance to insulin.

Figure 6-II: Oxidative stress: a common ground for insulin resistance and its therapeutic implications.



Modified from reference [358].

Abbreviations: CCB = calcium channel blockers; OHAs = oral hypoglycaemic agents

6.1.4 Insulin sensitization by perhexiline: current relevance

Perhexiline was previously reported to cause hypoglycaemia [239,242], particularly when utilized in conjunction with diabetic treatment regimens. The literature in this regard consists largely of case reports, many dating back to the period of initial clinical experience with perhexiline [242]. There is little information concerning the effects of perhexiline in patients receiving ACE-inhibitors or ARB; both of which insulin sensitizers. This is potentially important because recent clinical studies have provided a strong basis for the routine use of ACE inhibitors in particularly in diabetics at risk of myocardial ischaemia [354].

There are several reasons that perhexiline may improve insulin sensitivity. First, it inhibits CPT-1 [176] and promote glucose uptake. In addition to that, in the myocardium of non-diabetic patients, perhexiline has also been shown to increase activation of AMPK [280], a pharmacological action shared by metformin. Additionaly, perhexiline reduces oxidative stress, via inhibition of NAD(P)H oxidase [208]; and as suggested previously (Chapter 1.2.4), reduction in oxidative stress activity could potentially lead to improved insulin sensitivity.

TXNIP was shown in a recent microarray study to be the most upregulated gene in diabetic patients [139]; and is implicated in the complications of diabetes. Calcium channel antagonists reduced the expression of TXNIP in the heart and pancreas [154], and therefore potentially could be utilized as "first-line" agents to prevent diabetes-induced heart disease (assumimg a primary role for TXNIP). Perhexiline, either by other mechanism(s) or by its action as a weak calcium channel antagonist [224], has also been shown to reduce the expression of TXNIP in non-diabetic patients [280].

Perhexiline, being a potent prophylactic anti-anginal and anti-failure agent, is increasingly utilized in wide range of cardiovascular disease states. Devoid of direct haemodynamic effects (and in particular negative inotropy), perhexiline presents itself as being more advantageous than other cardiovascular agents currently available in this regard, especially when used in elderly patients. Should it be proven to also improve insulin sensitivity, it is likely to be preferred in patients with diabetes and cardiovascular disease.

A further dimension of interest in this regard is added by the increasing need to treat patients with concomitant diabetes, renal insufficiency and refractory cardiovascular disease. For example, in recently reported large series of patients with advanced heart failure, type 2 diabetes is present in approximately 20 % of the population studies [361,362], while approximately 16 to 40 % have moderate to severe renal insufficiency [363,364].

The implications of such renal insufficiency are considerable: there is an incremental risk of precipitating bradyarrhythmis through hyperkalaemia if aldosterone antagonist are used, and the safety of ACE-inhibitors is not clear-cut. On the other hand, perhexiline can be used safely in such patients [277] and is therefore increasingly prescribed in diabetics with renal insufficiency.

We now report an investigation to determine both the extent and mechanism(s) of putative insulin sensitization in a population of diabetic patients treated with perhexiline.

6.2 Objectives of current study

This study tests the hypothesis that perhexiline is an insulin sensitizing agent in a cohort of individuals receiving prior pharmacotherapy for insulin resistance.

Specifically, we sought to examine the putative insulin-sensitizing effect of perhexiline in the circumstance of extensive pre-treatment with ACE-inhibitor or angiotensin receptor blockers and/or metformin.

Furthermore, we wished to determine whether the utilization of perhexiline in this cohort was:-

(1) associated with NO sensitization (as in patients wiht unstable angina)

(2) associated with suppression of platelet TXNIP expression, given that:

(i) ACE inhibitors and metformin suppress TXNIP

(ii) TXNIP suppression represents a possible mechanisms for NO sensitization [138].

6.3 Methods

The trial was approved by The Queen Elizabeth Hospital/ Lyell McEwin Hospital human ethics committee (HREC/13/TQEHLMH/220) and registered in the Australian New Zealand Clinical Trials Registry (ACTRN12615000497505).

Patients were recruited from the outpatient clinic of Cardiology Department, The Queen Elizabeth Hospital. Criteria for inclusion were:

1. Known coronary artery disease, heart failure or symptomatic aortic stenosis
2. Diagnosed with diabetes mellitus or pre-diabetes

Exclusion criteria include:

1. <18 years old
2. Pregnancy, or women of childbearing age who are not using effective contraception
3. Concurent treatment with any P2Y12 antagonist or perhexiline

The regimen for initiation of perhexiline maleate was 600mg as loading dose on day 1, then 100mg bd from day 2 onwards or dose adjusted according to plasma concentration. Fasting blood sample were taken before and 2 weeks after perhexiline treatment (for following analyses); additional plasma samples were also taken on day 2 and day 14 for determination of plasma perhexiline concentrations. Investigations performed were as follow (details refer to Chapter 5.3.1):

1. Fasting glucose and insulin concentrations
2. Platelet response to anti-aggregatory effect of NO donor sodium nitroprusside
3. Platelet content of TXNIP
4. Plasma thrombospondin-1
5. Asymmetric dimethylarginine
6. Myeloperoxidase, determined by enzyme-linked immunosorbent assay (ELISA; Mercodia, Sweden)
7. Hepatic function tests

Patients were divided into two groups, depending on their prior treatment with an ACE-inhibitor or angiotensin receptor blocker. Power calculation for patient sample was based on previous result within the group [138] using platelet expression of TXNIP as primary endpoint. In the previous study, it was shown that compared to control, treatment with ramipril for two weeks reduced platelet TXNIP expression from 286 ± 95 (SD) down to 172 ± 76. Therefore, in order to detect 30% change with ACE-inhibitor treated patients, n=18 will provide ~80% of power at p=0.05.

6.4 Results

Thirty patients were recruited and completed 2 weeks of perhexiline therapy. Dosage of perhexiline was adjusted based on plasma drug level to achieve therapeutic concentration of 0.15-0.6 mg/L. Baseline characteristics were described in Table 6-I. In general, these are well-controlled type 2 diabetics; majority were already on ACE-inibitors or ARB. Most of the patients were on ≥ 1 hypoglycaemic agent.

None of the subjects experienced significant adverse effects, including symptomatic hypoglycaemia after perhexiline treatment.

Of the 30 patients, 83% had been formally diagnosed with type 2 diabetes. All were receiving some form of insulin-sensitizing therapy, and 27% were receiving both metformin and an ACE-innibitor or angiotensin receptor blocker.

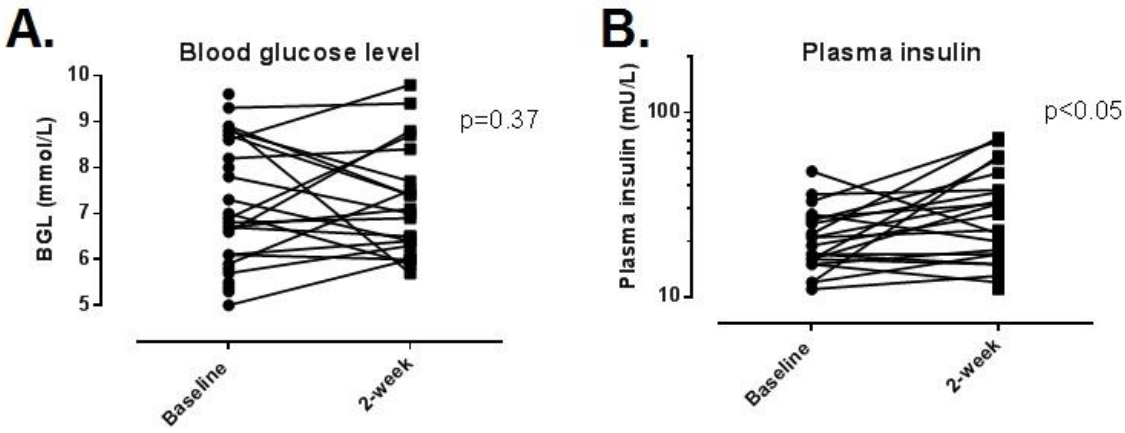
Given that the primary indication for perhexiline treatment was angina pectoris in 70% of patients, it is not surprising that 17% were receiving calcium antagonists (notably verapamil or diltiazem in all cases).

Extent of diabetic control was variable among these patients but none had required admission for management of hyperglycaemmia within the preceding three months.

Table 6-I: Baseline characteristics of the patients in this current cohort (n=30).

Patient characteristics	
Age (years: mean ± SD)	69 ± 11
Female (%)	37
HbA1c (%)	7.4 ± 1.5
Principal indication for perhexiline therapy	
Refractory angina (%)	70
Systolic heart failure (%)	23
Symptomatic aortic stenosis (%)	20
Concurrent Pharmacotherapy	
ACE-inhibitors/ ARB (%)	73
Metformin (%)	37
Insulin (%)	23
Calcium antagonist (%)	17

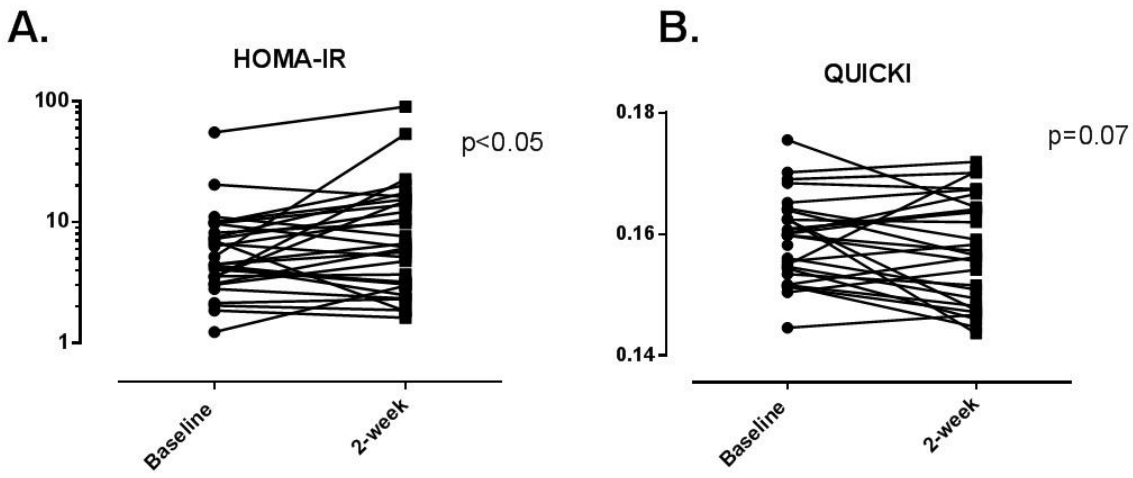
Figure 6-III: Effect of perhexiline on (A) fasting blood glucose level and (B) fasting plasma insulin concentration after two weeks of therapy.



Perhexiline concentrations were within therapeutic range (0.30 ± 0.04 mg/L) after 2 weeks of perhexiline therapy. 4 of the patients were poor metabolisers of perhexiline; hence requiring weekly dosing of perhexiline only. As shown in Figure 6-III, fasting blood glucose level did not change significantly after perhexiline therapy; from 6.8 (95%CI: 5.7, 8.6) to 7.0 mmol/L (95% CI: 6.0, 8.7). Plasma insulin level increased marginally but significantly from 18.5 (95% CI: 11.8, 25.3) to 19.0 mU/L (95% CI: 11.8, 37.3; $p<0.05$).

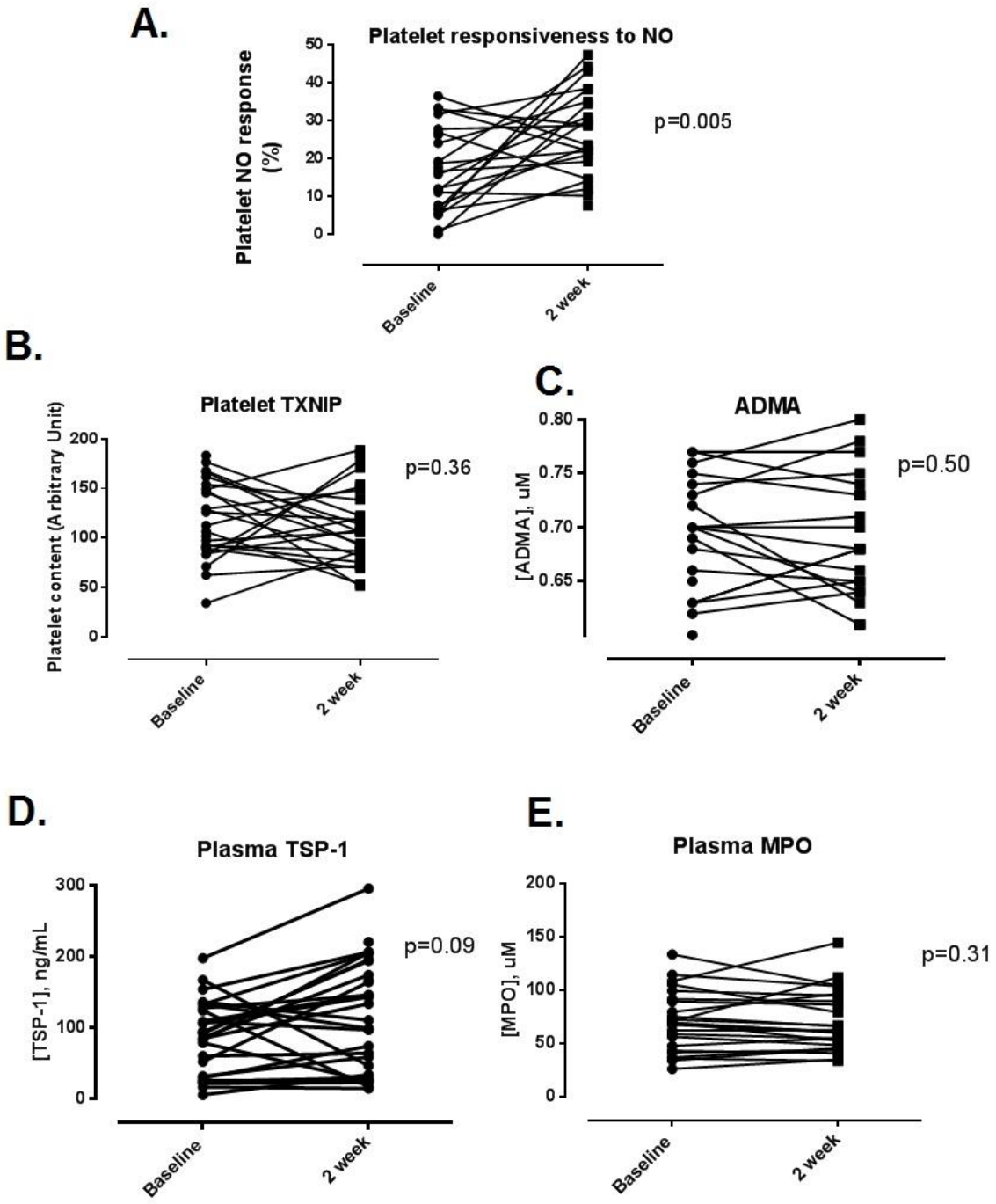
Insulin sensitivity increased marginally: when measured by HOMA-IR (Figure 6-IV A) this increase was statistically significant (median 4.5 to 6.2; $p<0.05$); while when measured by QUICKI there was a fall in overall score from 0.158 to 0.156 ($p=0.07$), consistent with a minimal degree of insulin sensitization (Figure 6-IV B).

Figure 6-IV: Effects of two weeks of perhexiline on insulin sensitivity, as measured by (A) HOMA-IR, and (B) QUICKI.



Perhexiline treatment was associated with a substantial improvement in platelet responsiveness to the anti-aggregatory effect of the NO donor sodium nitroprusside (5 μM), from 16.7 ± 16.0 to 27.3 ± 19.8 % ($p<0.01$; see Figure 6-V). There was no significant change in platelet content of TXNIP [127.6 (95% CI: 91.1, 154.4) vs 113.9 AU (95% CI: 86.3, 154.4); p=0.36], plasma ADMA concentrations (0.69 ± 0.09 vs 0.68 ± 0.09 μM; p=0.48), plasma thrombospondin-1 concentrations [94.64 (95% CI: 47.45, 133.0) vs 138.7 μM (95% CI: 43.69, 198.3); p=0.09], and plasma MPO concentrations [69.16 (95% CI: 46.35, 93.27) vs 61.47 (95% CI: 47.51, 95.00); p=0.31].

Figure 6-V: Effects of perhexiline on (A) platelet responsiveness to sodium nitroprusside; (B) platelet content of TXNIP; (C) NOS inhibitor, ADMA; (D) plasma thrombospondin-1; and (E) myeloperoxidase.



6.5 Discussion

The objectives of this study related to the emerging clinical roles of perhexiline in conditions such as severe angina pectoris and congestive heart failure. During the development of perhexiline, the management of patients with concomitant diabetes was different from that which applies today, with a central role for sulphonylurea derivatives in the majority of aptients who were not well-controlled with diet, or who did not require insulin therapy. Furthermore, ACE-inhibitors were not routinely used in type 2 diabetics for cardiovascular protection as they are today following the results of the HOPE [354] and EUROPA [365] studies.

Thus we have arrived at a modern setting where diabetic patients with myocardial ischaemia or heart failure are very likely to be receiving, inter alia, metformin, an inhibitor of AMPK which appears to be cardioprotective [87] and ACE inhibitors. Both of these (group of) drugs are not only insulin-sensitizing, but also have been shown to inhibit TXNIP expression, perhaps via the insulin sensitization [138,148].

Perhexiline has many actions, but perhaps the best established is CPT-1 (and CPT-2) inhibition [176]. The class of CPT-inhibitors is well known to act as insulin sensitizers: indeed one of this group, etomoxir, was originally evaluated for efficacy in the treatment of type 2 diabetes [366,367]. Hence it is not surprising that perhexiline has been reported both to lower blood sugar level as a “routine” finding in diabetics, and occasionally to induce symptomatic hypoglycaemia [239,368].

The question, which was addressed in the current study, was whether this effect would persist in the context of “modern” treatment of diabetes, with concomitant use in many cases of metformin and ACE-inhibitors. Indeed, in the group studied, 37% of patients were treated with metformin, and 73% with ACE-inhibitors or angiotensin receptor blockers.

Furtermore, we were interested, not only whether perhexiline might be an insulin-sensitizer in this setting, but whether the “accessory” actions of the drug might be relevant in this therapeutic context. As regards these “secondary” effects, we tested for some, but not all. Specifically:-

(1) TXNIP suppression has been observed in human myocardium with perhexiline [280], but only reported thus far in the non-diabetics. This could theoretically be less prominent in an environment including metformin, ACE-inhibitors, and insulin, all of which themselves suppress TXNIP [81].

(2) Potentiation of platelet NO signaling has consistently been reported with perhexiline [209]. The mechanism of the effect is uncertain, but could be:

(a) TXNIP suppression

(b) Decreased NOX expression

(c) Decreased thrombospondin-1 expression, given the NO-suppressing effects of thrombospondin-1 [311].

Additionally, there is preliminary evidence that perhexiline may increase activation of AMP kinase [280], but this was not tested in the current experiments.

The results of the secondary objectives of the study therefore can be summarized as follows:- *While perhexiline is a marginal insulin sensitizer in the context of normal diabetic therapy, it retains a clear-cut NO-potentiating effect, and the mechanism of which is uncertain.*

This should be clarified on the basis that the study was of only 30 patients, so that small changes might have been subject to Type 2 error. Nevertheless, as regards the other “accessory” actions of perhexiline:-

(1) TXNIP content tended to decrease (mean fall 6%; 95% CI -36, +27%). Thus a major degree of TXNIP suppression can be excluded, but involvement of TXNIP in insulin sensitization cannot.

(2) NOX expression was not measured, but given the role of NOX in generation of reactive oxygen species [369], this remains a possible contributor to the observed effects. A follow-up study should evaluate changes in NOX expression in both platelets and leukocytes in this population.

(3) Thrombospondin-1 levels in plasma tended to rise, rather than fall. Thus it seems most unlikely that the NO-suppressing effects of thrombospondin-1 [311] are relevant in this context.

Overall, the study augments evidence for the safety of perhexiline in diabetics. There should be less concern about severe hypoglycaemia, and reassurance that the major therapeutic actions of the drug remain intact.

6.6 Acknowledgement

In this study, Institute of Medical and Veterinary Sciences (South Australia) measured the concentrations of blood glucose and insulin. Tamila Heresztyn and Saifei Liu measured the concentrations of plasma ADMA. Irene Stafford and Saifei Liu provided the internal standards used for the measurements of TSP-1 and MPO.

Chapter 7: Evolving therapeutics: Can we do better than perhexiline?

7.1 Improving on perhexiline: a therapeutic holy grail?

Manipulation of myocardial metabolism has proven not only to improve cardiac efficiency but also to exert cardioprotective effects. Currently, there are very few cardiovascular pharmacological agents on the market that are known to be "metabolic". Amongst these are perhexiline, trimetazidine, etomoxir, oxfenicine (all with variable adverse effects) and to a lesser extent, amiodarone and potentially ranolazine. Therefore investigation of other agents with increased therapeutic efficiency and reduced adverse effects is desired.

First, previous pharmacokinetics and toxicology studies from our laboratory suggested that the enantiomers of perhexiline are different: not only that the pharmacokinetic profile of the enantiomers was significantly different [297,370,371], but the toxicology profile, especially that of (-)-perhexiline [286], suggested that it could potentially be utilised with less risk of known long-term adverse effects of racemic perhexiline. Rats that were treated with (-)-perhexiline displayed significantly lesser accumulation of hepatic lipid droplets and neurovascular toxicity [286]. Such observations prompted further investigation of the pharmacology profile of these agents.

Parallel to the development of perhexiline enantiomers, biochemists from Kosterlitz Institute, University of Aberdeen are also investigating new forms of perhexiline. Analogues were designed to modify the structures of perhexiline with two principal objectives: (1) to reduce importance of metabolism by CYP2D6 and (2) to facilitate metabolism by other less variable, more rapid, pathways.

Thus it was hoped that an analogue with more convenient and predictable pharmacokinetic profile could be developed. However, the chemical structures of these agents will not be revealed in this *thesis*.

7.1.1 CPT- inhibition by anti-arrhythmic agents: dronedarone and amiodarone

7.1.1.1 Amiodarone: effect beyond anti-arrhythmia

Amiodarone is known to be an antiarrhythmic agent that inhibits the sodium channel, β-adrenoceptors, L-type calcium channels and prolongs action potential duration via potassium channel blockade [372-374]. Interestingly, it was originally introduced as an anti-anginal agent. However, like perhexiline, amiodarone *per se* did not exert significant haemodynamic effects [375], although some may result from its dissolution in polysorbate 80 [376,377]).

In 1996, Kennedy *et al* hypothesized that like perhexiline, amiodarone was a CPT-1 inhibitor [176]. Indeed, it was found that amiodarone is a weak CPT-1 inhibitor. The IC_{50} for cardiac CPT-1 by amiodarone was approximately 200 μM, as compared to approximately 70 μM by perhexiline. Therefore, amiodarone was less potent (on a molar basis) than perhexiline in potentially decreasing fatty acid metabolism in favour of carbohydrate metabolism and thereby increasing cardiac efficiency.

The finding that amiodarone inhibited CPT-1 had a number of implications:

(1) It provided an explanation for the occasional development of hepatitis [378-381], and of peripheral neuropathy [382-385] on amiodarone.

(2) It provided additional evidence for the known anti-anginal effects of the drug.

(3) It provided a possible basis for the beneficial effects of amiodarone in systolic heart failure in the GESICA trial [386].

(4) It raised the issue of potential risk of incremental toxicity if amiodarone and perhexiline are co-administered. However, an audit of a small number of such patients suggested that risk is slight [240].

Finally, these findings were potentially of significance beyond amiodarone, given that other benzofuran derivatives are being developed for potential clinical use [387]. One such agent is dronedarone.

7.1.1.2 Dronedarone and amiodarone: similarities and differences

Dronedarone was developed by modifying the structure of amiodarone, removing iodine but retaining the benzofuran component, in an effort to reduce toxicity and improve pharmacokinetic profile. Indeed, iodine-related thyroid toxicity do not occur with dronedarone [388]. In the first placebo-controlled, double-blinded, parallel arm clinical trial of dronedarone to assess the efficacy in preventing cardiovascular hospitalization or all-cause mortality in patients with atrial fibrillation (the ATHENA trial [389]), dronedarone was shown to reduce the agglomerate end-point of hospitalization from cardiovascular events and death from cardiac

arrhythmia. After ATHENA, many drug regulatory authorities around the world approved its usage and it rapidly became part of the algorithm of treatment guidelines for the management of atrial fibrillation. However, the subsequent PALLAS trial showed (strikingly and confusingly) an increased incidence of heart failure with dronedarone therapy in patients with sustained atrial fibrillation [390]. Indeed, two other randomized studies suggested that there might be substantial problems with dronedarone: in the ANDROMEDA study [391], it was shown that dronedarone increased mortality rates in patients with severe systolic left ventricular dysfunction, while in the DIONYSOS study [392], it emerged that dronedarone was less effective than amiodarone in preventing AF recurrence. Such conflicting results have sparked various speculations around its relative efficacy versus toxicity profile. Additionally, a few cases of dronedarone hepatotoxicity have been reported [393,394]. Indeed thus far, dronedarone has not been released in Australia, largely because of concerns regarding its toxicity.

The latter finding bags the question of the precise mechanism(s) of effect of dronedarone: it has been labelled a “multi-channel blocker”, but the issue of concern here was whether it retained the CPT-1 inhibition effect seen with amiodarone.

7.2 Purpose of the study

This study represented a component of the therapeutic screening process for: (a) enantiomers of perhexiline, (b) newly synthesized perhexiline analogues, and (c) dronedarone.

The major issues to be investigated were the potency of these various therapeutic candidate drugs as inhibitors of cardiac and hepatic CPT-1, the main known mechanism of benefit and toxicity of perhexiline respectively. Although clinical use of perhexiline is associated with low incidence of adverse events in the context of regular therapeutic drug monitoring (Chapter 3), its clinical usage is partially hindered by the need of plasma concentration monitoring, accurate interpretation of results and variable dosage adjustment. It is expected that the newer agents (enantiomers of perhexiline or the newly synthesized analogues) would eliminate the need of drug monitoring and the use of a more straightforward dosing regimen will promote its clinical use. In the context of dronedarone, on the other hand, investigation of CPT-1 activity could potentially provide mechanism of benefit and toxicity associated with the drug, and serve as a basis for future drug development.

As regards to the four newly synthesized analogues, which are fluorinated derivatives of perhexiline, the primary objective of it was to derive a compound with similar efficacy with that of racemic-perhexiline, in terms of CPT-1 inhibition, but more predictable metabolism. However, given that the intellectual properties of these compounds reside within collaborators at the University of Aberdeen (UK), their structural analogues are not revealed in the context of this thesis, and are therefore only abbreviated as FPER1, FPER2, FPER3 and FPER4.

Studies were performed in healthy rat tissues under normoxic conditions. The CPT-1 activity developed by Kennedy *et al* [176] (which originally adopted from McGarry et al [16]) was employed with modifications. Essentially, enzymatic activity was measured by the formation of palmitoyl-carnitine from palmitoyl-CoA and *l*-carnitine (to be discussed further in this Chapter).

7.3 Methods

7.3.1 Animals utilized, reagents and materials

Male Sprague-Dawley rats aged 6 – 8 weeks old were used. Ethics approval was obtained from both the University of Adelaide (M-2012-039) and SA Pathology (12/12).

All reagents and materials were purchased from Sigma-Aldrich®. The only exceptions were D_3-palmitoyl carnitine (Cambridge Isotope Laboratories®), methanol (Scharlau®), acetonitrile (VWR Chemicals®), and ethanol (Chem-Supply®).

7.3.2 Sample preparation procedure: Tissue preparation and enzymatic assay

In studies examining the IC_{50} for CPT-1 inhibition of investigational drugs, rats of 6-8 weeks were euthanised under inhaled isoflurane 2 to 3%. Once removed, hearts and livers were placed in ice-cold isolation buffer (pH 7.4) consisting of 10 mM Tris-HCl, 250 mmol/L sucrose and 1 mmol/L EDTA immediately 1:5 (w/v). The tissues were homogenised using a Polytron PT-3000 (Kinematica®), with great care taken to avoid overheating by using half-maximal speed for three to five bursts of three seconds, separated by thirty seconds. Then supernatant was obtained by centrifuging the solution for ten minutes at 600 *g* and 4°C. In some experiments the supernatant (homogenate) was used as such; with protein concentration of 2 mg/ml was used in enzyme reaction assay. In some other experiments where mitochondria were needed, further centrifugation at 7000 *g* and 4°C for ten minutes was performed. The pellet was then resuspended to the original volume with isolation buffer and re-centrifuged at 7000 *g* for ten minutes at 4°C. The subsequent pellet was then resuspended. Following protein estimation, a solution with protein concentration of 1 mg/ml was used in enzyme reaction assay.

The incubate consisted of 400 µl of incubation buffer (pH 7.4), which contained 6.25 µmol Tris-HCl, 72 µmol KCl, 2.85 µmol of reduced glutathione, 1.45 µmol of KCN, 1 µmol of $MgCl_2$, 5 µL of ethanol, and 2.7 mg of bovine serum albumin without free fatty acid. 50 µl of homogenates containing CPT-1 (2 mg/ml of protein concentrations if whole tissue homogenate were used; or 1 mg/ml of protein if mitochondria was to be isolated) were added per assay to the incubate in a shaking water bath at 37°C for 5 minutes. Pilot experiments were conducted with

perhexiline to replicate the method of Kennedy *et al* [176], with 5 µl of the inhibitor (final concentration of 100 µM) with 15 minutes of pre-incubation would employed. The enzymatic reaction was commenced by adding 50 µL substrate mix, consisting of 100 µmol/L palmitoyl-CoA and 400 µmol/L of [^{3}H]-*l*-carnitine (prepared in milliQ water). After 1.5 minutes, the reaction was terminated by adding 50 µL of concentrated HCl. Samples were then diluted with 1.45 mL of distilled water, and the product palmitoyl-[^{3}H]-carnitine was extracted with 1 mL of *n*-butanol and measured by scintillation counting (Wallac Trilux 1450 Microbeta® liquid scintillation and luminescence counter).

7.3.3 Preparation of investigational drugs

7.3.3.1 Perhexiline and perhexiline enantiomers

Racemic perhexiline maleate (obtained from Sigma®) was dissolved in sodium hydroxide solution. It was then extracted with ether, washed with water and dried with magnesium sulphate, to obtain pure racemic perhexiline. Pure (+) and (-)-perhexiline maleate were available in the Clinical Pharmacology department and had been prepared according to the method of Davies *et al* (2006) [298].

(+), (-) or racemic-perhexiline (dissolved in ethanol) at concentrations of 10, 20, 50, 80, 100, 150, and 200 µM were used. Fifteen minutes pre-incubation of investigation drug with the incubate buffer at 37 °C shaking water bath was employed [176]. Malonyl-CoA (1 µM) was used as a positive control to confirm enzyme inhibition. Hepatic tissue without the addition of any drug (vehicle only) was used as a measure of 100% CPT-1 activity; and hepatic tissue without the addition of enzymatic substrate (i.e. palmitoyl-CoA and *l*-carnitine) was used as a negative control.

7.3.3.2 Fluorinated perhexiline analogues

Known weights of the four analogues (FPER1, FPER2, FPER3, FPER4) were received from Kosterlitz Institute, University of Aberdeen, UK. Except for FPER2, all other analogues were dissolved in ethanol.

FPER1 appeared as visible powder, and a 10mM stock solution was prepared. FPER3 appeared as brown stain on bottle with no visible powder, and hence 500 µl of ethanol was added into the bottle and a stock solution of 70.4 mM was prepared. No visible powder appeared with FPER4, and hence 50 µl of ethanol was added into the bottle to make a stock concentration of 74.9 mM.

FPER2 was originally dissolved in 600 µl ethanol (to make a concentration of 46.7 mM). However the powder did not dissolve even after extensive vortexing. After consultation with Dr Iain Greig (Kosterlitz Institute, Aberdeen), the solution was left to dry under room temperature and dissolved later in 600 µl of DMSO.

Racemic perhexiline (final concentration of 100 µM) was used as comparator. Similar to the previous experiments, malonyl-CoA 1µM was used as a positive control to confirm enzyme inhibition; hepatic tissue homogenate with vehicle only was used as a measure of 100% CPT-1 activity, and hepatic tissue homogenate without substrate mixture was used as negative control.

For an experiment involving tissue homogenate (n=1) involving FPER1, FPER3 and FPER4, concentrations used were 10 μM and 100 μM only.

For experiments involving isolated mitochondria (n=2) involving FPER1, FPER2 and FPER3, concentrations used were as followed:

FPER1: 10, 50, 100, 150 and 200 μM.

FPER2: 10, 50, 100, 150 and 200 μM.

FPER3: 10, 50, 100, 150 and 200 μM.

7.3.3.3 Amiodarone and dronedarone

Dronedarone was introduced to homogenates at concentrations 5, 10, 50, 100 and 500 μM was compared to that of amiodarone at concentrations 100, 200 and 500 μM, and malonyl-CoA at 1 μM.

7.3.4 Data analysis

Concentration-response curves for each inhibitor were expressed constructed by plotting the log of inhibitor concentration versus the percentage inhibition of palmitoyl carnitine formation. The IC_{50} for inhibition of palmitoyl carnitine formation was determined by fitting the data to a sigmoidal concentration response curve, using GraphPad Prism 6®, with the following equation:

$$Y = 100 / (1=10^((LogIC_{50}-X)*HillSlope)))$$

7.4 Results

7.4.1 Assay validation

For pilot studies, cardiac or hepatic tissues scavenged from adult rats utilized in other studies (euthanized under carbon dioxide) were sourced to confirm the validity of the assay, adapted from Kennedy *et al* [176]. After several failed experiments (to be explained later), it was thought that potential "ischaemic" time of tissues might be likely to be too long for animals euthanised under such method, which could potentially jeopardise the retention of enzymatic activity. Therefore, subsequent studies used only tissues scavenged from other studies whereby rats were euthanised under anaesthesia.

7.4.1.1 Difficulties with extraction process

In view of the principle for pathology board-certified laboratory equipment (non-radioactive) that equipment is not to be shared with radioactive work, an extraction process using rotational-vacuum-concentrator (Christ RVC 2-33 CD*plus*®) (which usually provides fast extraction and drying process) was deemed to be unsuitable and other methods had to be explored. First, allowing samples to be air-dried under a fume hood in a radioactive room had proven to be way too time-consuming, needing several days to extract the final product. Then, drying under nitrogen gas under fume

hood in the radioactive room was trialled and adopted, with extraction time shown to be around 1 hour.

7.4.1.2 Difficulties with scintillation counts

Initial experiments (n=3) were performed without the addition of any potential CPT-1 inhibitor. A typical experiment would have the following substrate sets of positive and negative controls:

(1) [^{3}H]-*l*-carnitine and palmitoyl-CoA: this interaction would yield palmitoyl-[^{3}H]-carnitine;

(2) [^{3}H]-*l*-carnitine without palmitoyl-CoA (replaced by equal volume of distilled water): after extraction, the water-soluble [^{3}H]-*l*-carnitine would expectedly be extracted and hence no or little radioactivity in the end-product would be expected; and

(3) palmitoyl-CoA without [^{3}H]-*l*-carnitine (replaced by equal volume of distilled water): the end product was expected to be similar to (2).

The yields, however, showed that there was still substantial radioactivity detected with (2) (see Table 7-I for example). This suggested that the extraction was potentially not specific enough.

Table 7-I: Scintillation counts (arbitrary units) providing evidence of poor sensitivity and contamination with unreacted *l*-carnitine.

	1	***2***	***3***
Incubate	[^{3}H]-*l*-carnitine and palmitoyl-CoA	[^{3}H]-*l*-carnitine without palmitoyl-CoA	palmitoyl-CoA without [^{3}H]-*l*-carnitine
A	102	60	34
B	82	57	32

Example of experiment. Rows A and B were duplicates of each other. Column 2 should (theoretically) have similar low levels of radioactivity as column 3, if extraction had been complete and accurate.

Table 7-II illustrates scintillation counts referable to incubations of tissue extract with labelled carnitine and unlabelled palmitoyl-CoA for periods of 5 to 30 minutes. Product of the reaction being measured was theoretically all labelled palmitoyl carnitine. The deficiency of the methodology from column 5 (scintillation counts is apparent without addition of palmitoyl-CoA): these should in theory be identical to counts for column 6 (water only).

The retention of radioactive signal from any source other than palmitoyl carnitine implies potential inaccuracy of the assay. Here for example, rates of apparent formation of palmitoyl carnitine appear non-linear. Hence this assay was regarded as unsatisfactory, and a detection method utilizing LCMS/MS was explored.

Table 7-II: Scintillation counts with variable protein concentration and incubation times.

		1	*2*	*3*	*4*	*5*	*6*
		Incubation time					
	Protein concentration	**5 min**	**10 min**	**20 min**	**30 min**	**Ctrl 1 (no CoA)**	**Ctrl 2 (H_2O only)**
A	**2 mg/ml**	113, 109	119, 125	229, 194	297, 296	75, 68	39, 40
B	**5 mg/ml**	176, 192	190, 228	395, 423	358, 386	74, 79	31, 45

Duplicate values from separate assays are provided.

7.4.1.3 Chromatographic conditions

The HPLC system consisted of an Agilent 1100 series® (Agilent Technologies, CA) pump, degasser, injector, and column oven; and an API3200 tandem mass spectrometer® (AB SCIEX Pty Ltd, MA). The assay was based on the method of Liu *et al* (2008) [395]. A C18 column (SunFire™ C18 3.5 µM; 2.1 x 50 mm column) and two mobile phase were adopted. Mobile phase A was water: 2mM ammonium acetate: formic acid at 100:154mg/L:0.1; mobile phase B was methanol: 2mM ammonium acetate: formic acid at 100:154mg/L:0.1. An injection volume of 20 µl was used. The final optimised conditions of both palmitoyl-carnitine and stable isotope d_3-palmitoyl carnitine were as shown in Table 7-III.

The total duration of the acquisition was 4.902 minutes with equilibration time set at 1 minute. During acquisition, only column effluent from 2.5 to 4.5 minutes was delivered to mass spectrometry. Eflluents before 2.5 minutes and after 4.5 minutes were delivered to waste. Binary flow mode was utilized at 0.4 ml per minute. From the start of acquisition up to 1 minute, the concentration of mobile phase B was set at 20%; with gradual increase to 95% during 1 to 2 minutes and maintained at 95% afterwards. Oven temperature was set at 50 ºC and rinsing volume of 200 µl before and after aspiration was employed.

Table 7-III: Mass-spectrometry conditions for the detection of palmitoyl-carnitine and its deuderated isotope.

	Palmitoyl-carnitine	D_3-palmitoyl carnitine
Q1 (Da)	402.4	405.601
Q3 (Da)	85	85.201
Time (milliseconds)	460	300
Declustering potential (V)	45	34
Entrance potential (V)	10	5
Collision exit potential (V)	40	15
Collision energy (V)	60	50
Collision cell exit potential (V)	30	3

7.4.1.4 Investigation of the solubility of palmitoyl-carnitine

Information from the supplier (Sigma-Aldrich®) indicated that palmitoyl-carnitine is soluble in water with heat or with sonication. Additionally, preliminary work suggested that its solubility in different solvents needed to be properly assessed. Therefore, a low and a high concentration (0.5 or 5 μM) of palmitoyl-carnitine were dissolved in different commonly used solvents on ice: acetonitrile, methanol, ethanol and propranol.

Essentially, 1450 μl of solvent were added to 50 μl of 0.5 μM or 5 μM of palmitoyl-carnitine and vortexed vigorously for about 30 seconds. Then 200 μl of sample was taken for injection (with injection volume to be 20 μl). Triplicates were performed.

First, it was found that palmitoyl-carnitine could not be dissolved in propranol. Then it was found that both concentrations of palmitoyl-carnitine dissolved in acetonitrile did not provide visible peak heights; whereas samples dissolved in methanol provided peak heights greater than that of ethanol, with retention times ranging from 3.2 to 3.8 minutes.

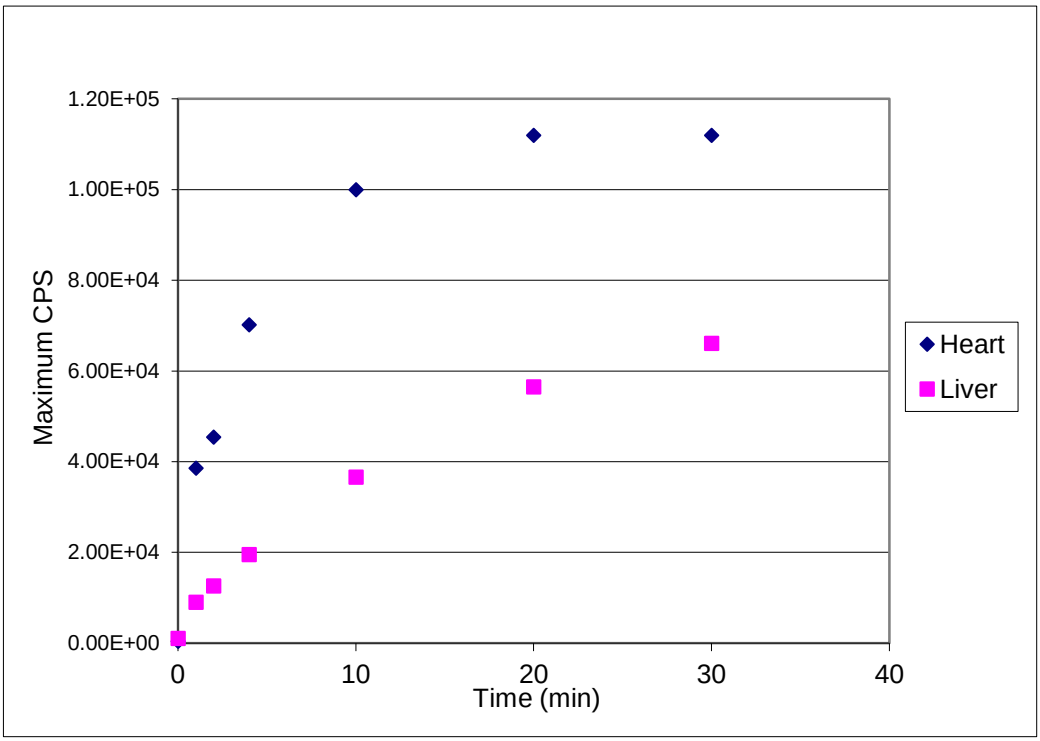
Therefore, methanol was selected for subsequent precipitation of incubates and samples.

7.4.1.5 Optimisation of protein concentration and reaction time

To confirm that the enzyme reactions occured at optimal protein concentration and duration of reaction, experiments with variable incubation time (0, 1, 2, 4, 10, 20 and 30 minutes; see Figure 7-I) and protein concentration (for isolated mitochondria: 0, 0,5, 1, 2, 4 mg/ml; for tissue homogenate: 0, 0.5, 1, 2, 4, 8 mg/ml; see 7-II A and B respectively) were performed.

In view of near linearity of rates of formation of product palmitoyl-carnitine under the first 5 minutes of incubation, this was utilized for subsequent quantitation. For all subsequent studies, enzyme reaction time was set at 1.5 minutes; isolated mitochondrial protein concentration was set at 1 mg/ml and whole tissue homogenate protein concentration was set at 2 mg/ml (same as Kennedy *et al* [176]).

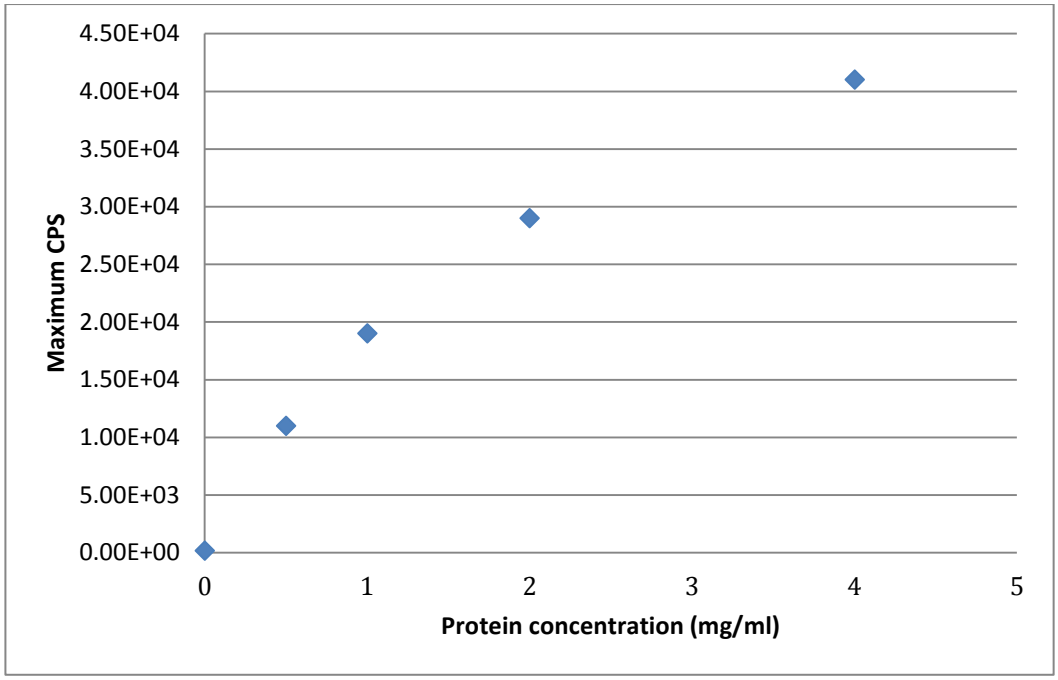
Figure 7-I: Optimisation of reaction time in tissue homogenate at 2mg/ml



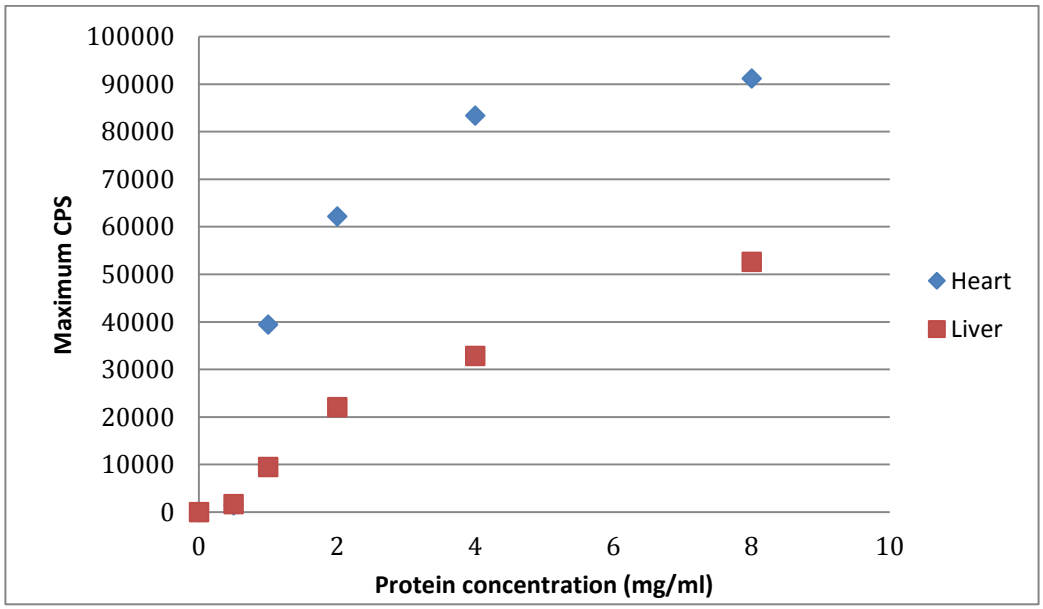
Abbreviation: CPS = counts per second

Figure 7-II: Optimisation of mitochondrial protein concentrations during 1.5 minutes of incubation.

A. Evaluation in isolated hepatic mitochondria



B. Evaluation in tissue homogenate: (a) rat heart (b) rat liver



7.4.2 Changes made to enzymatic assay and quantitation method

Due to difficulty in a lack of sensitivity of quantitation by scintillation (as explained earlier), changes were then made to quantitation via LCMS/MS. As the detection method had been changed to non-radioactive detection by LCMS/MS, some changes are made to the final stages of sample preparation after solubility of palmitoyl-carnitine was determined.

Instead of stopping enzyme reaction with concentrated HCl and extracted desired sample, 500 µl of ice-cold methanol was added to the incubate to precipitate and to stop reaction. The final product was then vortexed vigorously and placed on ice. Another 500 µl of methanol containing d_3-palmitoyl-carnitine (for final concentration of 1.67 nM) was added and the solution was vortexed vigorously for approximately 30 seconds. The samples were then centrifuged at 10,000 rpm for 10 minutes, and 200 µl of the supernatant was placed in a blank tube ready for injection into LCMS/MS. A series of calibration curves, ranging from 0 to 500 nM of palmitoyl-carnitine were prepared.

The use of internal standard and calibration curves were employed in some initial studies. However, subsequent studies abolished the use of calibration curve. Instead, concentration-response (expressed as percentage of inhibition) was established with each study: 100% of palmitoyl-carnitine production was established when no agent of interest was added (i.e. vehicle control only; or 0% of inhibition); and subsequent production of palmitoyl-carnitine by various concentrations of inhibitors were expressed as percentage of production relative to vehicle control.

7.4.3 Comparison of the CPT-1 IC_{50} of perhexiline enantiomers, fluorinated analogues and dronedarone

Inhibition of hepatic and myocardial CPT-1 activity by each potential inhibitor was firstly compared with racemic perhexiline in tissue homogenates (n=2 to 3 per compound), and subsequently in isolated mitochondria (n=3 to 4 per compound). Examples of concentration-response curves for each inhibitor were shown in Figure 7-III (for hepatic mitochondria) and Figure 7-IV (for myocardial mitochondria). Relative inhibitory potentcies were estimated by comparing the percentage of inhibition for each compound at a concentration of 100μM. Using this measure the relative potencies were dronedarone (most potent) > racemic or (+)- or (-)-perhexiline > amiodarone > FPER1 > FPER 3 > FPER2 > FPER4 (least potent). Overall, (+) and (-)-perhexiline were equipotent to that of racemic perhexiline; dronedarone was approximately 3-fold more potent than amiodarone, and equipotent in both heart and liver. On the other hand, at 100 μM, FPER4 inhibited cardiac CPT-1 by only approximately 15%.

Formal calculation of IC_{50} values was only performed in hepatic and myocardial mitochondria for racemic, (+)- and (-)-perhexiline, FPER1 and dronedarone, as shown in Table 7-IV. The small amounts available, poor solubility and relatively low potency of the remaining compounds meant I was unable to attain high enough concentrations during incubations to accurately estimate IC_{50} values.

Figure 7-III Comparison of concentration-response curves of perhexiline enantiomers, fluorinated perhexiline analogues and dronedarone in rat hepatic mitochondria.

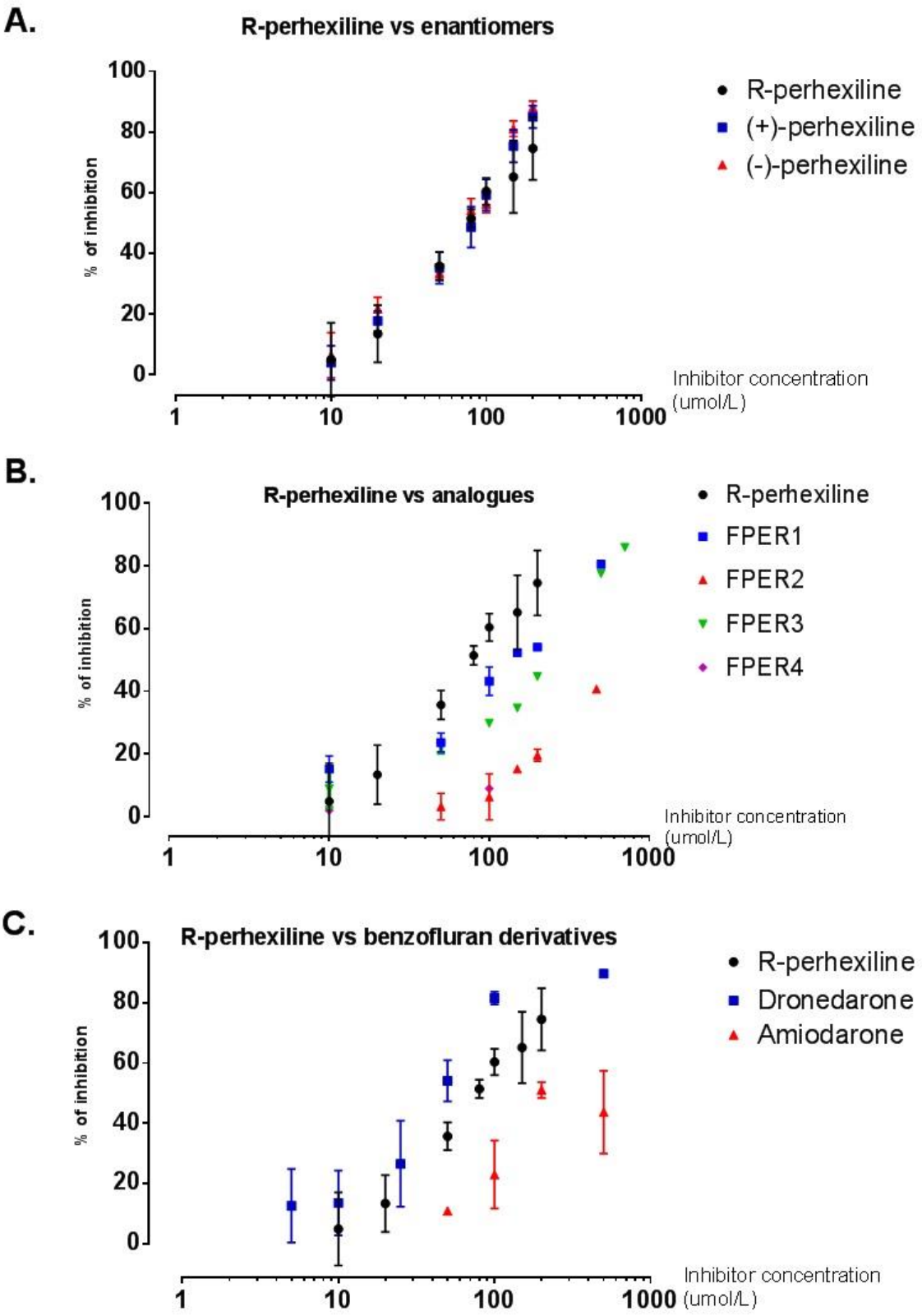


Figure 7-IV: Comparison of concentration-response curve of perhexiline enantiomers, fluorinated perhexiline analogues and dronedarone in rat cardiac mitochondria.

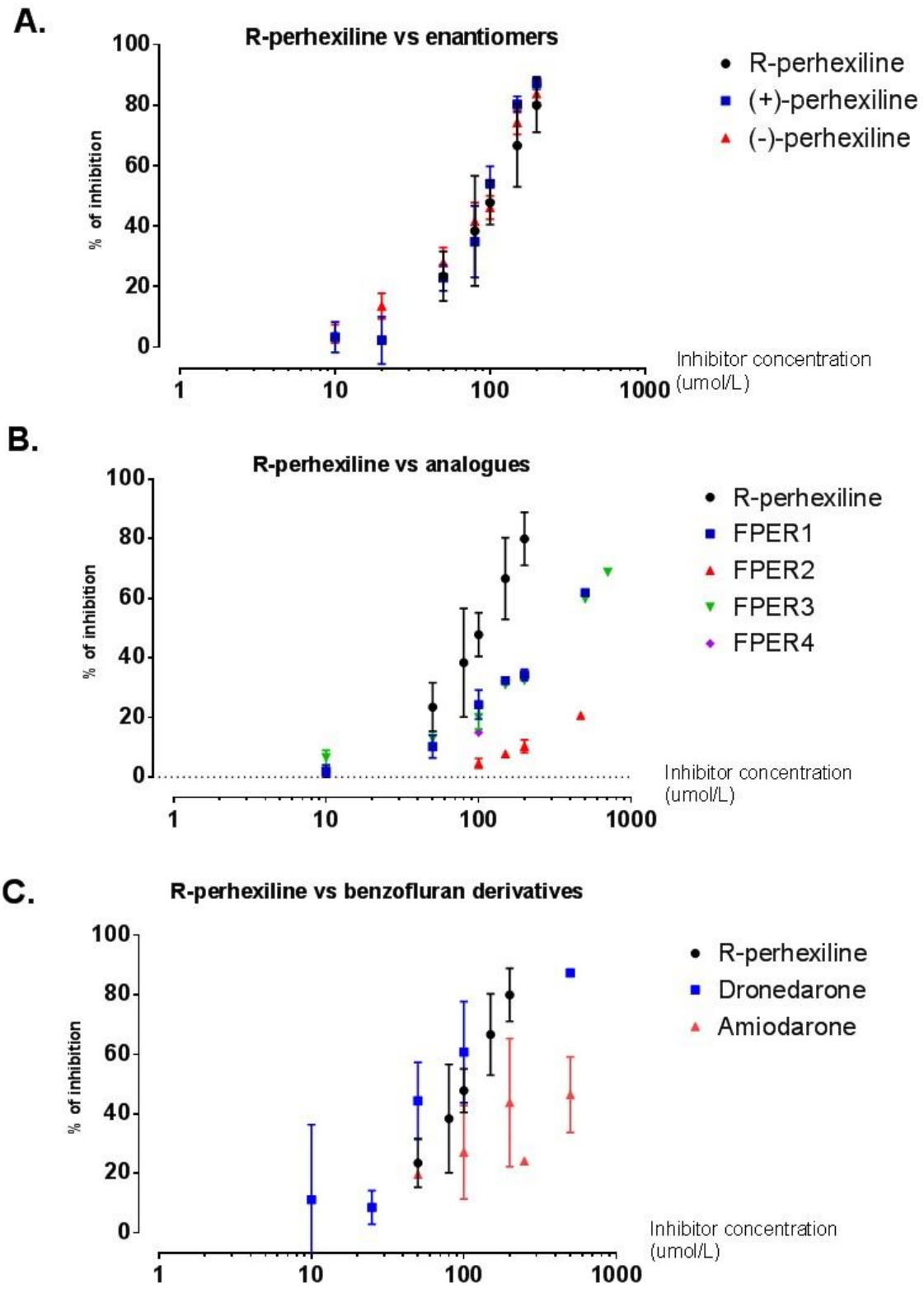


Table 7-IV: Comparison of the CPT-1 IC_{50} of perhexiline enantiomers, FPER1 and dronedarone with racemic mixture

	Mitochondria (n=4)	
	Hepatic	**Cardiac**
Rac-Px	49 (35 - 69)	63 (51 - 79)
(+)-Px	54 (43 - 69)	65 (55 - 76)
(-)-Px	45 (35 – 58)	56 (45 – 69)
FPER1	179 (130 - 247)	344 (272 – 435)
Dronedarone	39 (28 - 54)	-

Data shown were best fit value (95% confidence intervals). * p = ns (+) vs (-) Px

Abbreviations: Rac = racemic; Px = perhexiline

Samples without inhibitor yielded maximal palmitoyl-carnitine during reaction time, whereas samples without substrate yielded undetectable palmitoyl-carnitine. Malonyl-CoA inhibited >90% of palmitoyl-carnitine formation in both heart and liver. Table 7-VI provides a summary of the IC_{50} of both racemic and perhexiline enantiomers in either hepatic or cardiac tissue. The results did not differ significantly between those for isolated mitochondria and tissue homogenate (result of latter not shown). The IC_{50} of racemic perhexiline did not differ significantly from those of of (+) and (-)-perhexiline and there was also no significant difference between CPT-1 of hepatic or cardiac origin.

7.5 Discussion and conclusions

This evaluation was based on the idea that the enantiomers of perhexiline might exhibit variable CPT-1 inhibition. Since CPT-1 inhibition probably contributes to hepato- and neuro-toxicity of perhexiline, identification of a poor substrate for at least hepatic CPT-1 would have been of theoretical interest.

In fact, the major findings were:

(1) There is no difference in potency of CPT-1 inhibition between (+) and (-)-perhexiline in either rat liver or heart.

(2) Perhexiline and its enantiomers are approximately equipotent in the heart and liver. This is different from previous findings by Kennedy *et al* (1996) which showed that perhexiline was less potent in the liver [176]. Such disparity may be explained by improved sensitivity of the currently employed quantitative method using LCMS/MS.

The obvious implications of this finding are both therapeutic and relevant to toxicity. Given that CPT-1 inhibition is almost certainly a major contributor to long-term toxicity, the implication is that differential inhibition of CPT-1 is not the basis for the apparently greater safety of (-)-perhexiline. It is far from clear that CPT-1 inhibition is central to clinical efficacy [207], but again there is no basis here for differential effects. Thus the preliminary observation of differential toxicity of enantiomers can be based only on accessory properties, e.g. NAD(P)H oxidase inhibition [208], or CPT-2 inhibition [396].

An extension to the issue examined with perhexiline enantiomers is the search for bioactive derivatives of perhexiline with predictable pharmacokinetics. Staffs of the Kosterlitz Institute (Aberdeen, UK) have synthesized a number of perhexiline derivatives, which should be poor targets for CYP2D6, by means of fluorinating sites for potential hydroxylation, and substituting methyl groups.

Preliminary analyses were performed on four of these compounds, using the same methodology as perhexiline enantiomers. One compound (FPER4) lacked detectable CPT-1 inhibition. The remainder were CPT-1 inhibitors but were all less potent than perhexiline, with FPER1 showing the greatest inhibition of the four. None appeared to differ in the tissue selectivity from perhexiline itself. However, these data must be regarded as semi-quantitative by virtue of low numbers of experiments. The experiments of the new fluorinated analogues therefore emphasize the value of assessing CPT-1 activity as a component of the evaluation of “new” perhexiline derivatives while being cognizant that CPT-1 is not the only target of such drugs.

As regards dronedarone, the data from these experiments establish that dronedarone acts at least in part, as a CPT-1 inhibitor, with approximately equal potency in heart and liver tissue. Theoretically, CPT-1 inhibition should provide incremental safety of the drug in patients with heart failure [235], assuming this is associated with increased efficiency of myocardial energetics [397]. However, this is not the case with dronedarone, which appears to be potentially lethal in such patients as shown in the ANDROMEDA trial [391]. Presumable the basis for this is sodium channel blockade, which appears from the CAST study [390], to be predictably associated with increased mortality in the presence of left ventricular dysfunction.

The occurrence of hepatotoxicity with dronedarone [393,398] has never been widely investigated. Felser *et al* (2014) reported that the pattern of toxicity was consistent with CPT-1 inhibition [399] but no studies thus far have evaluated whether the appearance of hepatotoxicity is predictable in terms of plasma and tissue dronedarone concentrations nor whether it is associated with hepatic phospholipid accumulation: this should now be a major priority.

In conclusion:

(1) The enantiomers of perhexiline do not display heterogeneity as regards CPT-1 inhibition.

(2) Structural analogues of perhexiline synthesised to be poor substrates for CYP2D6 retain some CPT-1 inhibition, which in the bases of at least two of the compounds tested (FPER1 and FPER3) are not markedly less than that for perhexiline.

(3) Dronedarone is a strong CPT-1 inhibitor: more potent than amiodarone, and slightly more potent than perhexiline. It is therefore to be anticipated that it will eventually be found to be predictably hepato- and neurotoxic with chronic use.

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Chapter 8: Summary and future perspectives

The experimental chapters in this thesis have originated from two concepts:

1. That energetic impairment within the myocardium is a commonplace occurrence in many forms of heart disease, including myocardial ischaemia (acute and chronic), hypertrophic cardiomyopathy, and systolic heart failure.

2. That perhexiline is now established as an acute treatment for all of the above problems, but it remains a potentially toxic agent which even now is incompletely understood. There is therefore a great need both to improve understanding and safety of perhexiline administration, and to seek less complex and more effective “metabolic” treatment.

The major findings from these experiments can be summarized as follows:

Chapter 3: Long-term follow-up of a large cohort of patients treated with perhexiline revealed that there was a very low incidence of definite hepato- or neurotoxicity, despite suboptimal monitoring of perhexiline levels in the majority of patients. There was a suggestion that symptomatic improvement might occur with plasma levels of perhexiline which would previously considered subtherapeutic. This finding should stimulate prospective investigation of the plasma level: effect relationship at lower levels than previously considered. Additionally, it was noted that survival in this group of patients with ischaemia and often impaired left ventricular systolic function was remarkably good. There are no control prospective studies of perhexiline effects on mortality, but if financially feasible, this should stimulate such studies. Furthermore, it is possible that this is a generic rather than specific finding: any agent with predominant energetic effects might theoretically have beneficial effects on patients’ survival.

Chapter 4 evaluated the determinants of acute myocardial uptake of perhexiline, utilizing cardiac biopsy approximately ten days after initiation of therapy. This study is virtually unique in literature, most other investigations of drug uptake being conducted via mass-balance principles with single dose of the drug concerned. It was shown that perhexiline uptake into the myocardium revealed some evidence of saturability, but is still closely related to plasma perhexiline levels. Thus the results were hardly surprising, even though strictly speaking uptake of perhexiline into the left ventricle has not quite reached steady state within ten days. The results of the study were also interesting in that despite apparent failure of perhexiline to alter metabolomic spectrum, it was found that in this non-diabetic group there was significant fall in blood sugar level and evidence of both activation of AMPK and suppression of TXNIP expression [280]. Thus, the results of this study, apart from validating cardiac uptake methodology of this type, also provide evidence that even non-diabetics perhexiline increases efficiency of glucose utilization possibly via AMPK activation and TXNIP suppression.

Follow-up investigations prompted by this finding would be to investigate the effects of perhexiline on energetics in the presence and absence of AMPK inhibitors, e.g. compound C, and of chronic TXNIP suppression, e.g. utilizing siRNA knockdown. It is important in particular to determine whether the beneficial effects of perhexiline are AMPK or TXNIP-dependent because in such a case, the effects of perhexiline would be diminished in the presence of agents which activate AMPK and suppress TXNIP respectively.

Chapter 5 evaluated the acute impact of reversal of severe hyperglycaemia at hte levels of autocoidal function and formation of endothelial progenitor cels (EPCs). The basis for this study was a previous observation [306] that insulin

infusion for 12 hours increased NO signaling and suppressed superoxide release. In view of a subsequent finding of a reciprocal relationship between NO signaling and platelet TXNIP content in the setting of (1) physiological ageing [400], and (2) two weeks' of ramipril therapy [138], and the known effects of hypoglycaemic agents in suppressing TXNIP expression [81], we sought to determine whether TXNIP might mediate the suppression of superoxide release. Furthermore, as EPC formation is partially NO-dependent, we examined changes in EPC function by monitoring colony-forming unit formation. The results confirmed that reversal of hyperglycaemia by insulin for 12 hours increased NO anti-aggregatory response and decreased whole blood reactive oxygen species. Furthermore, EPC function was markedly augmented. However, neither platelet TXNIP content, nor plasma concentrations of the NO/sGC inhibitor TSP-1, fell significantly. Therefore the latter factors are unlikely to mediate the acute beneficial effects of insulin on platelet reactivity in this setting.

The study has a number of limitations. First, it is well established that the gene for TXNIP has a glucose-responsive element. Therefore, it is virtually certain that long-term reversal of hyperglycaemia would suppress TXNIP expression. The explanation for the negative result here might be:-

(1) Type 2 errors, but 95% confidence interval excludes any large effect;

(2) Insufficient time for change to occur; and/or

(3) A pecularity of platelet homeostasis is, depending on the lack of DNA in non-nucleated platelets.

To clarify these issues, the following might be considerd in the future:

(1) A substantially larger study of similar design

(2) A more prolonged study period (say, 24 hours); and most importantly

(3) Evaluation of these principles *in vitro* utilizing nucleated cells (e.g. endothelial cells and megakaryocytes).

From a practical point of view, the results of the study reinforce the clinical priority of rapidly achieving normal blood sugar levels in patients with acute coronary syndrome.

Chapter 6 evaluated the impact of 2 weeks of perhexiline therapy on diabetic control and platelet reactivity. In many respects, this study was analogous to the short-term insulin protocol in Chapter 5. However, it sought to manipulate the entire Randle cycle (using CPT-1 inhibitor with perhexiline), rather than just stimulate glucose utilization as a primary effect (with insulin). The results confirmed the previously known NO-dependent anti-aggregatory effect of perhexiline [209]. Two weeks of perhexiline therapy significantly improved platelet responsiveness to the anti-aggregatory effects of sodium nitroprusside in this group of diabetic patients, even in the setting of modern pharmacotherapy, where the majority of patients were already on ACE-inhibitors, which were also shown to improve nitric oxide responsiveness [138].

Of equal importance were the results of the main aim of the study: to evaluate the possible impact of perhexiline on diabetic control in patients already receiving appropriate pharmacotherapy. Although both perhexiline itself and also other CPT-1 inhibitors have well-documented hypoglycaemic effects, in the setting of frequent use of metformin and of ACE-inhibitors the insulin-sensitizing effects of perhexiline were marginal. No patient exhibited marked falls in blood sugar level.

Therefore the main two conclusions of the study, one “positive” and the other “negative” pose intrinsic challenges as regards the underlying biochemical mechanisms. Given that there is minimal insulin sensitization, it would be surprising if TXNIP expression fell markedly: indeed there was only a non-significant fall in TXNIP in platelets. We were also able to exclude a substantial decrease in TSP-1 release into plasma. Therefore, a major future priority is to determine exactly how perhexiline potentiated platelet NO signaling in this population. The only potential answer on the basis of the previous literature is inhibition of NAD(P)H oxidase, perhaps primarily in neutrophils, an effect demonstrated as an NO/perhexiline interaction by Liberts *et al* [210]. However, this hypothesis remains to be tested.

In chapter 7 the issue of CPT-1 inhibition as a therapeutic principle was explored in rats’ myocardium and hepatocytes. Perhexiline and malonyl-CoA were utilized as index compounds to quantitate inhibiton of labelled palmitic acid metabolism, and CPT-1 inhibition kinetics were compared with:

(1) Those of putative CPT-1 inhibitors (dronedarone and amiodarone)

(2) Derivatives of perhexiline: (a) enantiomers; (b) conjugates

It was confirmed that amiodarone exhibits moderate CPT-1 inhibtion, which may have contributed to its potential cardioprotective effects. Interestingly, dronedarone was also a moderate CPT-1 inhibitor. This might contribute to the reputed “soft landing” effect noted in AF patients in the ATHENA trial, but it is surprising in this sense that dronedarone is poorly tolerated in systolic heart failure.

As regards the enantiomer studies, these suggest that there is no significant difference in potency between (+) and (-) perhexiline: thus differences in effect between enantiomers must have other causes.

Experiments with conjugates revealed that these were all less potent than perhexiline. However, two conjugates (FPER1 and FPER3) had reasonably CPT-1 inhibitory effects, and might be suitable for clinical development subject to pharmacokinetics advantages over perhexiline.

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Addenda and Corrigenda

A number of additions and alterations have been made within the body of the text of this thesis in response to comments made by the examiners of the thesis. These changes are summarized below, together with the location within the revised text of the various alterations.

Chapter 1

1. An inclusion of the benefit of GIK in viable myocardium with dobutamine echocardiography is now added on page 43.
2. Measurements of cardiac function, apart from ejection fraction, in patients with "preserved ejection fraction" is now provided on page 45.

Chapter 3

3. The introduction section of Chapter 3, on page 100, is now shortened to avoid repetition of previous chapters.
4. The limitations of this study, is now re-written, as well as how the study could be designed in the future (page 114 and 115).
5. Discussion of patients' symptom control is omitted due to the lack of available objective definition of clinical assessment and availability of record in all of the case notes. However, this is acknowledged on page 115.
6. The Cox and Snell R^2 is now provided in Table 3-IV (page 112).

Chapter 4

7. The method section of Chapter 4 is now amended, to clearly provide the readers information of my contribution.

Chapter 5

8. The potential for type 2 errors regarding the involvement of known modulators of NOgeneration and/or effect is now discussed (p164-165).

Chapter 6

9. Part of the Introduction section is now re-written to provide basis for using a myocardial metabolic agent (which is used for angina or heart failure clinically) to restore systemic metabolic homeostasis (see pages 167-169).
10. The word “commonly” is omitted to describe the frequency of hypoglycaemia associated with perhexiline on page 177.

Chapter 7

11. The information of the newer analogues of perhexiline and the limitations on revealing their structures are discussed on page 197-198.
12. The purpose of the current study is now being elaborated (see page 197).

Overall

13. Typos, errors in tenses, inconsistent spelling, and the use of “diabetic” as adjective have now been corrected.
14. An acknowledgement is added at the end of Chapters 3 to 7 to provide clearer views of contribution from others.

Appendix

Cardiovasc Drugs Ther
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ORIGINAL ARTICLE

Drugs that Affect Cardiac Metabolism: Focus on Perhexiline

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Abstract Approaches to the pharmacotherapy of angina pectoris have previously centred on the concept that a transient imbalance between myocardial oxygen "demand" and supply within the myocardium can best be addressed by reducing demand (for example, with β–adrenoceptor antagonist) or by increasing availability of blood (via coronary vasomotor reactivity adjustment or coronary revascularization). However, this principle is potentially challenged by the emergence of cases of angina unsuitable for such therapies (for example because of concomitant severe systolic heart failure) and by the recognition that impaired myocardial energetics may precipitate angina in the absence of fixed or variable coronary obstruction (for example in hypertrophic cardiomyopathy). The past 20 years have seen the re-emergence of a class of anti-anginal agents which act primarily by improving efficiency of myocardial oxygen utilization, and thus can correct impaired energetics, simultaneously treating angina and heart failure symptoms. We review the principles underlying the safe use of such agents, beginning with the prototype drug perhexiline maleate, which despite complex pharmacokinetics and potential hepato- or neuro-toxicity has emerged as an attractive management option in many "complicated" cases of angina pectoris.

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Introduction

Myocardial ischaemia, irrespective of the severity of underlying coronary artery disease, is a process whereby part or all of the myocardium exhibits reversible impairment of oxygen uptake, with resultant limitation of a number of processes of aerobic cellular metabolism [1–3]. A central aspect of ischaemia is the coincident appearance or aggravation of disturbed myocardial relaxation, that is, diastolic heart failure: this in turn may result in further compromise of coronary blood flow, resulting in a "vicious cycle" of further reduction in oxygen delivery, particularly to the subendocardium [4].

In recent years, it has become practicable to monitor the process of ATP generation, largely within cardiac mitochondria, and the activity of the creatine kinase pathways, which facilitate the transfer of ATP to the cytoplasm via the production of phosphocreatine. Investigations of cardiac energetic status of this type, typically utilizing ^{31}P-magnetic resonance spectroscopy, have demonstrated that in both myocardial ischaemia and also heart failure associated with congestive or hypertrophic cardiomyopathies, there is a fall in phosphocreatine to ATP ratios [5–7]. The data indicate an overlap in the myocardial metabolic consequences of ischaemia (for example due to coronary stenosis and increased cardiac workload) and various forms of heart failure, and suggest reciprocal relationships between heart failure and ischaemia are possible [8]. Indeed, it is common for conditions such as aortic valve stenosis and hypertrophic cardiomyopathy to present with ischaemic-type chest pain, despite the absence of large vessel coronary stenosis.

If myocardial ischaemia is fundamentally linked with the development of energetic impairment, it is important to

understand exactly how such energetic impairment may be produced. In fact, the heart is able to generate ATP, largely within the mitochondria, primarily via metabolism of either glucose or fatty acids. Conversely, the development of deficiency of high energy phosphates results from extensive failure of interlinked generation pathways.

Cardiac Metabolic Pathways: Reciprocity of Glucose and Fatty Acid Utilization: Randle Cycle

Approximately 70 % of ATP generation by the myocardium results from oxidation of fatty acids (under fasting conditions), but there are a large number of modulating mechanisms which control the process of tissue uptake, which are potentially subject to considerable variability [9]. Importantly, extent of fatty acid oxidation may be modulated both acutely and chronically.

Fatty acid uptake into the heart is initiated via a number of fatty acid binding proteins and a fatty acid translocase (CD 36), and is facilitated by the actions of lipoprotein lipases in releasing fatty acids from triglycerides. The transfer of long-chain fatty acids (LCFAs) across mitochondrial membranes utilizes the "carnitine shuttle", whereby LCFAs are conjugated with carnitine on the outer mitochondrial membrane via the enzyme carnitine palmitoyltransferase-1 (CPT-1) [10]. The resultant acylcarnitine derivatives cross the mitochondrial membrane and are deconjugated from carnitine on the inner mitochondrial membrane by CPT-2 (see Fig. 1). Activity of CPT-1 represents the rate-limiting step in LCFA metabolism: once traversing the mitochondrial membrane, the LCFA undergoes β-oxidation within the mitochondrion: the latter process generates acetyl CoA, which enters the tricarboxylic acid (TCA) cycle and thus contributes to cellular ATP production [10].

There are a number of important modulating factors relevant to rates of LCFA metabolism. First, acetyl-CoA generated in the process of LCFA metabolism is partially converted to malonyl-CoA (by the enzyme acetyl-CoA carboxylase). Malonyl-CoA is a potent inhibitor of CPT-1 (Fig. 1), but accumulation of malonyl-CoA is usually limited by malonyl-CoA decarboxylase, which catalyses breakdown of malonyl-CoA [11, 12]. Second, a number of other ligands and enzymes modify the expression of genes involved in fatty acid metabolism. For example, the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) alters expression of several genes involved in fatty acid metabolism [13]. Furthermore, both sirtuin 1 and adenosine monophosphate kinase (AMPK) function as energy sensors, with the ability to modulate fatty acid metabolism [14, 15] (see Fig. 2). Thus even in isolation the control of fatty acid metabolism is complex, and potentially subject to pharmacological modulation at several points.

Critically, glucose utilization is also subject to a number of physiological controls, although in the fasting state this accounts for only 10–30 % of myocardial ATP generation [9]. The uptake of glucose into the myocardial cytoplasm is partially insulin-dependent: in the cytoplasm glucose is converted to pyruvate (Fig. 1), which undergoes active transport into the mitochondria to enter the TCA cycle. A critical role is played by pyruvate dehydrogenase (PDH), an enzyme complex located within mitochondrial membranes, which represents the rate-limiting step in glucose utilization. A major inhibitor of PDH activity is acetyl CoA, which is generated from LCFA metabolism [16]. Conversely, increased activity of the TCA cycle as a result of extensive glucose utilization results in cytoplasmic accumulation of citrate, and eventually increased malonyl CoA production, inhibiting CPT-1.

In 1963, Randle et al. proposed what has come to be called the Randle Cycle [17]: a reciprocity of utilization of long-chain fatty acids and of glucose by the heart. It has emerged that the key controls of this reciprocity are the activities of CPT-1 and PDH, together with generation of the relevant endogenous inhibitors malonyl CoA and acetyl CoA.

β-oxidation of fatty acids and glucose utilization via the TCA cycle represent the two major sources of ATP generation within mitochondria [9]. Glucose utilization carries a small (approximately 13 %) advantage in terms of ATP generation per unit oxygen consumption. In addition, smaller quantities of ATP may be generated within the cytoplasm under anaerobic conditions via glycolysis, a process which is accelerated when PDH is inhibited by hypoxia-inducible factor 1 under hypoxic conditions [18].

Other Modulation of Myocardial Energetics: Mitochondrial Dysfunction and Energetic Depletion

Production of reactive oxygen species (ROS), within mitochondria in particular, increases under a number of pathological conditions, including anoxia and hyperglycaemia [19]. This may lead to dysfunction of the mitochondrial membrane and of the mitochondrial electron transport chain, with an associated further increase in ROS production [19].

During stressful conditions, the excessively generated superoxide will react with nitric oxide to form peroxynitrite [20]. Peroxynitrite triggers DNA single strand breakage and subsequent activation of poly(ADP-ribose) polymerase (PARP). Once activated, PARP cleaves NAD^+ into nicotinamide and ADP-ribose, a process that depletes NAD^+ and consumes ATP. Therefore, overactivation of PARP has been shown to not only deplete its substrate NAD^+, but also slow the rate of glycolysis, reduce ATP formation and cause eventual cell death [20]. In animal models, PARP-1 knock-out was associated, surprisingly, with enhanced energy expenditure, together with increased glucose clearance, but also protection against

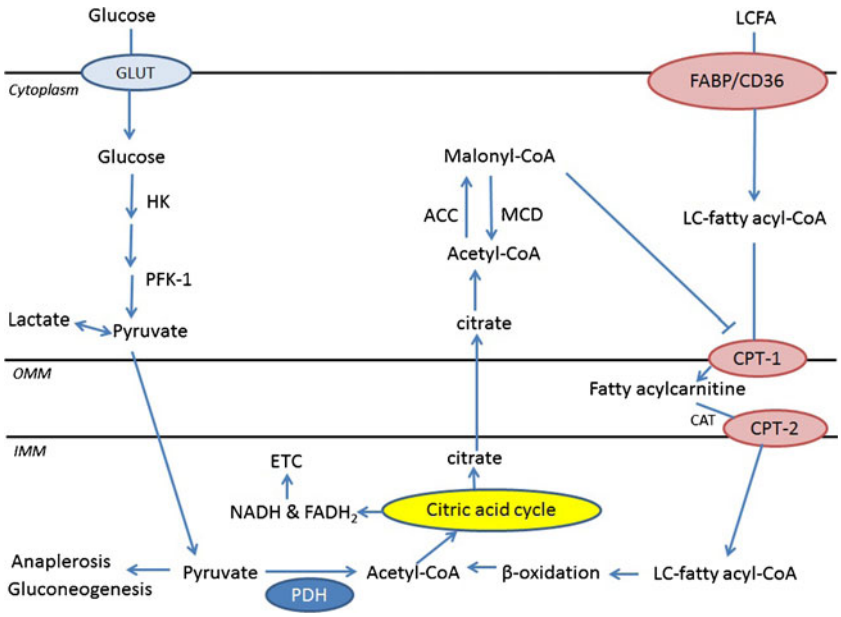

Fig. 1 The reciprocal regulation between glucose and fatty acid metabolism – Randle cycle. Abbreviations: GLUT = glucose transporter; HK = hexokinase; PFK-1 = phosphofructose kinase-1; OMM = outer mitochondrial membrane; IMM = inner mitochondrial membrane; PDH = pyruvate dehydrogenase; ETC = electron transport chain; ACC = acetyl-coA carboxylase; MCD = malonyl-CoA decarboxylase; LCFA = long-chain fatty acid; FABP = fatty acid binding protein; CPT-1/2 = carnitine palmitoyl transferase-1 (or 2)

diabetes while maintaining normal pancreatic insulin content and islet cell morphology [21].

Potential Targets for Therapeutic Modulation of Myocardial Metabolism

A large number of strategies have been proposed to increase generation of ATP in disorders of cardiac energetics, as summarized in Table 1.

Among the various strategies for which data are available indicating beneficial effects on myocardial energetics are infusion of glucose and insulin together with potassium (GIK),

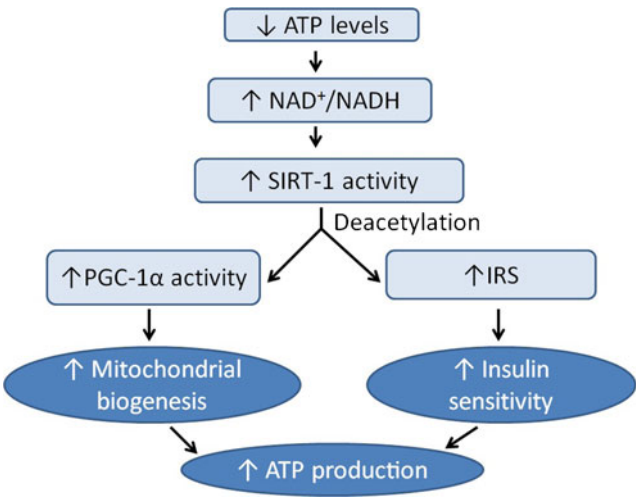

Fig. 2 Sirtuin-1 (SIRT-1) functions as an "energy sensor". Through deacetylation of post-translational proteins, SIRT-1 increases PGC-1α activity and the expression of insulin receptor substates, both of which eventually modify fatty acid or glucose metabolism, and lead to increased ATP production. Abbreviation: IRS = insulin receptor substrate

which in theory increases availability of glucose for myocardial metabolism. However, clinical data on this type of strategy are inconsistent: perhaps the most encouraging finding is of stabilization of haemodynamics during valve replacement surgery for aortic stenosis [22].

Inhibition of CPT-1 represents a component of the anti-ischaemic effects of perhexiline and amiodarone. However, excessive or irreversible CPT-1 inhibition tends to cause tissue accumulation of lipoprotein deposits, including the development of apparent myocardial hypertrophy with oxfenicine [23] and etomoxir [24–26].

Partial fatty acid oxidation inhibiton, via the enzyme long-chain 3 ketoacyl-CoA-thiolase, is probably the main action of trimetazidine [27], and a component of the effects of ranolazine [28]. The net effect is a reduction in LCFA metabolism:- indeed recent studies have suggested that trimetazidine

Table 1 Categories of proposed therapeutic strategies to accelerate myocardial ATP generation in the presence of ischaemia and/or other energetic impairment

1. Increased substrate utilization
   - Glucose-insulin-potassium (GIK)
   - Dichloroacetate: Pyruvate dehydrogenase activation
2. CPT-1 inhibition
   - Perhexiline
   - Amiodarone
   - •? Trimetazidine
   - Etomoxir
   - Oxfenicine
3. Partial fatty acid oxidation inhibitors
   - Trimetazidine
   - Ranolazine
4. Malonyl-CoA decarboxylase inhibitors
5. Coenzyme Q10

may have similar efficacy in heart failure to that of perhexiline [29]. Comparisons between CPT-1 and partial fatty acid oxidation inhibition have not yet been reported.

To date, no clinical studies of malonyl-CoA decarboxylase inhibitor therapy have been reported, but in theory their effects should be similar to those of CPT-1 inhibitors. Coenzyme Q10, an important factor in mitochondrial respiration, was recently reported to exert beneficial effects on outcomes in patients with chronic heart failure [30].

Perhexiline

(1) Studies of anti-anginal efficiency: Initial experience

Perhexiline was developed by Richardson-Merrell Pharmaceuticals (Cincinnatti, Ohio, USA) in the 1960s as a prophylactic anti-anginal. Early animal studies suggested that the antianginal properties of perhexiline may have arisen on the basis that it exerted systemic and coronary vasodilator effects, increased coronary arterial and venous blood flow, slowed the heart rate and increased pulmonary vascular compliance [31, 32]. Perhexiline was subsequently marketed essentially outside the USA in the 1970s, both as monotherapy [33, 34] and also later as incremental therapy beyond β-adrenoceptor antagonists [35], typically in doses between 200 to 400 mg daily. It is important to recognize that perhexiline was released for general clinical use in many countries despite little understanding at that time of its mechanisms of action, pharmacokinetics in humans and potential toxicity. As an example of the level of understanding, the drug was thought to be unsafe in the presence of renal disease, but in fact that is not the case [36].

(2) Experience regarding toxicity

In the 1970s, reports of hepatotoxicity and peripheral neuropathy associated with long-term use of perhexiline emerged [37–40]. These adverse effects were poorly understood at that time, apart from the fact that the toxicity was associated with phospholipid deposition in hepatocytes and Schwann cells [41–43], but soon led to the global gradual withdrawal of the drug during the 1980s, except in Australia and New Zealand. However, several observations during the late 1970s and early 1980s paved the way to better understanding of the role that pharmacokinetics and pharmacogenetics of perhexiline play in its potential toxic effects.

First, Singlas et al. (1978) observed that plasma concentrations of perhexiline were elevated in patients with long-term therapy who experienced hepatotoxicity and neuropathy compared to unaffected individuals [44]. It was also noted that there was a large interindividual variation in the apparent plasma half-life of perhexiline, and therefore that perhexiline-induced toxicity was secondary to some inborn metabolic variability which increased individuals' susceptibility to drug accumulation [45, 46]. Shah et al. (1982) later noticed that a genetic mutation in the metabolism of debrisoquine and perhexiline led to impaired metabolism and potential toxicity [47]. Together with these findings, Horowitz et al. (1986) demonstrated that the metabolism of perhexiline in unselected individuals with angina was non-linear (or "saturable"), and established that maintenance of concentrations of perhexiline at steady-state between 0.15 to 0.6 mg/L was effective in avoiding clinically overt toxicity [48]. The achievement of therapeutic drug concentrations corresponds to daily perhexiline dosage ranging from approximately 10 mg for poor metabolizers through to 500 mg for ultra-rapid metabolizers [49].

It is now known that cytochrome P450 2D6 (CYP 2D6) plays a major role in the metabolism of perhexiline, and that monitoring the concentration ratio between hydroxyperhexiline and perhexiline provides an indication of patients' metabolic capacity and need for further dosage adjustment [49]. Through continual research to better understand the cellular mechanisms of the drug and careful plasma drug concentration monitoring, perhexiline is now regarded as potentially conferring great benefits to patients with refractory angina [35] but also to other cardiovascular disease states (see below). It has been re-registered in several European countries, due to favourable clinical trials and has also very recently been projected as a treatment for symptomatic non-obstructive hypertrophic cardiomyopathy.

(3) How does perhexiline work?

The initial postulate that perhexiline was essentially a coronary vasodilator was rapidly replaced by the idea that it might be an L-type calcium antagonist [50, 51]. However, this was a relatively weak effect [52]. In 1980, Vaughan Williams first proposed that perhexiline's mechanisms of action might be based upon changes in myocardial metabolism improving efficiency of energy generation, but did not specify the specific changes involved [53]. Jeffrey et al. (1995) also provided evidence that perhexiline might increase efficiency of myocardial oxygen utilization, consistent with the presence of such an effect [54]. In 1996, over 20 years after perhexiline had first been utilized clinically, it was found to be a potent inhibitor of the carnitine shuttle [55]. Inhibition of CPT-1 [55], and to a lesser extent CPT-2 [29] would result in secondary activation of glucose utilization via increased activity of the pyruvate dehydrogenase complex (the "Randle cycle") [56]. Like oxfenicine, which was initially introduced primarily for the treatment of diabetes, perhexiline exerts moderate hypoglycaemic effects [57], potentially mediated by insulin sensitization.

However, perhexiline clearly exerts effects beyond CPT-1/CPT-2 inhibition. In 2005, Unger and colleagues found that in

isolated non-ischaemic working rat hearts, pre-treatment with perhexiline increased cardiac efficiency by about 30 %. However this was independent of changes in palmitate oxidation [58]. It remains quite possible that effects other than CPT-1 inhibition contribute to the therapeutic efficacy of perhexiline.

Indeed, perhexiline was subsequently found to inhibit pre-assembled neutrophil NADPH oxidase [59], which is responsible for the inflammatory process known as the "neutrophil burst". This anti-inflammatory effect of perhexiline could contribute to the related finding of potentiation of platelet responsiveness to nitric oxide by perhexiline when platelet aggregation was evaluated in whole blood [60].

Finally, in a recent study, perhexiline was found to activate Kruppel-like factor 14 and increase high density lipoprotein in animal models of atherosclerosis [61]. This finding raises the potential for an anti-atherogenic effect of perhexiline, which has not yet been evaluated formally.

(4) Integration of clinical experience with pharmacokinetics: Recent experience

Since the introduction of routine therapeutic drug monitoring of perhexiline, several clinical studies have provided evidence that perhexiline conferred significant clinical improvement with minimal risk of adverse effects. In 1990, Cole et al. first consolidated the effectiveness of therapeutic drug monitoring of perhexiline, demonstrating that in patients with otherwise refractory angina unsuitable for coronary revascularization, perhexiline treatment substantially improved anginal symptoms [35].

Perhexiline has also been shown to be useful in the management of patients with unstable angina pectoris, with resolution of symptoms correlating with attainment of therapeutic drug levels [62]. As regards long-term safety of the drug, a recent audit of 170 patients treated for a median period of 50 months revealed no hepatotoxicity and only 3 cases of peripheral neuropathy [63].

Given that perhexiline's therapeutic effects are independent of changes in coronary vasomotor tone, a number of studies have recently investigated its possible therapeutic effects in conditions associated with impaired myocardial energetics (with or without angina) but in the absence of underlying severe coronary disease-related ischaemia. An initial report on symptomatic response in elderly patients with aortic stenosis [64] has not been followed up with controlled data. However, Lee et al. demonstrated in a double-blind, placebo-controlled trial that perhexiline was markedly beneficial in chronic systolic heart failure, irrespective of the presence or absence of associated coronary disease [65]. This has led to widespread use of the drug in this context, particularly in patients with limitations to alternative pharmacotherapy, such as those with severe renal insufficiency [36]. Finally, Abozguia et al., in an elegant double-blind study showed that perhexiline improved symptomatic status in patients with non-obstructive hypertrophic cardiomyopathy, with concomitant improvement both in left ventricular relaxation, maximal oxygen consumption and myocardial energetics [66].

Conclusions and Future Directions

In summary, perhexiline is now established as a relatively safe treatment for myocardial ischaemia and other cardiac conditions associated with impaired cardiac energetics. On the other hand, it is a relatively difficult drug to use, requiring individual dose titration on the basis of plasma level monitoring. Efforts are currently being made to develop derivatives of perhexiline with more predictable pharmacokinetics in order to facilitate more widespread utilization of the drug.

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Editorial

Effects of acute hyperglycaemia on cardiovascular homeostasis: does a spoonful of sugar make the flow-mediated dilatation go down?

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Introduction

Patients with diabetes mellitus are at substantially increased risk for adverse outcomes in association with occurrence of acute coronary syndromes (1) and in the presence of atrial fibrillation (2). Although the occurrence of acute myocardial dysfunction in the presence of hyperglycaemia has been shown to be associated with poor short-term outcomes, the issue of the contribution of instantaneous (or recent) elevation of blood sugar level (BSL) to this risk remains incompletely evaluated. Currently there is only fragmentary understanding of the potential nexus between elevation of BSL and thrombotic diathesis.

A number of studies in the literature have evaluated the cardiovascular effects of transient increases in BSL, whether in normal subjects or in patients with underlying cardiometabolic disease states in virtually all cases focusing on effects on vascular reactivity, and in particular vascular endothelial function. We now examine the significance of these findings, their implications regarding nitric oxide (NO) signalling in other tissue such as platelets, and the potential mechanisms underlying these physiological changes. Finally, we review the arguments for rapid reversal of hyperglycaemia during cardiovascular crises as a form of ancillary therapeutic measure.

Impact of hyperglycaemia on the generation and signalling of NO

Acute elevation of BSL is associated with increases in oxidative stress [for review see (3)], and hence has the potential to result in disordered vascular, myocardial and platelet physiology. In practice, effects of hyperglycaemia on vascular function might theoretically involve impairment of generation of NO, for example via increased tissue concentrations of the NO synthase inhibitor asymmetric dimethylarginine (ADMA) (4) and/or via increased tissue arginase activity (5), either of which might also be associated with "uncoupling" of NO synthase. On the other hand, increased oxidative stress in association with hyperglycaemia might well contribute to "scavenging" of NO by superoxide anion (O_2^-) and/or partial inactivation of soluble guanylate cyclase (sGC), resulting in attenuation of tissue responses (6) to NO (see *Figure 1* for schematic representation).

Assessment of vascular function using flow-mediated dilatation (FMD)

FMD represents one of several techniques in common clinical use which can quantitate vascular endothelial function (7), in this case via measuring post-ischemic reactive hyperaemia (largely NO-independent). Investigation of FMD physiology suggests that the hyperaemic response of the circulation to a period of relative ischemia is mediated largely by formation and release of NO (8). On the other hand, few investigations have addressed the extent to which FMD responses reflect changes in NO generation versus integrity of NO signalling: indeed it has been found that there is only a moderate correlation in individual patients between magnitude of

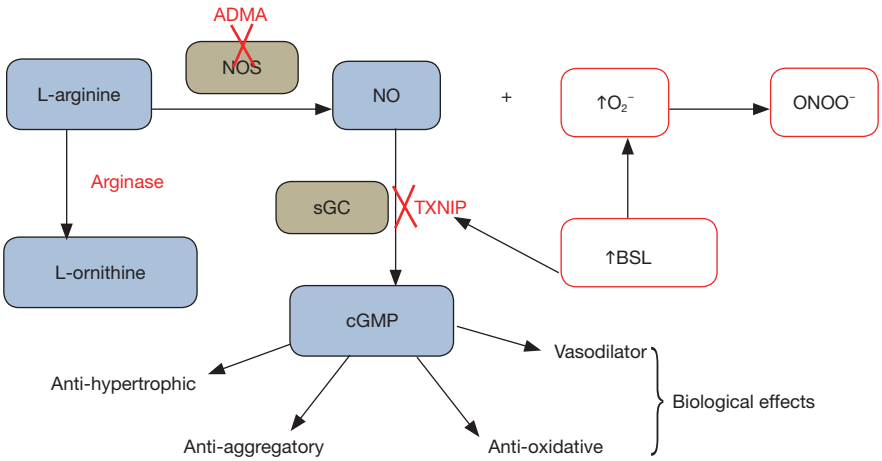

Figure 1 Schematic for impact of hyperglycaemia on nitric oxide (NO)/soluble guanylate cyclase (sGC) pathways. Under normal physiological conditions, NO is generated mainly from L-arginine under the influence of nitric oxide synthase (NOS), which is negatively regulated by asymmetric dimethylarginine (ADMA). Via activation of sGC/cyclic GMP pathway, NO exerts various physiological effects such as anti-aggregation, anti-oxidation and vasodilation. However, during acute hyperglycaemia (BSL ↑), the excessively generated superoxide "scavenges" NO, contributing to attenuation of tissue responsiveness of NO and formation of peroxynitrite ($ONOO^-$). Furthermore, increased expression of the pro-inflammatory protein thioredoxin-interacting protein (TXNIP) increases oxidative stress, potentially contributing to dysfunction of sGC. Major sites of resultant impairment of NO effect are: (I) "scavenging" of NO; (II) sGC oxidative dysfunction. BSL, blood sugar level; GMP, guanosine monophosphate.

FMD and extent of response to NO donors (9), as a probe of integrity of NO signalling pathways. Recent studies have also raised some doubts about the reproducibility of FMD data for individual subjects (10), somewhat limiting the clinical utility of this measure.

The significance of findings from Loader *et al.* [2015]

A recent study (11) examined the impact of acute glucose loading on FMD, utilizing a design involving meta-analysis of the published literature, focusing on 39 articles. The vast majority of these studies had utilized changes in FMD (as a "macrovascular" test of endothelial function) in healthy subjects treated with a single oral glucose load (usually of 75 grams). A minority of studies had evaluated similar changes in type 2 diabetic subjects. Few studies had evaluated "vascular smooth muscle function" simultaneously. However, as this evaluation was achieved via infusion of either sodium nitroprusside or glyceryl trinitrate (GTN) (both NO donors), the process was actually an evaluation of integrity of vascular NO signalling, rather than vascular smooth muscle function. In summary, the available data suggested a decrease in FMD of approximately 1.5% in both normal subjects and type 2 diabetics in the presence of acute hyperglycaemia. On the other hand, there was no consistent change in responses to NO donors during acute hyperglycaemia.

Superficially, this analysis argues that the adverse effects of acute hyperglycaemia on vascular function are mediated largely or entirely by decreased formation of NO. Therefore it is appropriate that we examine the known effects of acute hyperglycaemia on factors such as kinetics of ADMA and of arginases, which might represent mechanisms for decreasing NO release.

Potential mechanisms affecting NO signalling during hyperglycaemia

There is some evidence that activation of tissue arginases may be insulin-dependent. For example, Kashyap *et al.* (12) showed a direct correlation between extent of hyperglycaemia in diabetics and plasma arginase activity, with insulin infusion decreasing arginase activity. Ishizaka *et al.* (5) also showed that hyperglycaemia in rabbits was associated with enhanced arginase activity. A number of studies have also linked hyperglycaemia with increased ADMA production. For example, Mah *et al.* (13) showed that ADMA concentrations increase with post-prandial

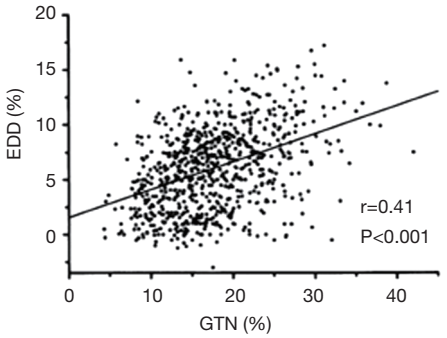

Figure 2 Relationship between FMD and vascular response to GTN. EDD, endothelium dependent dilation; FMD, flow-mediated dilatation; GTN, glyceryl trinitrate. [Reprinted with permission (9)].

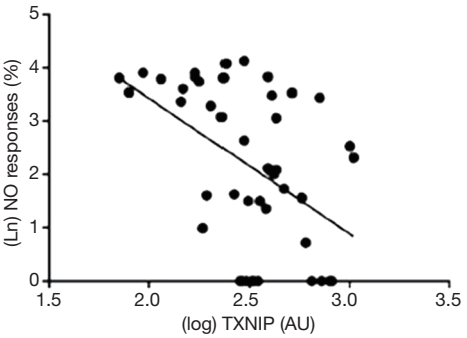

Figure 3 The anti-aggregatory response to NO is negatively correlated with the platelet content of TXNIP, r=–0.5, P<0.0001. NO, nitric oxide; TXNIP, thioredoxin-interacting protein. [Reprinted with permission (17)].

hyperglycaemia. Therefore the finding that FMD decreases with increasing BSL is easily explained by data of this type, although it is somewhat surprising that glucose loading in diabetics, which would be expected to more markedly increase oxidative stress, does not lead to greater changes in FMD.

The total failure of this meta-analysis to document variability in vascular responses to NO according to BSL is, however, surprising. For example, Adams *et al.* (9) previously documented (*Figure 2*) that FMD responses are directly correlated with extent of vascular response to NO donors, a finding which suggests partial commonality of controlling factors. In order to understand this more fully, it is appropriate to consider the literature related to NO responses in platelets, where influence of variable NO generation tends to be less important than integrity of signalling mechanisms.

Given the known mechanistic overlap (*Figure 1*) and the previously demonstrated nexus between FMD and NO response (9), it is possible that the failure of some studies to document changes in vascular responses to NO donors in response to hyperglycaemia results from the common practice of utilizing drug doses which induce near-maximal responses.

Studies in platelets: impact of hyperglycaemia

The major stimulus for evaluation of the impact of changes in BSL on platelet responsiveness to NO and its determinants has been a series of clinical findings which indirectly implicate hyperglycaemia as a focus of impaired NO signalling. Hyperglycaemia represents a basis for increased mortality risk in acute myocardial infarction (14) and the results of the DIGAMI-I trial suggest that rapid reversal of hyperglycaemia by intravenously infused insulin might also reverse this risk (15).

Is there a need to reverse hyperglycaemia during cardiovascular crisis?

In 2007, Worthley *et al.* (16) reported that in diabetic patients with acute coronary syndromes there was an inverse relationship between instantaneous BSL and extent of inhibition of platelet aggregation by the NO donor sodium nitroprusside. This reflected primarily incremental "scavenging" of NO by O_2^- release. With insulin infusion leading to rapid reversal of hyperglycaemia, there was also a fall in O_2^- generation, together with marked improvement in NO response.

More recently, we have noted that the pro-inflammatory protein thioredoxin-interacting protein (TXNIP) appears to control platelet NO signalling under chronic conditions irrespective of hyperglycaemia: there was a reciprocal relationship between NO response and platelet TXNIP content at steady state (17) (*Figure 3*), while treatment with ramipril simultaneously suppressed TXNIP expression and potentiated platelet NO signalling (17,18). It would be expected that TXNIP expression would also change in response to variability in BSL: after all, there is a glucose response element on the gene coding for TXNIP expression (19). However, platelet TXNIP content did not fall significantly over 12 hours of insulin infusion in hyperglycaemic patients (20), despite restoration of NO

responses, suggesting that the associated falls in O_2^- release were TXNIP-independent. This evidence of relatively slow changes in TXNIP expression may be relatively specific for platelets by virtue of limited DNA content. Previous studies suggest that TXNIP expression may be more rapidly adjusted in vasculature (21). It seems more likely that insulin-induced suppression of protein kinase C-dependent activation of NAD(P)H oxidase (22) may have been critical to decreases in O_2^- formation affecting NO "scavenging" in platelets.

Conclusions

It therefore appears that acute hyperglycaemia markedly impairs vascular endothelial function, primarily via diminished NO formation, and also impairs NO signalling, mainly in platelets. These findings constitute a compelling argument for limiting hyperglycaemia (for example via insulin infusion) at the time of all cardiovascular crises. The failure of the CREATE-ECLA trial (23) to improve outcomes in acute myocardial infarction should remind us that the latter was not really a study of reversal of hyperglycaemia, but rather evaluation of a strategy of increasing myocardial glucose utilization.

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Footnote

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New Developments in Anti-Anginal Therapy: Roles of Ivabradine, Allopurinol and of Agents Modifying Myocardial Metabolism

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Abstract

Over the last 20 years, it has emerged that, while surgical revascularisation of extensive ischaemic heart disease may have prognostic advantages, the main issues considered regarding individual management are usually those of symptomatic improvement only. The major impetus towards invasive intervention is therefore failure of prophylactic anti-anginal therapy. On the other hand, many patients, especially the elderly, now present the clinical problem of ongoing angina without residual invasive options. There is an ongoing need for more effective anti-anginal therapies. Of the currently available major classes of prophylactic anti-anginal agents, neither nitrates, *β*-blockers nor calcium antagonists generally produce marked improvements in exercise duration. Three areas of new therapeutic development in anti-anginal therapy are worthy of note. These involve the sinus node inhibitor ivabradine, high dose allopurinol (xanthine oxidase inhibitor) and a new class of "metabolic modulators" represented by perhexiline, trimetazidine and probably ranolazine. The current review addresses the therapeutic potential of these agents. Notably, all of these "new" drugs are potentially suitable for management of angina in the setting of impaired left ventricular systolic function, and they may also be utilized in patients with angina independent of the presence of coronary disease (for example in hypertrophic cardiomyopathy). The current evidence for efficacy and potential future development in this area are reviewed.

Keywords

Anti-Anginal Therapy, Myocardial Metabolism, Stable Angina Pectoris, Ivabradine, Allopurinol

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1. Introduction

"If I were an ischaemic cardiac cell, and someone offered me drugs or blood, I think I'd take blood!"
—Dr. W. Paulus, 1986

The history of development of anti-ischaemic therapy for angina pectoris has been bedevilled by the notion that a strategy of predominantly medical treatment is essentially palliative, while interventional restoration of normal coronary haemodynamics is "curative". Interestingly, evidence to support this philosophy in patients with stable angina is very limited, beyond the findings of the CASS study more than 25 years ago [1]. Notably, the results of the COURAGE trial [2] challenge the idea that coronary stenting should play a primary role in such patients.

If therefore the major efficacy of drugs of surgical/percutaneous intervention relates to amelioration of symptoms in patients with angina, how effective are our "core" medical anti-anginal therapies? The major groups of prophylactic anti-anginal agents are long-acting nitrates, β-blockers and calcium antagonists (especially non-dihydropyridine agents such as verapamil and diltiazem). The key findings from studies of mono-therapy with these agents are summarized in **Table 1**, and the "core" mechanisms of action of these agents are schematized in **Figure 1**.

In general, these show that long-acting nitrates and β-blockers induce only small prolongations of exercise duration, while both verapamil and diltiazem are a little more effective. On the other hand, there is no evidence that any of these forms of therapy improves long-term outcomes in patients with stable angina (although admittedly β-blockers have prognostic utility if patients have concomitant heart failure). Indeed, there is a concern that long-acting nitrates may have a number of important disadvantages, including potential aggravation of endothelial dysfunction [3] and also the precipitation of "rebound" ischaemia [4]. Interestingly, nicoradil, an organic nitrate/potassium channel activation, appears to reduce risk of hospitalization during chronic therapy [5] although the mechanism(s) of this beneficial effect remain uncertain.

The clinical characteristics of patients treated medically for angina pectoris have also evolved with the relatively widespread availability of coronary surgery/stenting. In most societies, such patients now tend to be elderly, with multiple comorbidities. Many such patients have undergone surgical interventions previously and are technically unsuitable for further invasive procedures: many have concomitant severe impairment of left ventri-

Table 1. Placebo-controlled studies of efficacy of long-acting nitrates, β-blockers, and calcium antagonists in prolonging exercise duration in stable angina pectoris.

Study	Agent	*n*	% exercise	*p*
(Category/Authors)	(Daily dose)		prolongation	
1. Nitrates				
Parker *et al*., 1995 [40]	Nitroglycerine (patches 0.4 mg/h for 12 hrs)	291	16	<0.001
Chrysant *et al*., 1993 [41]	Isosorbide-5-mononitrate (240 mg)	313	18	<0.01
2. β-blockers				
Jamal *et al*., 1987 [42]	Carvedilol (50 mg)	12	24	<0.05
Schnellbacher *et al*., 1986 [43]	Bisoprolol: 10 mg	12	22	<0.05
	20 mg		31	
DiBianco *et al*., 1980 [44]	Acebutolol (1155 mg)	44	14	<0.01
Schwartz *et al*., 1981 [45]	Atenolol (100 - 200 mg)	12	32	<0.01
3. Calcium antagonists				
Hung *et al*., 1983 [46]	Diltiazem (360 mg)	12	31	<0.05
Pine *et al*., 1982 [47]	Verapamil (240 - 480 mg)	18	42	<0.001
Andreasen *et al*., 1975 [48]	Verapamil (240 mg)	47	20	<0.05

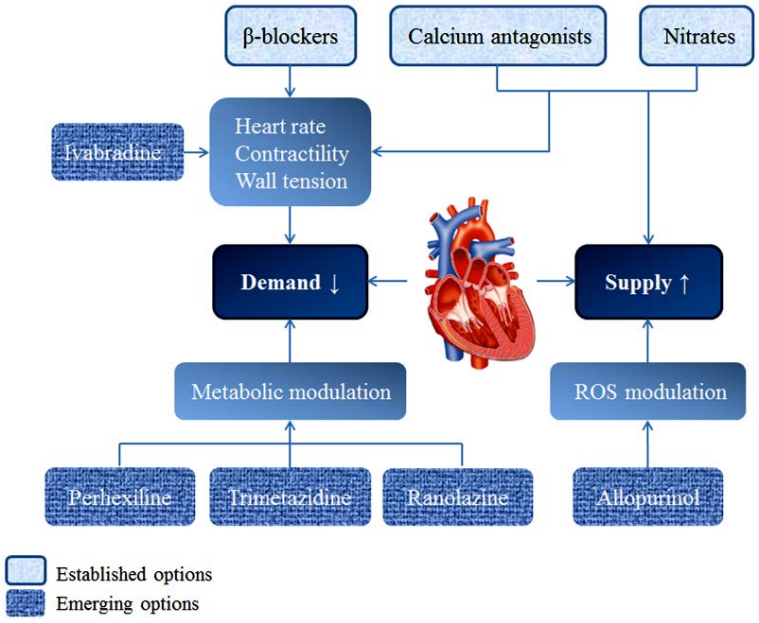

Figure 1. Determinants of therapeutic amelioration of myocardial ischaemia: emerging options (ROS: reactive oxygen species).

cular systolic function. In such individuals, there is a clear-cut need for a transition to "new" anti-anginal therapies. Notably, treatment with verapamil or diltiazem is contra-indicated in the presence of severe systolic heart failure [6].

This review addresses the evolving role of a number of newly recognised/developed anti-anginal agents in the setting of "difficult" angina pectoris. The major sites of action of these agents in ameliorating ischaemia are schematized in **Figure 1**. A potential role for all of these agents in angina pectoris refractory or unsuitable for *β*-blockers or calcium antagonists was also included in current textbook recommendations [7].

2. Ivabradine

Ivabradine is a sino-atrial node inhibitor which specifically inhibits the *If* channel of pacemaker cells, leading to a reduction in both resting and exercise-induced heart rate and improvement in myocardial O_2 balance. It has no effect on other intracardiac conduction system as well as on blood pressure and myocardial contractility. Because of these limited sites of action, it will not induce bronchospasm (unlike *β*-blockers), it is devoid of interactions with diabetic status, and does not adversely affect symptomatic status in the presence of systolic heart failure. On the other hand, it is totally ineffective in patients with no sinus node activity (e.g. atrial fibrillation).

To date, ivabradine has been evaluated as an anti-anginal agent both in monotherapy and in combination with *β*-blockers, as summarized in **Table 2**. In both circumstances, there was a moderate increase in exercise duration. The only common adverse effects seen were those of phosphenes (flashing lights) development, due to *If* current inhibition in the retina. Interestingly, recent clinical trial developments with ivabradine have focused mainly on its role as a secondary treatment in heart failure [8]. No long-term data are available regarding effects on cardiovascular events.

3. High Dose Allopurinol

One other agent that has been proposed as an anti-anginal recently is allopurinol, a xanthine oxidase inhibitor. Allopurinol has been traditionally used in the treatment of gout. The application of allopurinol in coronary artery disease is based on previous evidence that xanthine oxidase is a potent mediator of oxidative stress. Indeed early studies have shown the potential of allopurinol to reduce vascular oxidative stress and thereby improve endothelial function [9]. Furthermore, alterations in cardiac metabolism, primarily in oxygen consumption and contractility have been demonstrated in heart failure models with allopurinol [10] [11].

Table 2. Placebo-controlled studies of anti-angina efficacy of ivabradine in prolonging exercise duration in stable angina pectoris: (a) monotherapy, (b) addition to β-blockers.

Study (Category/Authors)	Antecedent therapy	Daily dose	*n*	% exercise prolongation	*p*
1. vs. placebo					
Borer *et al*., 2003 [49]	-	10 mg for 2 weeks then	360	9	0.02
	-	20 mg for 2 - 3 months		18.5	-
2. Add-on therapy					
Tardif *et al*., 2009 [50]	Atenolol 50 mg	10 mg for 2 months then	889	4	0.02
		15 mg for 2 months		5	<0.001

The first randomized double-blind placebo controlled trial of the use of allopurinol in the management of stable angina, offered promising results. In this study, 65 patients with documented coronary artery disease and positive exercise test were randomized to receive high dose allopurinol, 600 mg a day on top of a standard treatment, in a double-blinded controlled trial [12]. Allopurinol use was associated with a median increase in exercise time to ST-segment depression of 43 seconds ($p = 0.0002$), a median increase in total exercise time of 58 seconds ($p = 0.0003$), and a median increase in time to chest pain of 38 seconds ($p = 0.001$). These findings were backed up by a second study by the same group, which explored the potential mechanisms of the benefits of allopurinol in 80 patients with stable coronary artery disease, on maximal anti-anginal treatment [13]. Endothelial function was assessed by forearm venous occlusion plethysmography, flow-mediated dilation, and pulse wave analysis and vascular oxidative stress was assessed by intra-arterial co-infusion of vitamin C and acetylcholine. They found that indeed allopurinol use was associated with improved endothelium-dependent vasodilation, by both forearm venous occlusion plethysmography (93% ± 67% vs. 145% ± 106%, $p = 0.006$) and flow-mediated dilation (4.2% ± 1.8% vs. 5.4% ± 1.7%, $p < 0.001$). Arterial stiffness was also improved significantly (central augmentation index improved by 2.6% ± 7.0%, $p < 0.001$), and furthermore, vascular oxidative stress was completely abolished by allopurinol.

However, these studies are small and although no adverse effects were reported, further data in regards to safety and impact on long term cardiovascular outcomes in larger studies are prudent. More importantly, it is as yet unclear whether addition of allopurinol to other anti-anginal drugs improves long-term prognosis in patient with CAD. We have also seen benefits in similar small animal and human studies with allopurinol in congestive heart failure [9], and yet the recent large clinical trial OPT-CHF in 405 patients failed to produce any clinical benefits [14].

4. Agents Modifying Myocardial Metabolism

The association of exertional ischaemia with impairment of myocardial energetics raises the option of addressing this problem without either limiting myocardial work (for example by preventing tachycardia and/or exercise-induced increases in contractile function) or by improving coronary haemodynamics.

In theory, the generation of high energy phosphates by the myocardium should be critical to maintenance of homeostasis irrespective of whether the underlying problem is limitation of myocardial perfusion, increased left ventricular afterload (for example with aortic valvular stenosis) or even impairment of systolic and/or diastolic left ventricular dysfunction. However, pharmacological manipulation of myocardial energetics has been a slowly evolving area.

Initial attempts to “feed the heart” were associated with treatment for evolving myocardial infarction, where the glucose-insulin-potassium (GIK) treatment algorithm was proposed and then tried extensively (for review see [15]). Interestingly, this idea of promoting glucose uptake and utilization has generally been unsuccessful, with the possible exception of prevention of myocardial ischaemia associated with open-heart surgery [16].

Subsequently, there have been extensive developments of drugs which potentially affect myocardial energetics during chronic oral administration. Some of these agents inhibit long-chain fatty acid catabolism, whether via inhibiting the “carnitine shuttle” (which controls their uptake into mitochondria) or fatty acid oxidases. Such

agents may activate the so-called "Randle shift": a process of transition to more oxygen-efficient ATP generation from glucose utilization [17].

However, it appears possible to improve efficiency of myocardial oxygen utilization by more than the 13% - 15% expected from activation of a "Randle shift" [18]. Many of the agents concerned exert multiple effects, including interactions with "energy sensor" such as AMP kinase (AMPK) and the pro-oxidant thioredoxin-interacting protein (TxNIP). Specifically, metformin, which reduces mortality in diabetics, is an AMPK activator, and ACE inhibitors, among their many effects, reduce TxNIP expression. Amiodarone, apart from its anti-anginal effects through β-blockade/calcium channel blockade, also blocks the "carnitine shuttle" [19].

However, of the numerous agents with "metabolic" anti-ischaemic actions, there are of particular significance: perhexiline, trimetazidine and ranolazine. These will be discussed more fully.

4.1. Perhexiline

Perhexiline is a myocardial "metabolic" agent that was introduced in the 1970s as a prophylactic anti-anginal agent which was initially presumed to be a coronary vasodilator. Perhexiline proved to be an extremely effective treatment for exertional angina, whether in monotherapy [20] or in combination with other agents [21]. Despite its early success, its use was rapidly declined after reports of serious hepato- and neurotoxicity associated with long-term administration, which was poorly understood at that time. In the mid 1980s, many countries removed it from their markets following those reports. However, hepato- and neurotoxicity was later recognised to be attributable to drug accumulation, by virtue of inter-individual variability in pharmacokinetics, due to genetic polymorphism of cytochrome P450 2D6. Subsequently, significant long-term side effects were shown to be largely eliminated by dosage adjustment according to plasma perhexiline concentrations [22] [23].

Although there were early proposals about the potential "myocardial metabolic" effects of perhexiline, it was not until in 1996 [19] (almost 30 years after it was used clinically as an anti-anginal) that it was found to be a potent inhibitor of the carnitine shuttle. Inhibition of carnitine palmitoyl transferase-1 (CPT-1), and to a lesser extent, CPT-2 [24] led to secondary activation of glucose utilization via increased pyruvate dehydrogenase activity, as a component of the "Randle cycle".

This metabolic shift in theory resulted in greater glucose utilization, and therefore increased ATP production. These findings therefore provide a potential explanation of the anti-ischaemic benefit of the drug. However, it was later shown that in working non-ischaemic rat hearts, perhexiline increased cardiac efficiency by approximately 30%, without reduction of long-chain fatty acid utilization under these conditions [25]. As inhibition of CPT-1 and 2 could not have accounted for all these effects, it has been suggested that there are additional mechanisms of therapeutic action. Indeed, subsequent studies showed that perhexiline possess some "anti-inflamematory" effects, including potentiation of nitric oxide [26]. It also inhibits superoxide formation in intact neutrophils, aortic valve interstitial cells, human umbilical vein endothelial cells and NADPH oxidase-2 [27] [28], and reduces expression of TXNIP in human cardiac tissue [29].

Clinically, beyond its well-known anti-anginal effect, clinical efficacy of perhexiline has also been demonstrated in symptomatic inoperable aortic stenosis [30], systolic heart failure [31] and in symptomatic non-obstructive hypertrophic cardiomyopathy [32]. In the latter study, despite the relatively short duration of treatment, perhexiline demonstrated improvement in myocardial energetic status (as measured by phosphocreatine to ATP ratios), oxygen consumption during peak exertion (peak VO_2), ejection fraction and symptomatic status.

4.2. Trimetazidine

Trimetazidine inhibits 3-ketoacyl thiolase, the terminal enzyme involved in β-oxidation. It has therefore been shown to inhibit fatty acid oxidation *in vitro* and *in vivo*. It exerts moderate effects on exercise duration in patients with stable angina pectoris both as monotherapy [33] and in combination with β-blockers [34] [35]. It is widely used in Europe and Asia as anti-anginal treatment, without compromising haemodynamics. Similar to perhexiline, it has also been investigated in some small and relatively short clinical studies of heart failure, with promising endpoints focusing on left ventricular functions. Some of the studies also showed reduction in plasma cytokines and brain natriuretic peptide, suggesting its potential anti-inflammatory effect. However, it is noteworthy that there has been increasing reports of development of Parkinsonism after long-term treatment with trimetazidine. Although the rate is relatively low and the effect is usually reversible, such events should be evaluated and distinguished as a consequence of the individual agent or potentially of a particular drug class.

4.3. Ranolazine

Ranolazine has been utilized as an anti-anginal agent only relatively recently, with evidence of moderate efficacy, rather similar to that of trimetazidine [36], and standard anti-anginal drugs [37]. Its main advantage has been freedom from major adverse effects, including safety in patients with heart failure. Initial studies suggested that ranolazine might act primarily as a partial fatty acid oxidation inhibitor [38]. However, more recently it has been suggested that inhibition of the late sodium current might contribute significantly to its anti-ischaemic effects [39]. Unlike perhexiline, human data on effects of ranolazine on myocardial energetic status are currently lacking. On the other hand, ranolazine appears to be devoid of major adverse effects.

5. Conclusions

There is an increasing evidence that elective coronary revascularization, except in the circumstance of surgical intervention for extensive ischaemia, lacks major prognostic impact. Furthermore, an increasing proportion of patients with severe stable angina pectoris are unsuitable for any form of coronary revascularization.

On the other hand, long-acting nitrates, β-blockers, and calcium antagonists are only moderately effective in improving angina symptoms, and there is therefore a considerable need for new anti-anginal drugs.

The emerging data suggest that ivabradine, allopurinol, trimetazidine, and ranolazine all offer some incremental effect, and can be utilized as "add-on" therapy in most patients, including those with impaired left ventricular systolic dysfunction. Perhexiline, while extremely potent as an anti-anginal agent, has potentially serious long-term toxicity and should be utilized only with therapeutic drug monitoring. There is no evidence that any of these agents is effective in treatment of angina induced by coronary vasomotor disorders: they appear to act essentially on the determinants of ATP availability to myocardium. The development of the "new" anti-ischaemic options serves to remind us of the need for further progress in this area.

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REVIEW ARTICLE

Thioredoxin-Interacting Protein: Pathophysiology and Emerging Pharmacotherapeutics in Cardiovascular Disease and Diabetes

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Abstract The thioredoxin system, which consists of thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR), has emerged as a major anti-oxidant involved in the maintenance of cellular physiology and survival. Dysregulation in this system has been associated with metabolic, cardiovascular, and malignant disorders. Thioredoxin-interacting protein (TXNIP), also known as vitamin D-upregulated protein or thioredoxin-binding-protein-2, functions as a physiological inhibitor of Trx, and pathological suppression of Trx by TXNIP has been demonstrated in diabetes and cardiovascular diseases. Furthermore, TXNIP effects are partially Trx-independent; these include direct activation of inflammation and inhibition of glucose uptake. Many of the effects of TXNIP are initiated by its dissociation from intra-nuclear binding with Trx or other SH-containing proteins: these effects include its migration to cytoplasm, modulating stress responses in mitochondria and endoplasmic reticulum, and also potentially activating apoptotic pathways. TXNIP also interacts with the nitric oxide (NO) signaling system, with apparent suppression of NO effect. TXNIP production is modulated by redox stress, glucose levels, hypoxia and several inflammatory activators. In recent studies, it has been shown that therapeutic agents including insulin, metformin, angiotensin converting enzyme inhibitors and calcium channel blockers reduce TXNIP expression, although it is uncertain to what extent TXNIP suppression contributes to their clinical efficacy. This review addresses the role of TXNIP in health and in cardiovascular and metabolic disorders. Finally, the potential advantages (and disadvantages) of pharmacological suppression of TXNIP in cardiovascular disease and diabetes are summarized

Keywords Thioredoxin-interacting protein · Thioredoxin · Diabetes · Cardiovascular diseases · Oxidative stress · Inflammation · Nitric oxide · Therapeutics

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Introduction

TXNIP Physiology: Original Observations and Relationship to Thioredoxin Activity

The thioredoxin system is a functionally intracellular and pivotal component of cellular anti-oxidant mechanisms, which includes thioredoxin (Trx) and thioredoxin reductase (TrxR). Trx functions by reducing oxidized cysteine groups on intracellular proteins, being itself simultaneously oxidized and inactivated. In turn, oxidized Trx is re-activated by TrxR. This inactivation of Trx occurs by sulphydryl oxidization, and capacity to regenerate active (reduced) Trx via TrxR is therefore critical (Fig. 1).

Distinct forms of Trx are found in different subcellular compartments: Trx-1 is a 12 kDa protein distributed between the nucleus and cytosol according to a variety of modulating stimuli, while Trx-2 is an 18 kDa protein found in the mitochondria. These play critical roles in the modulation of cellular physiology, including limitation of apoptosis, anti-oxidant effects, modulation of DNA synthesis and repair, modulation of cell membrane signaling and control of the cell cycle (for review see reference [1]). This subcellular compartmental regulation of the redox state is critically dependent on regional integrity of Trx activity in these cellular compartments.

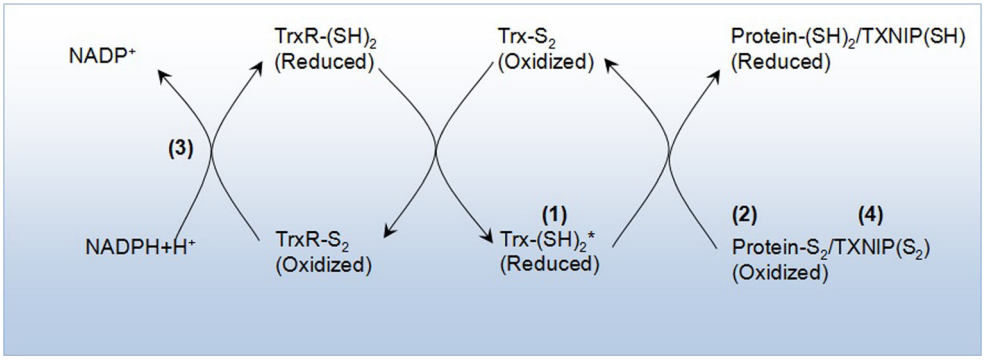

Fig. 1 Redox cycling interactions between Trx and TXNIP. Trx in reduced form acts as a reductase **1** for oxidized proteins **2**, being itself oxidized to Trx-S_2. Reactivation of Trx is achieved by TrxR **3**. TXNIP in oxidized form binds to Trx **4**, resulting in the formation of a reduced TXNIP/oxidized Trx complex

In 1999, Nishiyama et al. [2] identified a number of Trx-binding proteins. One of these, designated thioredoxin-binding protein-2 (TBP-2) was found to be identical to a previously described protein termed vitamin D-upregulated protein 1 (VDUP-1), so named because its expression in isolated cells was increased by 1α,25-dihydroxyvitamin D_3. It was observed that this protein, subsequently re-designated as thioredoxin-interacting protein (TXNIP), acted as a physiological inhibitor of Trx expression and activity [2]. It is now appreciated that the interaction between (reduced) Trx and the oxidized form of TXNIP results in Trx inactivation [3]. Subsequent investigations have demonstrated that TXNIP exerts a wide variety of physiological (and potentially pathophysiological) effects, but that not all of these result from attenuation of Trx effect.

TXNIP Structure

TXNIP has been found to conform to the structural characteristics of the α-arrestin family [4]. Within the scaffold-like structure of the molecule, distinct binding sites are found for a number of proteins. For example, two SH3-binding domains adjacent to the N-terminal amino acid bind apoptosis signaling kinase-1 (ASK1) and Src, while binding sites for ubiquitin ligase (involved in endocytosis) are adjacent the C-terminal [5, 6]. Overall, the importance of the α-arrestin structure of TXNIP is that irrespective of its intracellular site it acts as a scaffold protein which expedites a number of signal transduction pathways (for review, see reference [7]).

Intracellular Localization of TXNIP: Physiological Translocation and Regioselective Effects

While TXNIP was originally thought to reside only in the cytoplasm [8, 9], several studies later found that TXNIP "shuttles" between various intracellular locations, exerting distinct effects according to intracellular site [10, 11]. Indeed, it now appears that the actions of TXNIP depend substantially on the pattern of intracellular distribution, with specific actions emerging in particular within mitochondria and at the cell surface. TXNIP is located entirely in the nucleus under basal conditions [12], and under such conditions it binds to a number of intranuclear proteins, such as poly [ADP-ribose] polymerase-1 (PARP-1).

"Shuttling" of TXNIP from nucleus to cytoplasm (including both mitochondria and plasma membrane) appears to be engendered by dissociation of TXNIP from its various intranuclear binding sites. For example, in pancreatic β cells, Saxena and colleagues [10] found that in response to oxidative stress, TXNIP translocates from the nucleus to mitochondria, potentially via binding to importin-α, and competes with the proapoptotic protein apoptotic signaling kinase-2 (ASK-2) to bind with Trx-2. This leads to subsequent mitochondrial dysfunction and activation of apoptosis. Furthermore, binding of TXNIP to (mitochondrial) Trx-2 is critical to development of intra-mitochondrial redox stress and inflammatory activation [10, 13].

Within the endoplasmic reticulum (ER), TXNIP limits protein ubiquination, and binds to protein disulphide isomerase, potentially contributing to ER dysfunction [14]. ER stress activates a signaling cascade called the unfolded protein response (UPR), resulting in activation of a number of sensor proteins, whose function is to promote synthesis of proteins which tend to restore ER homeostasis. However, marked UPR activation induces inflammation and potentially cell death. Two of these sensor proteins, PERK and IRE1, stimulate increased synthesis of TXNIP [15]; in the case of IRE1 activation, there is also an additional effect of stabilization of TXNIP mRNA [16].

Within the nucleus, TXNIP binding to PARP-1 is critically important in maintaining its "quiescent" state. On dissociation from PARP-1, TXNIP translocates to the plasma membrane [12], where it plays a major role in a number of effects including activation of the vascular endothelial growth factor (VEGF) pathway [11]. Components of the physiological role of TXNIP with regard to VEGF signaling include facilitation of Trx transport to the cell membrane where it tends to activate the interaction between VEGF and its receptor (VEGFR2), as well as promotion of VEGFR2 internalisation, which

potentiates the initiation of angiogenic change in endothelial cells [11, 17].

Therefore, it is likely that regulating the intracellular localisation of TXNIP as well as modulating its expression may prove to be potential strategies to alter its biological effects.

Trx-Dependent and Independent Actions of TXNIP

The "classical" (redox-dependent) effect of TXNIP is binding to Trx. The reduced form of Trx, via cysteine 32, binds to the oxidised form of TXNIP via cysteine 247 [3]. It was initially considered that such interaction with Trx not only limits TXNIP degradation, but also constitutes the basis for all biological effects of TXNIP. Such interaction also appears to be critical to the role of TXNIP in limiting protein denitrosylation, thus contributing to nett nitrosative stress [18]. It has been shown that the apoptosis-signaling kinases (ASK-1 and 2) normally are tightly bound to Trx-1, under which circumstances they are inactive [19]. TXNIP displaces ASK-1/2, and binds to Trx itself. This process frees ASK within cells, initiating proapoptotic signaling [see Cardiovascular disease: key mechanisms section].

However, TXNIP interacts with numerous other proteins, largely or entirely in a Trx-independent manner. The structural similarity of TXNIP with that of the α-arrestin group of proteins facilitated delineation of Trx-independent from Trx-dependent effects. Arrestin-mediated effects of TXNIP are redox-independent and are mediated by the "scaffold-like" structure of the α-arrestin protein. Table 1 summarises redox-dependent versus independent actions of TXNIP. For example, inhibition of glucose uptake into adipocytes and skin fibroblasts occurred to a similar extent in cells with wild-type TXNIP and in those with the mutant TXNIP C247S (which is incapable of binding Trx), thereby establishing independence of this effect from binding to Trx. Furthermore, other α-arrestins, none of which can bind to Trx, also inhibited glucose uptake [4]. These findings led to a modification of understanding of the TXNIP-Trx interaction: not only does binding of TXNIP inactivate Trx, but also this complex is relatively stable, being resistant to clearance via ubiquination-proteasomal pathway [20, 21]. On the other hand, the duration of effect of "free" TXNIP in inhibiting glucose uptake is limited by rapid clearance [7].

Finally, there is recent evidence that the α-arrestin structure of TXNIP is critical to its role in expediting the transport of Trx from cytoplasm to the nucleus, where Trx suppresses expression of genes associated with hypertrophic responses [22]. Interestingly, the implication of this effect is that TXNIP exerts both direct pro-hypertrophic and indirect anti-hypertrophic effects.

Table 1 Summary: major redox-dependent (Trx-associated) and independent (scaffold protein-associated) effects of TXNIP

Redox dependent actions
Inhibition of Trx activity
Stimulation of hypertrophy and angiogenesis
Stimulation of inflammation
Increase in oxidative stress
Redox independent actions
Initiation of apoptosis*
Stimulation of insulin secretion
Regulation of cell growth
Control of response to hypoxia

* This includes displacement of ASK-1/2 from binding to Trx

TXNIP Pathophysiology

The potential involvement of TXNIP in cardiovascular and non-cardiovascular pathological processes is predicated on its extensive physiological significance. Thus the major physiological effects of TXNIP all have potential pathophysiological connotations. Additionally, TXNIP appears to function as a general metabolic sensor and modulator of cellular energetics, a stimulus to abnormal calcification, with impact as an immune modulator and vascular mechanosensor.

For the purpose of this review, however, we will concentrate in particular on the critical roles played by TXNIP in modulating various forms of cardiovascular disease and in the (cardio)-vascular complications of diabetes mellitus.

Cardiovascular Disease: key Mechanisms

The major physiological effects of TXNIP relevant to its roles in cardiovascular pathophysiology are summarised in Table 2. Critical to these effects are:

(i) the interactions between TXNIP and activation of inflammatory pathways
(ii) the impact of disordered patterns of flow as a stimulus for TXNIP expression
(iii) the potential activation of apoptotic signaling pathways by TXNIP
(iv) increases in TXNIP expression during prolonged hypoxia
(v) interactions between nitric oxide (NO) signaling and TXNIP expression

TXNIP and Inflammatory Activation

It is now widely appreciated that within mitochondria TXNIP amplifies inflammatory response to redox stress: in this aspect

Table 2 Major pathophysiological effects of TXNIP relevant to cardiovascular diseases

1. Cardiac
Regulation of hypertrophy
Activation of apoptosis/ischaemia-reperfusion injury
Pro-inflammatory effect in myocardium and valves
Modulation of myocardial metabolism
2. Vascular
Suppression of angiogenesis/interaction with VEGF
Induction of inflammation with non-laminar flow
?Pro-atherogenesis
?Suppression of NO effect
3. Platelet
?Suppression of NO effect

it functions as a physiological antagonist to the anti-oxidant transcription factor nuclear factor erythroid factor-2 related factor 2 (Nrf2) [23]. Specifically, TXNIP expression is increased by superoxide (O_2^-), for example after NAD (P) H oxidase activation [24], or in association with hyperglycaemia. In turn, this results in activation of the nod-like receptor protein 3 (NLRP3) inflammasome [see Diabetes Mellitus/induction of islet cell inflammation section], and activation of a number of downstream inflammatory mediators, including interleukin (IL)-1β, as summarised in Fig. 2. Similar mechanisms have been demonstrated to underlie TXNIP release and inflammatory activation with high fat diets [25].

Nrf2 is activated by a range of oxidants and electrophiles, and has also recently been identified to negatively regulate TXNIP expression in diabetic murine models, via binding to the antioxidant response element of the TXNIP promoter [23]. Following dissociation from its cytosolic adaptor protein Keap1, Nrf2 accumulates in the nucleus and activates a wide range of genes encoding antioxidant proteins. In Nrf2 knockout mice, both basal and glucose-induced expression of TXNIP was increased, associated with reduced Trx activity. This suggests that Nrf2 controls both basal and stimulated TXNIP expression [23]. Future therapeutic agents targeting the Nrf2 system may therefore serve to modulate TXNIP expression.

Mechanosensor Effects: Disturbances of Flow and TXNIP Expression

There is substantial evidence that the maintenance of "normal" laminar flow facilitates eNOS expression, with apparent associated suppression of TXNIP, and the creation of an "anti-inflammatory" milieu [26–28].

Non-laminar or low flow exerts opposite effects: there is markedly increased TXNIP expression and that of vascular/intercellular cell adhesion molecules, resulting in increased recruitment of leukocyte adhesions [26, 29]. Although the precise interaction is unknown, it is thought that TXNIP represses Kruppel-like factor 2, an important anti-inflammatory transcription factor induced by steady flow [26]. The potential consequences of TXNIP expression within the endothelium include inflammatory activation and a pro-atherogenic state, [as described in Cardiovascular disease: Implications of TXNIP physiology in myocardial ischaemia and reperfusion section].

It is also possible that stimulation of TXNIP expression with non-laminar flow influences cardiac hypertrophy. TXNIP knockout animals developed less cardiac hypertrophy during the first 4 weeks of pressure overload, suggesting that the development of abnormal myocardial contractile function may normally be associated with increased TXNIP expression, which may contribute to the hypertrophic process. However, such effect was attenuated after 4 weeks with the development of left ventricular remodeling [30].

TXNIP and Apoptosis Signaling Pathway

Increased TXNIP can cause apoptosis via several mechanisms. It disrupts the Trx-ASK1 complex [as described in Trx-dependent and independent actions of TXNIP section] activating the ASK-1-c-jun-N-terminal kinase (JNK) and p38 MAP kinase pathway. It also disrupts antioxidant defence mechanisms, induces the release of pro-inflammatory mediators such as TNF-α or IL-1β, and induces cell cycle arrest [31] (see Fig. 2).

TXNIP During Hypoxia: Implications on Angiogenesis

The effect of hypoxia on TXNIP is biphasic: during the initial stage, TXNIP expression is decreased. However, during prolonged hypoxia, TXNIP expression is increased, as a result of inhibition of the mTORC1 signaling pathway [32]. Additionally, overexpression of TXNIP is known to reduce hypoxia-inducible factor activity and to inhibit VEGF signaling, independent of its binding to Trx via cysteine 247 [33]: this may contribute to inhibition of angiogenesis. However, translocation of TXNIP from nucleus to plasma membrane includes promotion of vascular endothelial growth factor signaling [12], which potentially leads to angiogenesis and promotion of cell survival.

TXNIP and NO Signaling

Mechanisms of Interaction There is increasing evidence for the existence of multiple interactions between TXNIP and nitric oxide (NO) signaling which result potentially both in suppression of TXNIP expression by NO and suppression of both NO generation and biological effects by TXNIP. For example, TXNIP-null mice exhibited increased NO production and increased iNOS expression in response to lipopolysaccharide [34]. Conversely, endothelial-specific over-

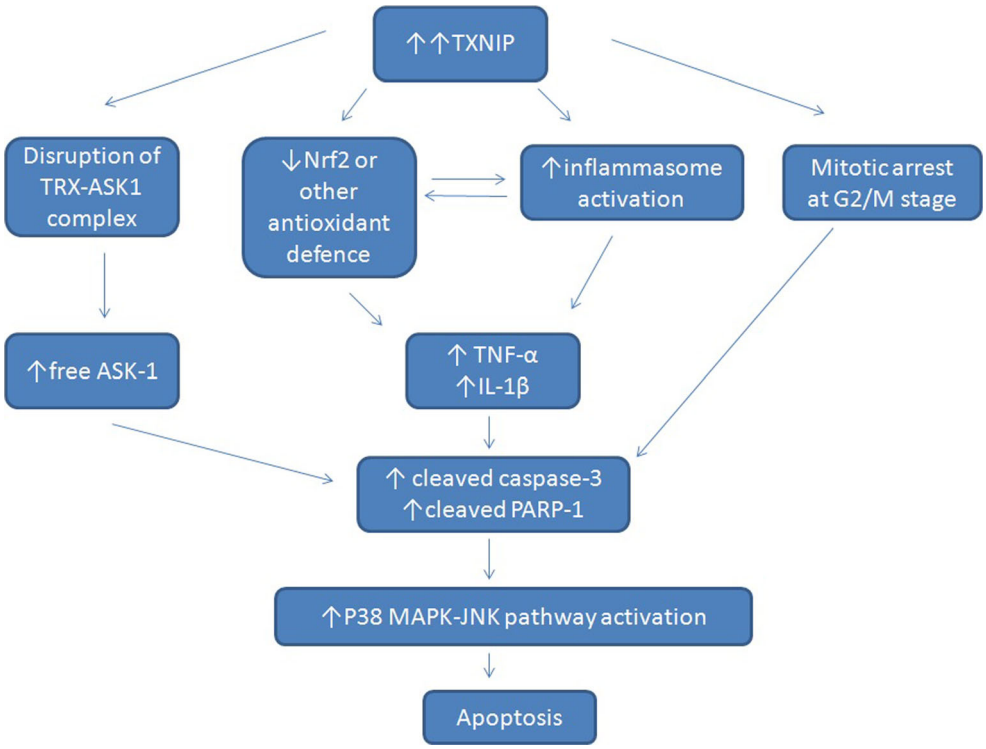

Fig. 2 Schematic: impact of increased TXNIP expression on inflammatory activation and that of pro-apoptotic pathways

expression of Trx-2 in ApoE$^{-/-}$ mice led to increases in NO availability (via decreased scavenging) [35]: increases in Trx-2 activity associated with impaired TXNIP expression or translocation to mitochondria would be expected to exert similar effects.

As regards the various sites of interaction, these might reflect direct effects of TXNIP (for example, inhibition of S-nitrosoprotein dethiolation [18]), thus increasing the relative extent of soluble guanylate cyclase (sGC)-independent effects of NO, or they might reflect TXNIP-induced augmentation of O_2^- production (e.g. inactivation of dimethylarginine dimethylaminohydrolase, partial inactivation of sGC, and "scavenging" of NO to generate peroxynitrite). The nett impact of all of these effects of TXNIP would be a diminution in NO signaling via inhibition of protein kinase G activity. Therefore, in theory, it would be expected that increased TXNIP expression, for example in diabetes, would be associated with impairment of endothelial function. A schematic for TXNIP effects on the NO signaling cascade is provided in Fig. 3.

Conversely, it has been demonstrated (both directly and indirectly) that NO suppresses TXNIP expression. The probability of a direct interaction with NO was demonstrated by Schultze et al. [36], who showed that in rat pulmonary artery smooth muscle cells the NO donor S-nitrosoglutathione suppressed TXNIP expression at the mRNA level. This was further supported by Shaked et al. [37] who demonstrated that the NO synthase inhibitor, L-NAME, not only abolished PKB/Akt phosphorylation and increased TXNIP protein expression in β-cells, but also attenuated the inhibition of TXNIP by glucagon like peptide-1 (GLP-1) agonists and phosphodiesterase inhibitors.

Impact of Interaction With NO Signaling More recently, a study was performed in order to delineate the relationship between TXNIP expression and efficacy of NO signaling in human platelets [38]. In this setting, NO functions as an inhibitor of platelet aggregation and tissue responsiveness to NO can be quantitated conveniently via inhibition of ADP-induced aggregation in whole blood. It was found that platelet TXNIP content varies inversely with platelet NO responsiveness, with a highly significant negative correlation between these parameters in otherwise normal aging subjects (Fig. 4). Furthermore, platelet TXNIP content increased with age in the normal population, corresponding to an increased incidence of platelet resistance to NO in aging subjects. These data further established evidence of a reciprocal regulation of TXNIP expression and integrity of NO signaling, without identifying the precise mechanism (s) involved. It remains possible that the suppression of TXNIP expression by NO (as demonstrated by Schulze et al. [36]) is dependent on the integrity of sGC activity, as is platelet NO responsiveness. It is also theoretically possible that the interaction may be mediated, in whole or part, by TXNIP-enhanced effects on activity of protein kinase G, although this putative effect has not yet been evaluated fully.

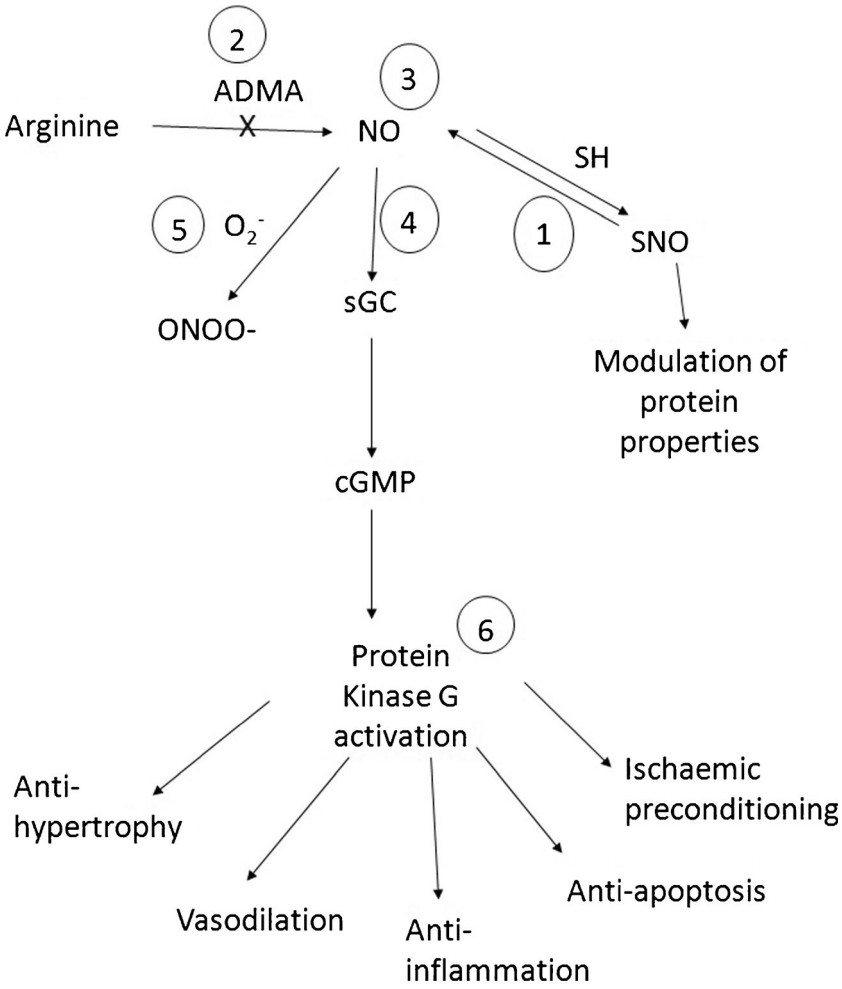

Fig. 3 Potential interactions between NO and TXNIP generation (1) Inhibition of denitrosylation. (2) ? TXNIP→ O_2^- ↑→inhibition of DDAH→ ADMA↑→NO production↓. (3) NO suppresses TXNIP expression. (4) Redox stress→ sGC inactivation→NO effect ↓. (5) TXNIP→O_2^- ↑→$ONOO^-$→ NO effect↓. (6) ? ↓ activity of PKG→effect ↓

Alternatively TXNIP may provide a form of protection against excessive NO production which could lead to nitrosative stress, for example during septic shock [34]. *However, the main issue which emerges from analysis of the NO-TXNIP interaction is whether some of the reported anti-inflammatory effects of NO, in particular, may be partially mediated by suppression of TXNIP effect* [39].

Cardiovascular Disease: Implications of TXNIP Physiology in Myocardial Ischaemia and Reperfusion

All of the various components of the cardiovascular physiological effects of TXNIP described above are relevant to its emerging roles in modulation of myocardial ischaemia and reperfusion injury. Initial evidence that TXNIP might play a critical modulating role came from a study by Yoshioka et al. [40] evaluating the effect of TXNIP knockout on post ischaemia recovery. TXNIP knockout mice appeared to have impaired mitochondrial function, but following periods of ischaemia-reperfusion they displayed better preservation of mitochondrial activity, with less generation of reactive oxygen species (ROS) and better recovery of adenosine triphosphate (ATP) generation.

It has emerged progressively that the complex potential effects of modulation of TXNIP in the context of myocardial

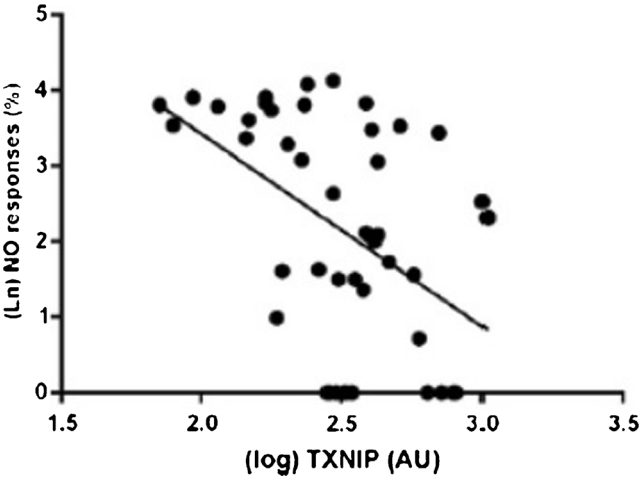

Fig. 4 Reciprocal regulation between platelet TXNIP content and platelet anti-aggregatory effect of NO (Reproduced with permission from reference [38])

ischaemia reflect the intracellular translocation of TXNIP under conditions of redox stress (for review, see reference [13]). In this context, the impact of TXNIP in impairing glucose utilization is likely to be of critical importance, thus reducing the capacity of ischaemic cells to limit mitochondrial damage by down-regulating β-oxidation and Krebs cycle activity while maintaining extra-mitochondrial production of ATP through anaerobic glycolysis [41].

Although human studies are currently lacking, the thrust of the available investigations is that the functions of TXNIP during myocardial ischaemia-reperfusion tend to be deleterious, contributing to mitochondrial ROS production, dysfunction and activation of apoptotic signaling. Indeed, it is possible that inter-individual variability in TXNIP production is a critical modulator of outcomes in ischaemia-reperfusion, for example in diabetics. The corresponding therapeutic implications are discussed in Potential for pharmacological manipulation: modulation of TXNIP synthesis and clearance section.

Diabetes Mellitus/Induction of Islet Cell Inflammation

As previously outlined, hyperglycaemia provides a stimulus for increased TXNIP expression, and TXNIP exerts a deleterious effect on pancreatic islet β-cell function inducing apoptotic changes. In isolated human pancreatic islet cells, TXNIP was the most significantly upregulated gene in response to glucose in an oligonucleotide microarray study [42].

The recently described critically important role of TXNIP in activation of the NLRP3 inflammasome [15, 16, 43–45] plays a central role in its modulation of the response to hyperglycaemia. The inflammasome is a macromolecular complex which is activated in response to redox stress and induces caspase-1 mediated secretion of IL-1β and tends to induce cell death (see Fig. 2). ROS (such as H_2O_2) caused TXNIP to dissociate from Trx [43]: the generation of "free" TXNIP enables activation of the NLRP3 inflammasome, leading to release of active caspase-1 and generation of IL-1β. This could potentially lead to pancreatic β-cell dysfunction or death [46]. Although there are inflammasome activators other than NLRP3, this form of activation appeared to be entirely TXNIP-dependent. Critically, it was also shown that inflammasome activation in response to hyperglycaemia and increased tissue uric acid concentrations utilized this TXNIP/NLRP3 pathway [47]. Conversely, TXNIP inhibits expression of the anti-inflammatory transcription factor Kruppel-like factor 2 [26].

TXNIP-related activation of the inflammasome also induces inflammation and endothelial dysfunction of intraocular vessels in diabetic models [48]. Increased TXNIP generation in association with hyperglycaemia has been implicated in the pathogenesis of diabetic cardiomyopathy [44, 45, 49], presumably in part via analogous inflammatory activation. In a rat model of diabetes, TXNIP mediated renal oxidative stress and fibrosis [50].

Hyperglycaemia is associated with incremental oxidative stress, and with impairment of NO signaling, as well as poor outcomes in patients with concomitant myocardial ischaemia [51]. Reversal of hyperglycaemia reduces oxidative stress and tends to normalise NO signaling [52]. However, it remains uncertain whether suppression of TXNIP plays a major part in this restoration of cardiovascular homeostasis:- there is evidence that activation of Nox4 in the presence of hyperglycaemia may play a more central role [53].

Clinical Monitoring of TXNIP Expression

Given the pivotal role of TXNIP in various metabolic and cardiovascular disorders, it is attractive to be able to evaluate changes in tissue expression in a clinical setting.

As the known physiological effects of TXNIP are all exerted within cells, it is questionable whether any assay for plasma TXNIP concentrations is relevant to its (patho) physiological actions. On the other hand, there is very limited clinical potential for determination of TXNIP concentrations in "target" tissues, such as myocardium and pancreas.

We have recently developed an immunohistochemical assay for platelet TXNIP content and validated this against immunoblotting technology [38]. While this technique has contributed to insights regarding the potential role of TXNIP in platelet physiology and in particular its relationship to platelet NO signaling, it remains uncertain to what extent this reflects its expression elsewhere in the body.

Potential for Pharmacological Manipulation: Modulation of TXNIP Synthesis and Clearance

Stimuli for TXNIP Synthesis and Clearance

In theory, modulation of TXNIP-dependent pathophysiological effects may be achieved by altering its rate of production and/or of clearance or even by modifying its binding to Trx and to other critically important proteins such as PARP-1 and ASK-1. This would also have major consequences on the intracellular translocation of TXNIP and the intensity of its potential effects outside the nucleus.

TXNIP Synthesis: Modulation

The established stimuli for increased TXNIP synthesis are schematised in Fig. 5. Several of these are worthy of specific comment in the context of potential for therapeutic manipulation of synthesis.

Vitamin D_3, the originally described stimulus for increased TXNIP synthesis, appears to interact with two CCAAT motifs

in the gene regulatory region [54]. Heat shock protein [8] and PPARα and γ ligands [55, 56] interact with distinct sites on the gene promoter to increase TXNIP expression.

Perhaps the most physiologically important stimulus for TXNIP synthesis is increased tissue glucose concentration. In this case, the mechanism of increased TXNIP expression revolves around a carbohydrate-response element binding protein (ChREBP) on the TXNIP gene promoter [57]. In diabetics, therefore, the combination of hyperglycaemia and ER stress contribute to TXNIP activation; there is also evidence that increased tissue concentrations of fatty acids may potentiate TXNIP secretion in such patients [58].

Other described stimuli for increased TXNIP expression include transforming growth factor-β1 (TGF- β1) [59]. Intriguingly it appears that TXNIP in turn suppresses TGF-β1 expression [60].

Mechanisms of Diminution of TXNIP Effect

As previously outlined, dissociation of TXNIP from its binding protein leads to its accelerated clearance. A fundamental mechanism for rapid TXNIP clearance involves the ubiquination-proteasomal pathway [61]. Inhibitory effects on promoter gene sites may also decrease TXNIP expression. It might also be postulated that Trx functions to "reciprocally regulate" TXNIP [58], but definitive evidence for this regulation is currently lacking.

There is extensive evidence that insulin rapidly decreases TXNIP protein levels in many, but not all, tissues. For example, Robinson et al. [61] showed that 100nM insulin accelerated TXNIP degradation in adipocytes but not in pancreatic β-cells, by a process involving proteasomal degradation. This effect was inhibited by wortmannin, suggesting that PI-3 kinase was involved in activating the ubiquitin/proteasome pathway.

Key experimental evidence that AMPK activation contributes to TXNIP degradation was recently provided [62]. Energetic depletion results in activation of AMPK, with a resultant increase in nett ATP production [63]. It had already been shown that AMPK regulated the responsiveness of ChREBP, with secondary effects on TXNIP expression [64]. However, Wu et al. [62] additionally found that glucose "starvation" in Hep G2 cells resulted in AMPK-activation-dependent phosphorylation of TXNIP (a process which could be mimicked with pharmacological activators of AMPK). Phosphorylation increased the rates of degradation of TXNIP, resulting in prolonged increases in rates of glucose utilization (and potential restoration of cellular ATP synthesis rates).

It is also probable that suppression of TXNIP expression may be associated with activation of histone deacetylases (HDAC), which are also critical to physiological hypoxic signaling. The relationship between hypoxic effects on TXNIP expression and its suppression in association with some cancers [65, 66] remains incompletely understood. However, tumour angiogenesis depends substantially on the fact that hypoxia-inducible factor-1α (HIF-1α), which is normally rapidly degraded via interaction with von Hippel-Lindau factor under normoxia, becomes stabilized in hypoxic conditions and facilitates angiogenesis [67]. A number of HDACs increase expression of HIF-1α, potentially mimicking "hypoxic" signaling. (Conversely, HDAC inhibitors are increasingly utilized to suppress tumour angiogenesis). It is increasingly clear that HDAC-10 in particular also functions as a suppressor of TXNIP expression [68]. Therefore the likelihood is that this interaction is pivotal to TXNIP suppression in association with carcinogenesis.

Therapeutic Manipulation: Current Status

Although no currently utilized therapeutic agents, whether for cardiovascular or other disease states, were specifically designed to interact with TXNIP expression, it is increasingly clear that many of the beneficial and potentially harmful effects of such agents are TXNIP-modulated.

Mechanisms of effect of agents which decrease expression of TXNIP are schematized in Fig. 5: these various classes of drugs will be discussed individually.

Insulin and Oral Hypoglycaemics

TXNIP overexpression increases rates of pancreatic β-cell apoptosis and also impairs glucose metabolism [57, 69]. Conversely, many hypoglycemic agents have been shown to reduce TXNIP expression [37, 64, 70]. However it is not entirely certain if the effects seen are direct or indirect: for example via reduction of circulating glucose levels.

The suppression of TXNIP by insulin was first demonstrated in human muscle biopsies and adipocytes in cell culture [71]. It was shown that there is a reciprocal regulation of TXNIP by insulin and glucose. Four-hour exposure to insulin was associated with reduction in mRNA expression of TXNIP. This effect was dependent on intact insulin receptor signaling, rather than simply a reduction in circulating glucose levels: in muscle insulin receptor knockout mice treated with streptozotocin (STZ), insulin failed to suppress TXNIP expression [71]. Although degradation of TXNIP by insulin seems to be cell and tissue-specific [61], given that TXNIP plays a significant role in the development of insulin resistance and diabetes, this has nonetheless provided a potential mechanism and therapeutic application for the restoration of insulin sensitivity in diabetics or pre-diabetics.

Insulin seretagogues such as GLP-1 agonists also suppress TXNIP expression. Similar ubiquination and proteasomal degradation pathway also seem to be at least partially responsible for the reduction of TXNIP expression by GLP [37, 70,

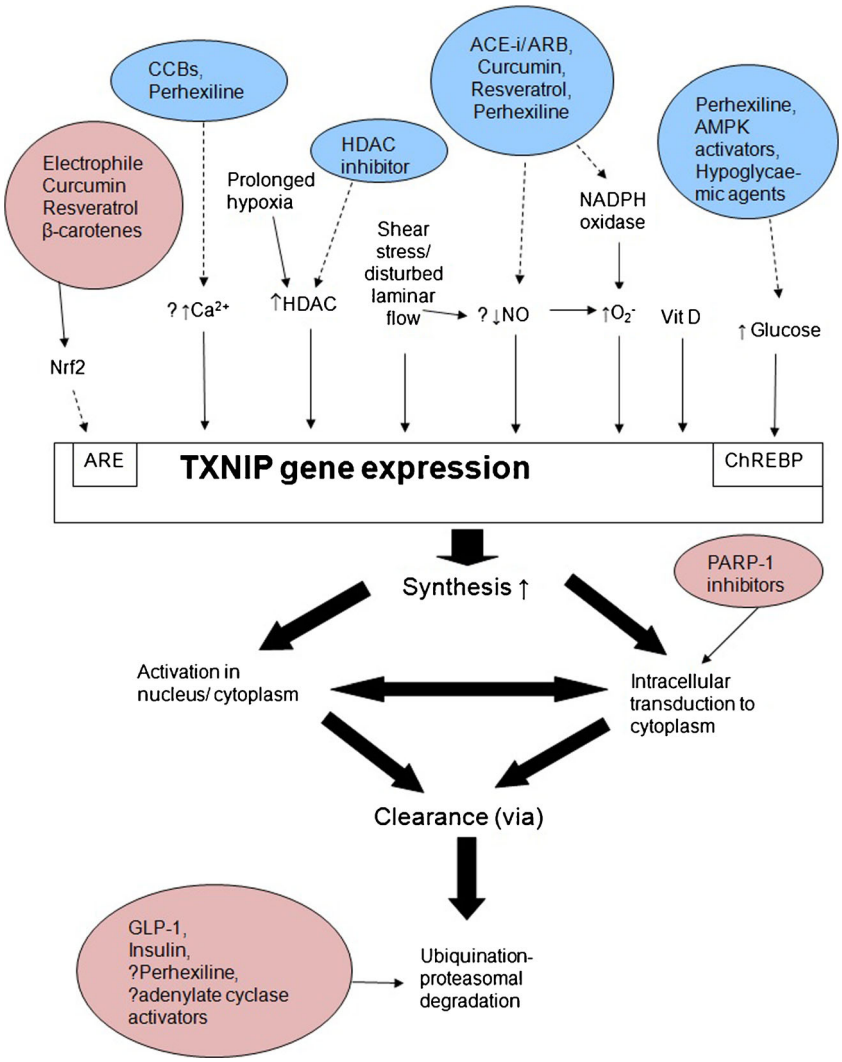

Fig. 5 Activation/Inactivation pathways of TXNIP: Potential sites for therapeutic manipulations. (Blue box or dash line arrows: inhibitory effects; pink box or solid line arrows: stimulatory effects). NO=nitric oxide; Nrf2=nuclear receptor factor erythroid 2-related factor 2; O_2^-=superoxide; CCBs=calcium channel blockers, ACE-i= angiotensin converting enzyme inhibitors; ARBs=angiotensin-II receptor blockers; ChREBP= carbohydrate response element binding protein, ARE= Antioxidant response element

72]. Treatment with exendin-4, a GLP-1 agonist, reduced pancreatic β-cell expression of TXNIP in vitro [37, 70]. This was associated with an increase in cAMP signaling, involving not only protein kinase A but also exchange protein activated by cAMP (Epac) signaling [70]. Forskolin, an adenylate cyclase activator, enhanced the effect of TXNIP ubiquination during hyperglycaemia, suggesting that cAMP enhancement may potentially be employed as a strategy to increase degradation of TXNIP [70].

Metformin, which functions as an insulin-sensitising agent, is a potent activator of AMPK signaling. The AMPK signaling cascade, which is activated during cellular depletion of ATP and plays a significant role in cellular metabolic homeostasis, has also been implicated in the regulation of TXNIP. Specifically, AMPK suppresses TXNIP expression in pancreatic β-cells [64] and also has been shown to accelerate degradation of TXNIP [62]. Recently cultured fibroblasts are utilized to demonstrate that metformin reduced transcription of the TXNIP gene, partially via AMPK-dependent mechanisms. [73] However to date, no studies have evaluated the extent to which this effect is manifested clinically.

Agents Potentiating NO Effect: ACE-Inhibitors, Angiotensin-II Receptor Blockers & Perhexiline

As discussed in Cardiovascular disease: key mechanisms section, potentiation of NO signaling, perhaps by virtue of reducing NO "scavenging", could potentially be another clinical strategy in targeting the suppression of TXNIP. NO signaling includes activation of sGC leading to the production of cGMP, which exerts its effect on a number of cGMP-effector proteins. Theoretically, increasing NO availability and effect could ameliorate endothelial dysfunction, and suppress platelet hyperaggregability [74].

There is substantial evidence that the angiotensin converting enzyme (ACE) inhibitor ramipril suppresses TXNIP expression. For example, in vitamin D-treated rabbits, aortic valve calcification was associated with increased

expression of TXNIP within valve matrix [75]. Concomitant treatment with ramipril diminished valve narrowing, and was associated with a reduction in intra-valvular accumulation of TXNIP (Fig. 6), as well as with salvage of NO signaling [76]. Furthermore, in humans at increased risk of coronary events, 2 weeks' therapy with ramipril decreased intra-platelet TXNIP expression while potentiating NO response [38]. This study represents the first "clinical" evidence that TXNIP expression can be suppressed by commonly utilized cardioprotective agents. However, it remains to be determined to what extent this contributes to the effects of ramipril in reducing frequency of acute cardiac events. It also remains to be determined whether:-

(a) The NO-potentiating effects of ACE inhibitors are mediated primarily via TXNIP suppression, as would be suggested by the data for platelet function [38].
(b) Angiotensin-II receptor blockers (ARBs) exert similar effects in humans. It has been reported that telmisartan also suppressed TXNIP expression in the kidneys of rats with STZ-induced diabetes [77].

Perhexiline, a myocardial energetic agent with clinical benefits in refractory angina, congestive heart failure, symptomatic aortic stenosis and hypertrophic cardiomyopathy, has also recently been shown to reduce myocardial content of TXNIP in patients awaiting coronary artery bypass surgery after approximately 2 weeks of treatment [78]. This could contribute to the beneficial effects seen with perhexiline treatment on energetic improvement and oxidative stress [79, 80], potentially via induction of autophagy [81].

Calcium Channel Blockers

A number of recent studies have provided strong evidence that verapamil and diltiazem suppress TXNIP expression. In 2008, Chen et al. [49] reported that verapamil and diltiazem reduced TXNIP transcription and protein levels in the presence of elevated glucose concentrations in cultured cardiomyocytes, and that 3 weeks' verapamil therapy duplicated this effect in STZ-diabetic mice. Subsequently, the same group demonstrated that verapamil protected pancreatic β-cell survival in STZ-diabetic mice, effectively via attenuation of the pro-apoptotic effects of TXNIP [82].

A key issue is whether this finding is clinically relevant. To date, there has been no definitive study to show that any calcium channel antagonist exerts particular nephroprotective effects in diabetics, nor any clinical evidence that verapamil or diltiazem may be clinically more effective in diabetics than dihydropyridine calcium channel antagonists. However, there is equivocal evidence that verapamil and diltiazem may be more effective than dihydropyridines in limiting proteinuria in diabetics [83]. Furthermore, verapamil treatment for hypertension appears to reduce the likelihood of development of diabetes [84], consistent with a protective effect on pancreatic β-cell function. Clearly further clinical correlative studies in diabetes would help to delineate this area.

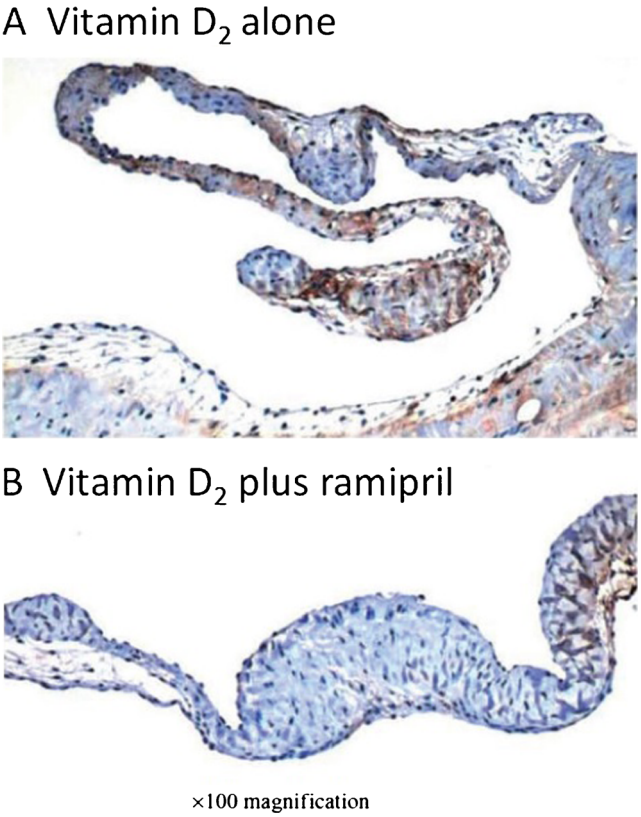

Fig. 6 Section of micrographs: rabbit aortic valve **A** treated with vitamin D_2 alone, and **B** treated with vitamin D_2 plus ramipril. Note suppression of TXNIP expression (brown pigment) with ramipril treatment (Reproduced with permission from reference [76])

Although not yet evaluated from a mechanistic point of view, one major clinical role of calcium channel antagonists is in the prophylaxis of coronary artery spasm. It has already been shown that disturbances of laminar vascular flow increase TXNIP expression [26, 27]. Furthermore, 5-fluorouracil, an anti-neoplastic agent which often precipitates coronary spasm [85–87], increases TXNIP expression [88]. Therefore, it is also appropriate to determine whether TXNIP suppression might contribute to the clinical effectiveness of calcium channel blockers in inhibiting coronary artery spasm.

Agents Under Current Investigation

Several other agents in various stages of investigation for treatment of cardiovascular and non-cardiovascular disorders have been shown under experimental (but not yet clinical) circumstances to potentially suppress TXNIP expression and/or signaling. Specifically:

a) PARP-1 inhibitors (e.g. rucaparib, olaparib) are currently under investigation predominantly as adjuncts to anti-neoplastic therapy (for review see reference [89]). However, they may also be effective in reducing reperfusion injury in the myocardium [90–92]. The potential relevance in this context is that PARP-1 inhibition increases intracellular translocation of TXNIP [12] and thus alters its effects on inflammatory activation.
b) Nrf2 activators, as described previously, could potentially be employed as a therapeutic target to augment cellular antioxidant capacity by enhancing the antioxidant gene expression. Such agents include many phytochemicals, e.g. isothiocyanates, dithiolethiones and cinnamaldehyde (for review, see reference [93]). The synthetic Nrf2 activator bardozolone has recently been investigated as a potential therapy for patients with diabetes and advanced renal functional impairment. In the absence of a substantial reduction in the incidence of end-stage renal disease, there was a significantly increased risk of onset of cardiac failure, causing premature termination of the trial [94].
c) Curcumin is a component of turmeric which is under investigation as an anti-inflammatory and nephroprotective agent. Amongst its documented effects in a mouse model was inhibition of TXNIP expression [95].
d) Resveratrol exerts anti-oxidant effects and is being developed as an adjunct to the treatment of acute myocardial ischaemia. Amongst the many effects documented for resveratrol is suppression of TXNIP expression [96, 97]. This may be a secondary rather than primary effect of the agent.

Does TXNIP Inhibition Increase Risk of Carcinogenesis?

As a growth suppressor that causes G1 cell cycle arrest, reduced expression of TXNIP has been implicated with various human cancers [65, 66, 98, 99]. Therefore the major theoretical concern deriving from pharmacological suppression of TXNIP is the potential for increased risk of carcinogenesis. Recently, administration of ARBs has been linked to increased risk of prostate cancer [100], although other larger studies found no such association [101–103].

Summary/Conclusions

This review presents evidence that TXNIP functions not only as an antagonist to Trx, but also as an α-arrestin-type scaffolding intracellular protein which modulates the function of mitochondria and ER in conditions of redox stress, counterbalancing anti-oxidant effects such as those of Nrf2. TXNIP exerts critically important effects in "amplifying" inflammatory activation and apoptotic pathways, as well as modulating metabolic responses to increases in glucose and fatty acid availability. It exerts profound influences on disorders of blood flow, tissue oxygenation/perfusion, particularly in the presence of diabetes. Its effects include physiological antagonism of NO. Hence development of therapeutic strategies to decrease TXNIP expression is potentially advantageous.

As regards the treatment of diabetes, it is not surprising that many hypoglycaemic agents suppress TXNIP: this effect should tend to render them protective against pancreatic and vascular injury, but cause-effect data remain incomplete.

There is experimental evidence that the calcium channel antagonists verapamil and diltiazem decrease tissue expression of TXNIP [104]. What remains to be ascertained is whether this occurs to an important extent clinically, and whether TXNIP suppression is critical to the salutary effects of these agents in patients with increased risk of cardiovascular events. Similarly, it appears that ACE inhibitors and ARBs also suppress TXNIP, but it is not clear precisely which receptor systems are involved, whether this action is NO-independent, or indeed whether this represents the main basis for cardioprotection.

Given the fact that all current therapeutic methods for TXNIP suppression in humans also exert other potentially important effects, the clinical advantages inherent in such strategies can only be extrapolated from the results of animal experiments involving TXNIP knock-down or mutation. At least from the point of view of evaluation of mechanisms of therapeutic effect, it would be theoretically desirable to develop more specific TXNIP-suppressing therapies for clinical use. This being the case, we can surmise that TXNIP suppression is likely to represent an important advance in the management of diabetes, ischaemic and valvular heart disease.

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Can Perhexiline Be Utilized Without Long-Term Toxicity? A Clinical Practice Audit

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Background: Perhexiline, originally used as a first-line prophylactic antianginal agent, is now regarded primarily as a treatment for otherwise refractory myocardial ischemia. Recent studies have also demonstrated its short-term utility in heart failure, hypertrophic cardiomyopathy, and inoperable aortic stenosis. Its benefits on myocardial energetics state are potentially counter-balanced by risk of hepatotoxicity and peripheral neuropathy during long-term treatment if drug accumulation occurs. Since perhexiline exhibits complex pharmacokinetics with wide inter-individual variability, its long-term use requires regular plasma concentration monitoring. In this study, the risk of neuro- and hepato-toxicity during long-term perhexiline therapy in relation to the intensity of therapeutic drug monitoring was investigated. Furthermore, determinants of mortality during perhexiline treatment were evaluated.

Methods: In 170 patients treated with perhexiline for a median of 50 months (interquartile range: 31–94 months), outcomes and relationship to plasma drug concentrations were documented.

Results: Rationale for treatment with perhexiline included myocardial ischemia in 88% and severe systolic heart failure in 38%. Plasma concentrations were within the therapeutic range of 150–600 ng/mL on 65% of assay occasions and toxic levels accounted for 8.8% of measurements. No patient developed hepatotoxicity attributable to perhexiline while 3 developed peripheral neuropathy possibly induced by treatment. Actuarial 5-year survival rate was 83% overall, and 76.3% in patients with associated systolic heart failure.

Conclusions: This first audit of a large population treated long-term perhexiline demonstrates the following: (1) Although the frequency of monitoring is less than ideal, therapeutic drug monitoring effectively limits occurrence of toxic drug concentrations and virtually eliminates long-term hepato- and neuro-toxicity and (2) Mortality rates during long-term therapy, notably for patients with concomitant heart failure, are surprisingly low.

Key Words: perhexiline, myocardial energetics, hepatotoxicity, peripheral neuropathy, safety

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INTRODUCTION

Perhexiline maleate was developed clinically as a first-line, prophylactic anti-anginal agent approximately 50 years ago, largely on the basis of its exerting coronary vasodilator effects in isolated heart models.[1,2] Indeed, clinical trials established a remarkable degree of efficiency in suppressing anginal symptoms, when used as monotherapy,[3,4] typically in dosage regimens of 200–400 mg per day. Subsequently, it was found that perhexiline also induced marked improvement of anginal status when added to other prophylactic anti-ischemic treatment regimens.[5]

From 1973 onwards, reports began to appear of development of hepatitis and/or peripheral neuropathy during long-term (>3 months) therapy with perhexiline.[6–9] As these reports coincided with the increasing clinical use of β-adrenoceptor antagonists, long-acting nitrates, and nondihydropyridine calcium antagonists as medical treatment options for angina pectoris, and with increased availability of coronary revascularization, they led to a decline in the use of perhexiline. Furthermore, it was demonstrated that perhexiline had calcium antagonist properties[10,11]; if it were merely a potentially toxic calcium antagonist, there seemed to be little reason to continue using the drug.

A number of critically important findings since that time have led to a gradual return in clinical interest of the drug. Firstly, it was demonstrated that in the majority of patients with perhexiline toxicity, plasma concentrations of the drug were unusually elevated.[12] Prospective studies demonstrated that adjusting drug dosage to maintain plasma perhexiline concentrations below 600 ng/mL markedly decreased the risk of hepato- and neuro-toxicity in the medium-term,[5,13] and led to the inception of routine therapeutic drug monitoring. Pharmacokinetic studies revealed nonlinear (saturable) metabolism,[14,15] and that perhexiline, a substrate of CYP2D6, tended to accumulate dramatically in "slow hydroxylators," representing 6%–7% of the White population.[16,17]

Finally progress was made regarding understanding both the mechanisms of effect of the drug and its potential spectrum of clinical utility. Concordant with Vaughan Williams' initial suggestion that perhexiline might modulate metabolism,[18] and findings by Malloy's group of increased efficiency of cardiac work per unit of oxygen consumption,[19]

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Kennedy et al showed that perhexiline inhibited carnitine palmitoyl transferase-1, a key enzyme in the "carnitine shuttle," thus limiting long-chain fatty acid metabolism, and potentially activating the "Randle Cycle" and associated increases in glucose utilization.[20] In view of these findings, the potential clinical utility of perhexiline extended to disorders of myocardial energetics without fixed coronary artery disease. Subsequent studies provided evidence that it might improve symptomatic status in patients with aortic stenosis,[21] systolic heart failure (ischemic or otherwise),[22] and hypertrophic cardiomyopathy.[23]

In the current analysis, we have evaluated the long-term safety of perhexiline in a cohort of Australian patients treated mainly for otherwise refractory ischemia and/or heart failure. We sought to determine the following:

1. The incidence and determinants of serious long-term perhexiline toxicity (ie, hepatotoxicity and/or peripheral neuropathy),
2. Mortality rates during perhexiline therapy for the entire patient cohort and for those with concomitant systolic heart failure, and
3. The relationships between long-term outcomes and
   - Achievement of therapeutic drug concentrations
   - Frequency of monitoring of steady-state perhexiline concentrations, given that trimonthly determination of concentrations is currently recommended.[24]

The results provide incremental evidence of the safety of perhexiline in long-term treatment. Furthermore, the survival data raise the possibility that this form of treatment may improve prognosis in the populations currently evaluated.

METHODS

Patient Selection

Patients were evaluated on the basis of at least 12 months of perhexiline therapy for a currently recommended indication with associated records of predominantly out-of-hospital follow-up including cardiac clinic review.

The only criteria for non-prescription of perhexiline in this cohort were

1. Severe chronic hepatocellular disease,
2. Previous severe acute adverse reaction to perhexiline, for example, dizziness, nausea, and vomiting, and
3. Anticipated inability to comply with monitoring requirements.

Diabetes mellitus (with or without associated peripheral neuropathy), concomitant renal insufficiency, and treatment with potentially interacting drugs (such as amiodarone,[25] selective serotonin reuptake inhibitors,[26] or antifungal terbinafine[27]) were not contraindications to perhexiline therapy at entry.

Therapeutic Drug Monitoring

Determinations of plasma perhexiline concentrations (and those of its major monohydroxylated metabolite) were performed via high-performance liquid chromatography as previously described.[14] Ideally, monitoring was repeated frequently until levels were stable within the previously described therapeutic range and then repeated trimonthly.[24] However, this was a suggested rather than strictly enforced frequency of monitoring. Actual frequency of monitoring was documented throughout the treatment period, together with results of these analyses.

Clinical Follow-Up

Patients were assessed symptomatically both by their general practitioners, and by treating cardiologists at regular intervals. Measurements of liver function were performed routinely with other biochemical tests; and formal neurological assessment was performed only on the basis of clinical symptoms or abnormal physical findings.

Hepatotoxicity, whether likely due to perhexiline or otherwise, was defined on the basis of at least 3-fold elevation of liver function tests beyond the upper limit quoted by the testing laboratory. Peripheral neuropathy, whether predominantly sensory, motor, or autonomic was diagnosed only in the presence of objective clinical abnormalities. In all cases, appearance of such anomalies was correlated with simultaneous plasma perhexiline concentrations.

Data regarding mortality rates were retrieved from the Registry of Births, Deaths, and Marriages of South Australia and presumptive causes of death recorded. Variables selected for multivariate backward logistic regression analyses to identify independent predictors of mortality were age, gender, duration of perhexiline therapy, history of coronary artery disease, presence of aortic stenosis, heart failure, diabetes mellitus, proportion of drug assays within therapeutic range, and proportion of drug assays over therapeutic range.

Statistics

All data for normally distributed parameters are described as mean ± SEM; for skewed data, medians and interquartile ranges are given.

Survival rates (overall and for patients who had concomitant heart failure) were quantitated by Kaplan–Meier analysis, and correlates of survival rate were evaluated by multiple logistic backward regression analysis. All analyses were performed with the SPSS version 20 software (SPSS, Chicago, IL).

RESULTS

Table 1 summarizes the clinical demographics of the 170 patients in this study cohort. In general, patients were elderly. Known coronary disease and therefore potential myocardial ischemia was present in 88% of cases while 37.6% had symptomatic heart failure. Furthermore, aortic stenosis represented an occasional component of the indication for perhexiline therapy (12.9%).

Figure 1 summarizes metabolizer phenotype. The majority of the patients were intermediate to extensive metabolizers (criteria[15] are shown in Fig. 1) and received daily doses of perhexiline maleate between 125 mg and 200 mg; 1.8% were poor metabolizers who received 50–100 mg weekly; and 4.12% were ultra-rapid metabolizers who received 100–250 mg daily. To determine if in practice steady-state perhexiline

TABLE 1. Baseline Characteristics of the 170 Patients in This Study Cohort

Male (%)	58
Age (yrs)	74 (65–81)*
Duration of treatment (mo)	50 (31–94)*
Cardiovascular history (%)	
Coronary artery disease	88.2
Previous myocardial infarction	58.8
Previous coronary artery bypass	37.6
Previous percutaneous coronary intervention	33.5
Aortic stenosis	12.9
Systolic heart failure	37.6
Atrial fibrillation	17.1
Non-cardiac disease (%)	
Diabetes mellitus	37.1
Known peripheral neuropathy (pre perhexiline)†	7.1
Known hepatic disease (pre perhexiline)‡	1.2
Significant alcohol intake	2.4
Concomitant cardiac pharmacotherapy (%)	
Antiplatelet therapy	78.8
Angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers	68.8
Nitrates	68.2
Beta-blockers	40
Calcium channel blockers	46.4
Digoxin	18.2
Amiodarone	1.7

*median (interquartile range).
†10 of 12 patients had diabetes.
‡Mild abnormalities of liver function tests at entry.

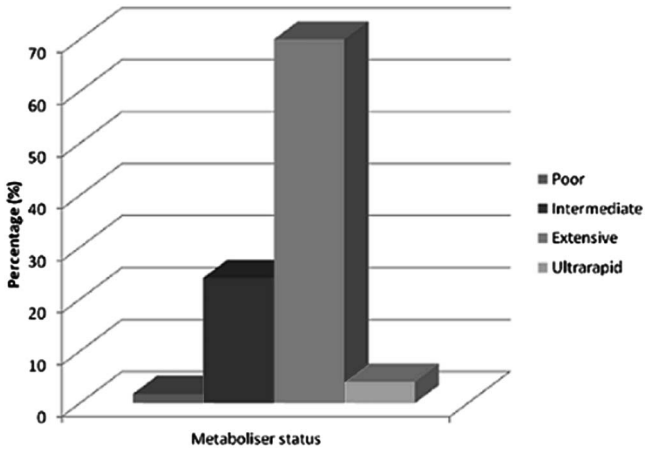

FIGURE 1. Distribution of metabolizer's phenotype in this cohort of patients. (Metabolizer status was defined as the ratio of plasma concentrations of cis-hydroxyperhexiline to perhexiline at steady-state. Poor metabolizer was defined by a ratio of ≤0.3; intermediate metabolizer was defined by a ratio of 0.3–2.5; extensive metabolizer was defined by a ratio of 2.5–20; and ultra-rapid metabolizer was defined by a ratio of ≥20).

concentrations might be varied according to metabolizer status, we tested the hypothesis that extensive and intermediate metabolizers would have identical probability of achieving >70% of plasma drug concentrations within the therapeutic range. It was found that the probability of such a degree of achievement of therapeutic levels did not differ by the metabolizer status ($\chi^2 = 0.10$; $P = 0.7$).

Over the period of follow-up, as shown in Table 2, 65% of drug concentrations measured were within the therapeutic range of 150–600 ng/mL[13]; the majority of other concentrations were subtherapeutic (26.2%) rather than potentially toxic (8.8%). Only 20% of patients experienced ≥2 consecutive toxic concentrations, and 16.4% had periods of toxicity extending ≥3 months. The overall frequency of monitoring appeared suboptimal; only 32% of patients had at least trimonthly drug monitoring, as currently recommended by the Australian guidelines.[24]

Elevation of hepatic enzyme levels beyond population norms, detected during routine monitoring in asymptomatic patients, were usually transient and occurred in 51 cases. Of these, potential causes included 21 cases of gastrointestinal disease (cholecystitis, pancreatitis, and gallstones), 2 of known hepatitis B or C, and 2 of metastatic hepatic infiltration. There were also 8 cases of ischemic hepatitis associated with acute heart failure. Two of the cases appeared to be related to other drugs such as HMG-CoA inhibitors (statins). In another 12 cases of hepatitis, although of uncertain cause, could not be attributed to perhexiline as the elevation of plasma levels of hepatic enzyme was transient and resolved despite continuation of therapy. Overall, none of the cases of abnormal hepatic function appeared likely to be related to perhexiline therapy. In particular, in no case "classical" perhexiline hepatotoxicity was there; that is, simultaneous elevation of perhexiline concentrations and development of abnormal liver function.[13,28,29]

There were also a total of 4 new cases of presumptive peripheral neuropathy in the course of the study, although in 1 case, objective signs were never elicited. In the 3 other suspected cases, perhexiline could not be excluded as

TABLE 2. The Distribution of Concentrations With Respect to Therapeutic Range of 150–600 ng/mL and the Frequency of Monitoring During Study Period

Quality of monitoring (%)	
Proportion of concentration within therapeutic range	65
Proportion of concentrations above therapeutic range	8.8
Proportion of concentrations below therapeutic range	26.2
Frequency of monitoring over total study period (%)	
At least trimonthly	32
Three to six monthly	48
Less than six monthly	20

TABLE 3. Serious Toxicity Potentially Caused by Long-Term Perhexiline

Patient	Other Risk Factors	Metabolizer Status	Monitoring Frequency	Monitoring Period (mo)	Proportion of Over-Therapeutic Values (%)
Peripheral Neuropathy					
1	Peripheral vascular disease	Intermediate	Less than 6-monthly	197	7.15
2	Nil	Rapid	3–6 monthly	58	0
3	(Dietcontrolled) Diabetes mellitus	Extensive	Less than 6-monthly	124	10

No cases of hepatotoxicity were deemed likely to be induced by perhexiline.

a potential cause of peripheral neuropathy. Of these 3 patients 1 had diabetes. In the 2 non-diabetic patients with defined peripheral neuropathy suspected to be perhexiline-induced, 1 had known peripheral vascular disease. Once again, none of these cases were associated with supra-therapeutic perhexiline concentrations and none occurred in poor metabolizers (Table 3).

As regards to mortality, actuarial survival of the entire cohort and that of patients with concomitant systolic heart failure is shown in Figure 2. Five and 10-year survival rates were 83% and 72.6% respectively for the entire cohort, and 76.3% and 63.4% for patients with heart failure. On multivariate analyses (Table 4), deaths on perhexiline tend to occur significantly more commonly during initial years of therapy ($P = 0.021$), and there were trends towards increased mortality with advanced age ($P = 0.064$) and for patients with associated coronary artery disease ($P = 0.068$).

DISCUSSION

The current data represent the most extensive documentation of long-term safety of perhexiline in the modern era of routine therapeutic drug monitoring. Initial experience with perhexiline, confined to its role as a prophylactic anti-anginal agent, suggested remarkable clinical efficacy, but the serious long-term complications of hepatitis and peripheral neuropathy were documented in as many as 40% of cases.[4] In the current series, presumptive long-term toxicity (limited to 3 cases of peripheral neuropathy) occurred with an incidence of only approximately 1 case per 236 patient-years' utilization.

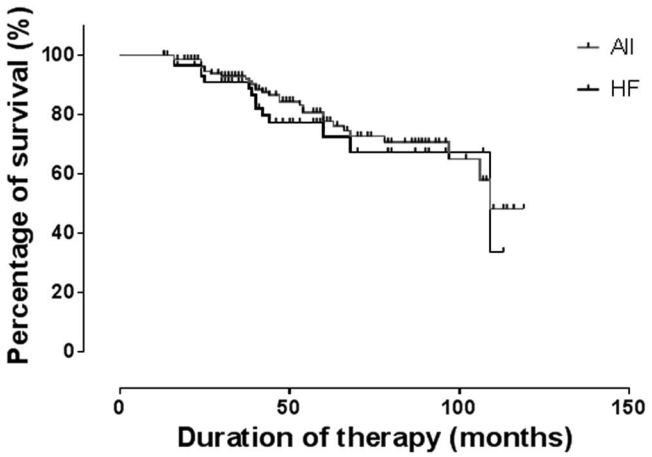

FIGURE 2. Kaplain–Meier curves over the first 10 years of therapy for all patients (n = 170), and for those who had concomitant systolic heart failure (HF) (n = 82).

The basis for this vastly improved safety profile was superficially routine monitoring of plasma perhexiline concentrations. There is ample evidence that most cases of previously reported long-term toxicity occur with prolonged (>3 months) elevation of plasma perhexiline concentrations, beyond 600 ng/mL.[13,29] The strategy of maintaining concentrations below that point has been successful in limiting toxicity, although this has been tested previously mainly in short-term exposure.[22,23] Plasma concentration monitoring at trimonthly intervals is recommended on the basis that no long-term toxicity emerges in shorter intervals,[13,30] and the upper limit of the therapeutic range has been set according to the lowest levels at which hepato- or neuro-toxicity has been observed. On the other hand, designation of 150 ng/mL as the lower limit of the range was essentially arbitrary, never being formally correlated with the extent of suppression of symptoms.[13]

Although the current data did not capture details regarding concomitant CYP2D6 inhibitor therapy, this study confirms the considerable inter-individual variability in the pharmacokinetics of perhexiline. As expected, daily dosage to achieve therapeutic levels ranged widely (from less than 100 mg per week to 400 mg per day). Less patients than expected were poor metabolizers of the drug. Furthermore, very few monitored concentrations (approximately 8.8% of total readings, occurring in 57% of patients) were above the therapeutic range, and concentrations of more than twice the therapeutic range were virtually never seen. Additionally, consecutive toxic drug concentrations and exposures to toxic concentration ≥3 months were also relatively low. Hence the avoidance of toxicity was less than startling.

Surprisingly this occurred despite apparently suboptimal frequency of monitoring in two-thirds of cases.

TABLE 4. Multivariate (Backward Stepwise Multiple Logistic Regression Analysis) Correlates of Mortality in the Current Study Cohort (n = 170)

Parameter	β-Coefficient	*P*
Age	0.026	0.064
Duration of perhexiline treatment	−1.447	0.021
History of coronary artery disease	1.258	0.068

Furthermore, patients apparently experienced adequate symptomatic response in some cases with associated plasma perhexiline concentrations below the therapeutic range (ie, <150 ng/mL). The issue of extent of therapeutic effect associated with such low concentrations should therefore be evaluated in placebo-controlled studies.

The other important change which has occurred in the general utilization of perhexiline in the last 20 years has been the transition from use only as a prophylactic anti-anginal agent to that of a second-line treatment for systolic heart failure,[22,31] particularly in patients with contra-indications to use of other agents. A substantial minority of the current patient series had heart failure, often with associated renal insufficiency. In a recent analysis of survival trends for chronic systolic heart failure among patients treated with extensive pharmacotherapy in a tertiary hospital, 5-year survival, free of heart transplantation was approximately only 55% irrespective of the presence or absence of underlying myocardial ischemia, despite a substantially younger mean age of patients than the currently evaluated population.[32] Additionally, in a recent systematic review in developed countries, the 5-year observed survival rate for heart failure was between 26% and 52%.[33] In this study cohort, the 5-year survival rate of the entire cohort was 83%, while in the presence of heart failure that fell only slightly. Although our current data are not placebo-controlled and are subject to limitations of interpretations, the very extensive survival of a group of elderly individuals with serious and multiple morbidities seems remarkable. On multivariate analyses, neither the presence of impaired systolic function nor the proportion of therapeutic or toxic assay results predicted increased death rates; these results are superficially surprising.

The study has several limitations. In the 3 cases of hepato- and neuro-toxicity of uncertain cause, perhexiline was potentially, but not definitely, implicated. Indeed, none of these cases had associated "toxic" drug concentrations. Secondly, the therapeutic efficacy of perhexiline cannot be adequately determined, given that the study was not placebo-controlled and the overall inability of the study to assess individual patient's compliance to prescribed therapy. Thirdly, CYP2D6 genotype was not determined given that previous study provided evidence of positive correlation between CYP2D6 genotype and its phenotype, expressed as the metabolizer ratio between cis-OH-perhexiline and perhexiline.[34] Nevertheless, the data demonstrate considerable safety, and suggest that therapy with perhexiline should now be regarded as an appropriate option for difficult cases of myocardial ischemia and/or heart failure.

CONCLUSIONS

This is the first large scale audit that investigates the incidence of adverse effects in patients on long-term perhexiline therapy. Although the drug monitoring frequency is less than ideal, this study showed that therapeutic drug monitoring is effective in limiting (and virtually eliminating) hepato- and neuro-toxicities. Approximately two-thirds of the drug concentrations measured were within therapeutic range, and among those drug concentrations which were nontherapeutic, they were mostly subtherapeutic rather than supratherapeutic. Additionally, the mortality rate in this current cohort of patients, particularly those with concomitant heart failure, is surprisingly low.

ACKNOWLEDGMENTS

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PHARMACOKINETICS AND DISPOSITION

Stereoselective handling of perhexiline: implications regarding accumulation within the human myocardium

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Abstract
Purpose Perhexiline is a prophylactic anti-ischaemic agent with weak calcium antagonist effect which has been increasingly utilised in the management of refractory angina. The metabolic clearance of perhexiline is modulated by CYP2D6 metaboliser status and stereoselectivity. The current study sought to (1) determine whether the acute accumulation of perhexiline in the myocardium is stereoselective and (2) investigate the relationship between duration of short-term therapy and the potential stereoselective effects of perhexiline within myocardium.
Method Patients (n=129) from the active arm of a randomised controlled trial of preoperative perhexiline in cardiac surgery were treated with oral perhexiline for a median of 9 days. Correlates of atrial and ventricular concentrations of enantiomers were sought via univariate followed by multivariate analyses.
Results Myocardial uptake of both (+) and (−) perhexiline was greater in ventricles than in atria, and there was more rapid clearance of (−) than (+) perhexiline. The main determinants of atrial uptake of both (+) and (−) perhexiline were the plasma concentrations [(+) perhexiline: β=−0.256, p=0.015; (−) perhexiline: β=−0.347, p=0.001] and patients' age [(+) perhexiline: β=0.300, p=0.004; (−) perhexiline: β=0.288, p=0.005]. Atrial uptake of (+) enantiomer also varied directly with duration of therapy (β=0.228, p=0.025), while atrial uptake of (−) perhexiline varied inversely with simultaneous heart rate (β=−0.240, p=0.015).
Conclusion (1) Uptake of both perhexiline enantiomers into atrium is greater with advanced age and displays evidence of both saturability and minor stereoselectivity. (2) Atrial uptake of (−) perhexiline may selectively modulate heart rate reduction.

Keywords Perhexiline · Metabolism · Pharmacokinetics · Drug uptake · Stereoselectivity

Introduction

Perhexiline is a metabolic modulating agent that was first introduced into clinical practice as a prophylactic anti-anginal drug in the 1970s. It exerts multiple effects which increase efficiency of myocardial metabolism: amongst these, it is known to inhibit mitochondrial carnitine palmitoyl transferase-1 [1], potentially resulting in a shift from fatty acid to glucose utilisation with more adenosine triphosphate production per unit oxygen consumption.

Despite the considerable clinical efficacy of perhexiline in prophylaxis of exertional angina [2, 3], its use declined because of the substantial risk of hepato- and neuro-toxicity during chronic therapy [4–6]. However, it emerged that this toxicity reflected drug accumulation in plasma [7] which in turn resulted from inter-individual variability in CYP2D6-mediated metabolism [8, 9]. With widespread availability of

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therapeutic drug monitoring [10, 11] and greater understanding of its potential widespread utility for disorders of cardiac energetics [12], the clinical use of perhexiline is now increasing.

Pharmacokinetically, perhexiline is a lipophilic drug well absorbed from the gastrointestinal tract. It is highly protein bound and has a large volume of distribution. As it is subject to hepatic metabolism by CYP2D6, the plasma half-life of perhexiline ranges from several hours to several weeks [7, 13].

A number of studies have specifically evaluated short-term utility of perhexiline in the management of potential cardiac crises, such as the management of high-risk patients with unstable ischaemia and for cardioprotection during coronary revascularisation [14–16]. The recently reported CASPER trial evaluated its use as an adjunct to myocardial protection in patients undergoing coronary artery surgery but found no clear-cut beneficial effect of prophylactic perhexiline therapy [17]. During this trial, atrial and ventricular myocardial biopsies were taken at the time of surgery. We have previously reported on analyses of these samples to evaluate the relationship between plasma and myocardial drug concentrations [18]. The objective of the current analysis stems from our recent observation that the effects of racemic perhexiline may result from unequal steady-state concentrations of its two enantiomers [19].

In human liver microsomes, the intrinsic clearance of (−) perhexiline is greater than that of the (+) enantiomer [20], which explains the greater clearance rate of (−) perhexiline at steady state in patients receiving racemic perhexiline [21]. Interestingly, it appears from studies in a rat model, that the safety of the two enantiomers may vary, with greater hepatotoxicity associated with the (+) enantiomer [22].

Therefore, we have re-evaluated the CASPER data in order to determine the following:

(1) Whether atrial and ventricular myocardial accumulation of perhexiline during short-term treatment is stereoselective, and whether this reflects similar trends in plasma enantiomer concentrations.
(2) The relationship between duration of (short-term) perhexiline therapy and the potential for stereoselective effects of perhexiline within the myocardium.

Methods

Data were mainly derived from patients in the active treatment arm of the CASPER (Coronary Artery Surgery with PERhexiline therapy) trial (NCT00845364). In brief, this was a double-blind, randomised, placebo-controlled clinical trial evaluating whether preoperative oral perhexiline improves myocardial protection in patients undergoing cardiac surgery. Non-diabetic patients, who were not taking CYP2D6 inhibitors, undergoing first-time coronary artery bypass graft surgery were randomised to either perhexiline maleate or placebo for at least 5 days prior to surgery. All patients received the following medication regimen: 200 mg twice daily for 3 days then 100 mg twice daily until the morning of the surgery [17]. On-treatment heart rate was determined by preoperative resting electrocardiogram.

Following induction of anaesthesia but prior to commencing surgery, the haemodynamic status of each patient, including arterial pressures and cardiac index, were determined. Plasma collected at this time point was centrifuged and stored at −80 °C, and had previously been used to phenotype patients for CYP2D6 metaboliser status [17] according to the plasma concentration ratios of perhexiline monohydroxylated metabolite to parent drug [13]. During preparation for cardiopulmonary bypass but prior to aortic cross-clamping, right atrial and left ventricular myocardial biopsies were obtained, as previously described [17]. These biopsies were initially snap-frozen in liquid nitrogen and stored at −80 °C, then later digested in 0.15 M of phosphate buffer solution (pH 6.0) using a homogeniser and tissue grinder to form a suspension (approximately 100 mg tissue in 5 mL buffer). Plasma and myocardial perhexiline enantiomer concentrations were determined utilising a modification of a previously described HPLC assay [23]. Thresholds for detection of myocardial (+) and (−) perhexiline were 0.01 mg/L with sensitivity and accuracy between 0.01 and 2.00 mg/L with intra-assay coefficients of variation and bias <20 % at 0.01 mg/L [23].

Analysis of results

1. Determination of relative uptake of enantiomers

   Concentrations of each enantiomer in atrial and ventricular myocardium were correlated with plasma enantiomer concentrations to derive tissue to plasma concentration ratios.

   Potential stereoselectivity of uptake was evaluated via determination of

   (a) Differential (+) to (−) enantiomer ratio in myocardium versus plasma (in order to determine whether uptake, rather than clearance, might engender stereoselective myocardial effects).
   (b) Percentage of (+) enantiomer concentrations in plasma and myocardium, in relation to time (in order to determine the net effects of stereoselective kinetics on the myocardial uptake of the drug).
2. Identification of determinants or correlates of myocardial (+) and (−) perhexiline uptake

   Univariate (evaluated utilising Spearman's correlation) followed by multivariate analyses were utilised for assessment of the atrial uptake of each enantiomer. Parameters

evaluated were plasma concentration, metaboliser status, age, weight, duration of therapy, resting heart rate, creatinine clearance, and cardiac index. Multivariate backward stepwise analyses were performed using statistical software SPSS (version 20, Chicago).

3. Data from poor metabolisers (n=7) were excluded from analysis (unless specified otherwise) because of potential for differential clearance mechanisms and non-attainment of near-steady-state kinetics.
4. Data are expressed throughout as mean±SD for normally distributed parameters and median (interquartile range) for skewed data.

Results

Patient demographics: metaboliser status and absence of dose titration

One hundred and twenty-nine patients from the active arm of CASPER trial were included and their clinical characteristics are summarised in Table 1. While patients had well-preserved renal function, their pre-treatment cardiac function is not known, with formal estimation of cardiac indices and heart rates performed following the induction of anaesthesia for surgery; these data therefore reflect the potential interaction of perhexiline and pre-treatment status. However, the generally low cardiac indices in these patients imply some degree of systolic left ventricular dysfunction at least at the time of measurement. The median plasma concentrations of perhexiline at the time of blood sampling were 0.27 mg/L (IQR 0.13–0.47), with approximately one third of patients having subtherapeutic levels (i.e. <0.15 mg/L).

Figure 1 examines the relationship between plasma cis-OH-perhexiline/perhexiline ratio (used to categorise metaboliser status) and plasma perhexiline concentration at the time of surgery. It is apparent that there was substantial variability in racemic perhexiline concentrations, such that therapeutic concentrations were not generally attained in rapid metabolisers. There were a total of seven poor metabolisers, all of whom attained therapeutic or potentially toxic perhexiline concentrations.

Table 1 Clinical characteristics of patients (n=129)

Parameters	
Age (years)[a]	66 (58–73) [a]
Weight (kg)	83±14
Creatinine clearance (mL/min)[a]	70 (60–85) [a]
Baseline cardiac index	2.04 (1.78–2.34) [a]
Resting heart rate (beats per minute)[b]	59±10
Duration of therapy (days)[a]	9 (6–12) [a]

[a] Expressed as median (interquartile range)

[b] On treatment, while under anaesthesia immediately prior to surgery

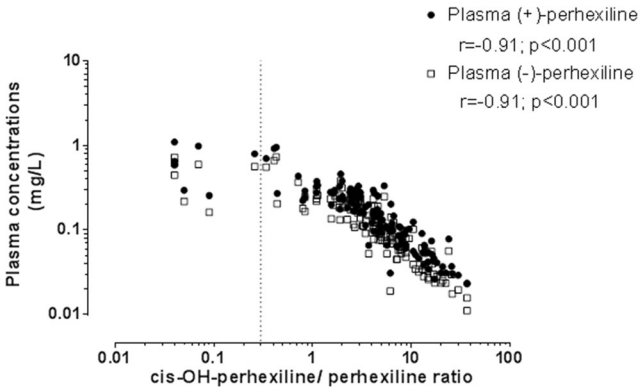

Fig. 1 The relationship between CYP2D6 metaboliser status (expressed as cis-OH-perhexiline/perhexiline ratio) and plasma perhexiline enantiomer concentrations at the time of surgery. *Data to the left of the dashed line* (ratio <0.3) correspond to "poor metaboliser" status

Myocardial concentrations of enantiomers

The relationships between plasma and atrial or ventricular concentrations of (+) and (−) perhexiline are depicted in Fig. 2a and b, respectively. There were strong direct correlations for both enantiomers, with slightly greater concentrations of both enantiomers in ventricle than in atrium (p<0.001 for both, Spearman's test).

The impact of metaboliser status on these plasma/myocardial concentration relationships is summarised in Table 2. All but two plasma enantiomer concentrations were >0.02 mg/L. In summary, plasma concentrations of (+) perhexiline tended to be greater than those of (−) perhexiline for both plasma and myocardium, irrespective of metaboliser status.

We next sought to determine whether stereoselective clearance of perhexiline and/or of its uptake into myocardium might vary with duration of therapy. The proportion of (+) perhexiline in plasma did not vary significantly with duration of therapy (Fig. 3a), while that in atria increased significantly (p=0.004) with time (Fig. 3b). Thus, the ratio of (+) perhexiline in the atrium to that in the plasma tended to increase progressively (r=0.19, p=0.07).

Concentrations of both (+) and (−) perhexiline into the ventricle increased significantly with time (p=0.005 and 0.004, respectively, Fig. 4), with no significant difference between the enantiomers.

Multivariate correlates of uptake of enantiomer

Table 3 summarises atrial/plasma concentration ratios, with all patients (excluding poor metabolisers)

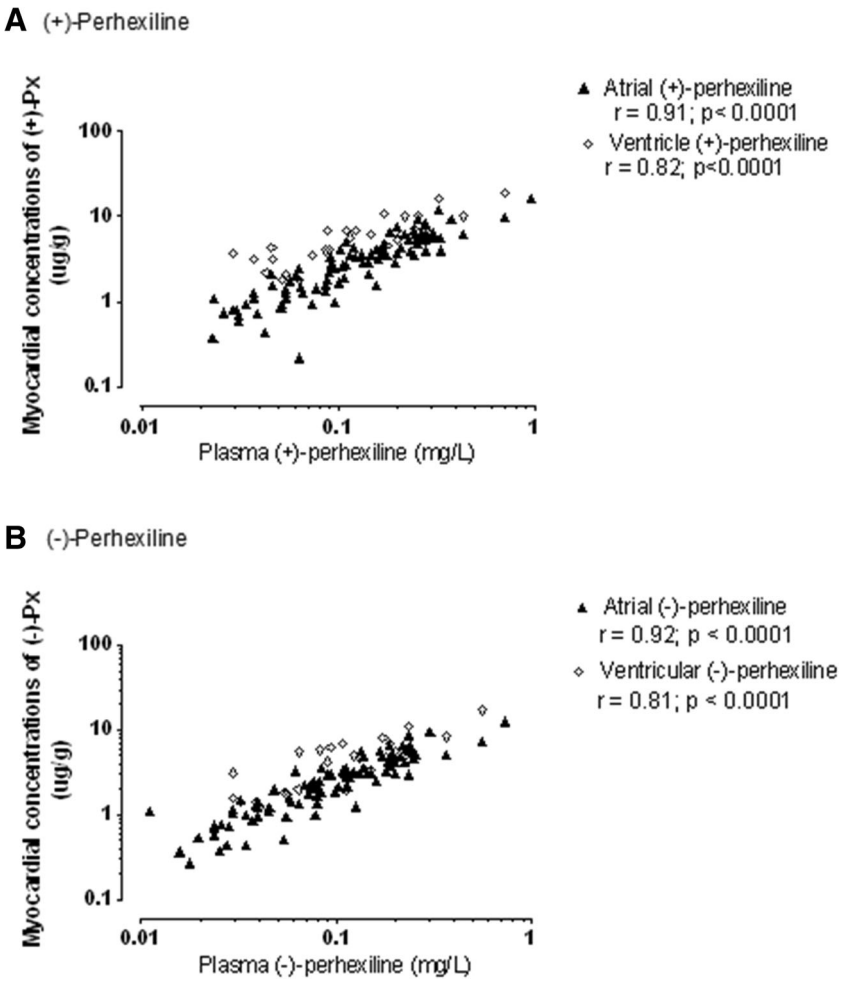

Fig. 2 Correlations between plasma and myocardial (atrial and ventricular) concentrations for **a** (+) perhexiline and **b** (−) perhexiline. Spearman's correlations are shown

evaluated. Plasma perhexiline concentration was a strong negative correlate and age was a positive correlate of this ratio for both enantiomers.

Uptake of (−) perhexiline also varied inversely with simultaneous heart rate. Similar trends were also apparent with univariate correlations (Fig. 5).

Table 2 Perhexiline enantiomer concentrations in plasma and atrial and ventricular myocardium across all metabolic phenotypes. Metaboliser status was defined by the ratio of plasma concentrations of cis-hydroxyperhexiline to perhexiline. Poor metaboliser was defined by a ratio of ≤0.3; intermediate metaboliser was defined by a ratio of 0.3 to 2.5; extensive metaboliser was defined by a ratio of 2.5 to 20; and ultrarapid metaboliser was defined by a ratio of ≥20

Metaboliser status	Plasma concentrations (mg/L)		Atrial concentrations (μg/g)		Ventricular concentrations (μg/g)	
	(+)	(−)	(+)	(−)	(+)	(−)
Poor metaboliser	n=7		n=3		n=1	
	0.67±0.32*	0.48±0.21	16.57±6.57	13.22±4.89	17.5	12.5
Intermediate metaboliser	n=28		n=19		n=6	
	0.28 (0.24–0.37)*	0.23 (0.18–0.29)	6.17 (5.45–8.16)*	5.11 (4.28–6.58)	11.07±5.41$^{\Delta}$	9.08±4.54$^{\Delta}$
Extensive metabolisers	n=85		n=64		n=21	
	0.11 (0.07–0.17)*	0.08 (0.05–0.13)	2.77 (1.50–3.99)*	2.17 (1.26–3.24)	5.16±2.48$^{\Delta}$	3.55±2.17$^{\Delta}$
Ultrarapid metabolisers	n=9		n=8		n=2	
	0.03 (0.026–0.037)*	0.023 (0.017–0.027)	0.84±0.31	0.623±0.314	3.41±0.41	1.56±2.21

Data expressed as mean±SD or median (interquartile range)

*p<0.05 vs antipode (Wilcoxon); $^{\Delta}p$<0.005 vs atrial/plasma ratio

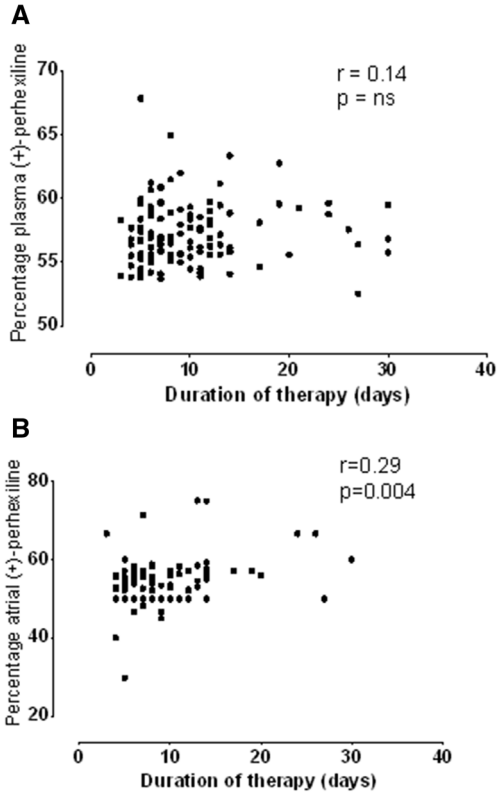

Fig. 3 Variations in the percentage of (+) perhexiline in **a** plasma and **b** atrial myocardium relative to duration of therapy. Spearman's correlations are shown

Discussions

The current analyses complement our previously published evaluation of the uptake of racemic perhexiline into the human myocardium [18]. It is apparent from the current studies that the perhexiline dosing regimen utilised, with inadequate time available for adjustment of dosage on the basis of metaboliser status, led to wide variability in plasma and myocardial drug concentrations but, nonetheless, allowed exploration of determinants of myocardial uptake of perhexiline enantiomers.

The main findings of the current analysis are that

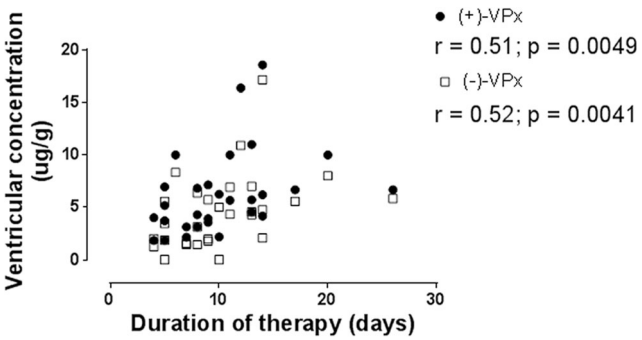

Fig. 4 Impact of duration of treatment on concentrations of perhexiline enantiomers in ventricular myocardium. Spearman's correlations are shown

1. Plasma concentrations of (+) or (−) perhexiline and the corresponding myocardial concentrations are closely and directly correlated.
2. Just as plasma (+) enantiomer concentration exceeds that of (−) enantiomer, similar trends are present in myocardium, especially atrial muscle.
3. Myocardial uptake of each enantiomer also depends on plasma drug concentrations, where a strong inverse relationship is present, and patients' age.
4. Atrial uptake of (+) perhexiline also varies with duration of therapy.
5. Atrial uptake of (−) perhexiline varies inversely with simultaneous heart rate.

These findings carry a number of important implications regarding the myocardial handling and effects of perhexiline enantiomers.

The higher concentrations of (+) than (−) enantiomer in plasma are consistent with more rapid clearance of the latter. These data are consistent with previous publications regarding the stereoselective clearance of perhexiline [20, 21]. On the other hand, myocardial concentration ratios of the enantiomers generally parallel with those in plasma, suggesting the absence of major stereoselectivity in myocardial uptake. The exception to this is the small but statistically significant increase in the proportion of (+) enantiomer in the atrial myocardium with time (Fig. 3b). These data suggest that myocardial uptake and efflux of (−) perhexiline may be slightly more rapid than that of the (+) enantiomer.

The relatively prolonged time required to reach steady-state perhexiline concentrations in the ventricular myocardium may reflect greater distribution into mitochondria [24], known to represent a site of intracellular drug accumulation for perhexiline in hepatocytes [25].

As regards the interactions between advanced age and cardiac uptake of the enantiomers, the current data suggest that uptake of both perhexiline enantiomers is greater in older patients, perhaps related to decreased skeletal muscle mass. Indeed we have previously also shown that steady-state dosage requirements for perhexiline tend to fall with age [26], and in the current study, plasma perhexiline concentrations increased with patient age (r=0.24, p=0.029). Together, these data suggest that elderly individuals may benefit from perhexiline therapy despite apparently borderline subtherapeutic plasma drug levels.

Finally, atrial uptake of (−) perhexiline varied inversely with concurrent heart rate. This is not consistent with the usual accelerating effect of tachycardia on myocardial drug uptake [27] and suggests another mechanism for the association. Perhexiline is a weak L-type calcium channel antagonist [28, 29], although its effects on the myocardium have undergone only limited study. The IC_{50} for inhibition of calcium fluxes in chick embryo ventricular myocardium by racemic perhexiline

Table 3 Correlates of the atrial/plasma ratio (net uptake) of each enantiomer on multivariate analyses (excluding poor metabolisers)

Determinant	Correlates	β coefficient	p value
Atrial/plasma ratio of (+) perhexiline	Plasma (+) perhexiline concentration	−0.256	0.015
	Duration of therapy	0.228	0.025
	Age	0.300	0.004
Atrial/plasma ratio of (−) perhexiline	Plasma (−) perhexiline concentration	−0.347	0.001
	Age	0.288	0.005
	On-treatment resting heart rate	−0.240	0.015

was 8.3×10^{-7} M [28]. In the current study, atrial concentrations of (−) perhexiline approximated to these values. It is therefore possible that the calcium antagonist effect of rac-perhexiline is mediated primarily by the (−) enantiomer, despite the current limitation of the understanding of pharmacological actions of the perhexiline enantiomers.

The current study has several limitations. Most importantly, correlation with cardiac effects of perhexiline is limited by the absence of true pre-treatment data and that evaluation of the effects of the enantiomers is only extrapolated on the basis of administration of rac-perhexiline. Second, myocardial drug content after a median of 9 days of therapy reflects both uptake into and efflux from the myocardium, and there is no way to determine the precise component of each, given that steady state has not been reached. Finally, the full implications of the widely variable plasma perhexiline concentrations on variability in drug uptake cannot be fully understood without a strategy of multiple drug dosing per patient.

The current results also need to be related to the clinical context. Recently, we have reported on acute loading of perhexiline in patients with severe ischaemia [14, 15]. The current data are consistent with the idea that there may be early onset of cardioprotective effects, especially in the elderly, at a time when plasma drug concentrations are notionally subtherapeutic. Furthermore, the data regarding heart rate correlations should stimulate evaluation of whether the calcium antagonist effects of perhexiline can be dissociated from its "metabolic" cardioprotective effects [30, 31], by selective administration of the (+) enantiomer.

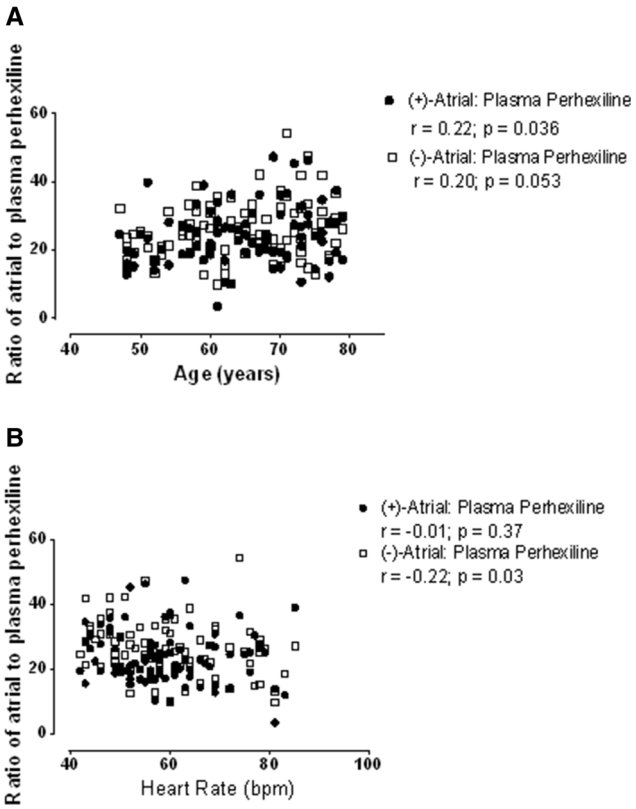

Fig. 5 Univariate comparisons (Spearman's correlation) between **a** patients' age and **b** resting heart rate and uptake ratio of each enantiomer into atrial myocardium

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Relationship between plasma, atrial and ventricular perhexiline concentrations in humans: insights into factors affecting myocardial uptake

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Perhexiline is a modulator of myocardial metabolism used for treatment of angina, and increasingly, heart failure and hypertrophic cardiomyopathy.
- Perhexiline has a small therapeutic index and highly variable pharmacokinetics (polymorphic and saturable), necessitating therapeutic drug monitoring to individualize dosage and minimize the risk of hepatotoxicity and peripheral neuropathy.

WHAT THIS STUDY ADDS

- Plasma perhexiline concentrations are highly predictive of myocardial concentrations, with net uptake into the left ventricle being more extensive than into the right atrium and requiring more time to reach steady-state.
- Net myocardial drug uptake also appears to be affected, to a lesser extent, by age and heart rate.

AIM

Little is known regarding the steady-state uptake of drugs into the human myocardium. Perhexiline is a prophylactic anti-anginal drug which is increasingly also used in the treatment of heart failure and hypertrophic cardiomyopathy. We explored the relationship between plasma perhexiline concentrations and its uptake into the myocardium.

METHODS

Blood, right atrium ± left ventricle biopsies were obtained from patients treated with perhexiline for a median of 8.5 days before undergoing coronary surgery in the perhexiline arm of a randomized controlled trial. Perhexiline concentrations in plasma and heart tissue were determined by HPLC.

RESULTS

Atrial biopsies were obtained from 94 patients and ventricular biopsies from 28 patients. The median plasma perhexiline concentration was within the therapeutic range at 0.24 mg l^{-1} (IQR 0.12–0.44), the median atrial concentration was 6.02 mg kg^{-1} (IQR 2.70–9.06) and median ventricular concentration was 10.0 mg kg^{-1} (IQR 5.76–13.1). Atrial ($r^2 = 0.76$) and ventricular ($r^2 = 0.73$) perhexiline concentrations were closely and directly correlated with plasma concentrations (both $P < 0.001$). The median atrial : plasma ratio was 21.5 (IQR 18.1–27.1), ventricular : plasma ratio was 34.9 (IQR 24.5–55.2) and ventricular : atrial ratio was 1.67 (IQR 1.39–2.22). Using multiple regression, the best model for predicting steady-state atrial concentration included plasma perhexiline, heart rate and age ($r^2 = 0.83$). Ventricular concentrations were directly correlated with plasma perhexiline concentration and length of therapy ($r^2 = 0.84$).

CONCLUSIONS

This study demonstrates that plasma perhexiline concentrations are predictive of myocardial drug concentrations, a major determinant of drug effect. However, net myocardial perhexiline uptake is significantly modulated by patient age, potentially via alteration of myocardial:extracardiac drug uptake.

Introduction

Little is known regarding the steady-state uptake of drugs from the blood into the human heart, their accumulation or partitioning, in part due to the inherent difficulty of obtaining heart tissue from patients. Although indirect measurement of uptake in humans has utilized paired arterial and coronary sinus blood sampling, this can only measure acute drug uptake following intravenous bolus injection [1]. On the other hand, animal studies have provided much information on the principles modulating drug uptake into the heart. In general, uptake is affected not only by plasma drug concentration but also by heart rate and the relative octanol : water partition coefficients [2]. Additionally, active transport mechanisms may be further modulators of the uptake process [1]. Therefore in theory, the ratio of plasma to myocardial drug concentrations may vary between patients.

Perhexiline is a modulator of myocardial metabolism that is effective in the treatment of patients with refractory angina unsuitable for revascularization [3]. More recently it has also been shown to improve myocardial energetics and function in chronic cardiac failure [4] and symptomatic hypertrophic cardiomyopathy [5]. Historically, there have been difficulties in balancing the clinical effectiveness of perhexiline with significant toxicity, due to marked inter-individual variation in its pharmacokinetics, principally differences in elimination resulting from genetic polymorphisms of CYP2D6. These polymorphisms give rise to approximately 100-fold inter-individual differences in apparent oral clearance and plasma half-lives that range from 1–2 days in most subjects and up to 40 days in poor metabolizers [6]. Severe adverse events, including hepatotoxicity and peripheral neuropathy, can be avoided by therapeutic drug monitoring to maintain plasma perhexiline concentration within a defined therapeutic range (0.15–0.6 mg l^{-1}) [7]. Perhexiline has been shown to inhibit carnitine palmitoyltransferase-1 (CPT-1), the key uptake enzyme for long chain fatty acids into mitochondria, and this is thought to be its principal mechanism of action [8]. This promotes a switch in myocardial energy metabolism from fatty acid to glucose utilization, providing a significant oxygen-sparing effect. Perhexiline has been shown in a number of studies in isolated tissues and animal models to be highly concentrated in many tissues, including myocardium and hepatocytes [8, 9], to an extent consistent with intracellular CPT-1 inhibition. This is despite the fact that the IC_{50} for CPT-1 inhibition in rat myocardium is approximately 50–100 fold greater than therapeutic plasma perhexiline concentrations [8]. No human tissue concentration data have previously been reported.

In the current study, we evaluated the relationship between plasma and myocardial perhexiline concentrations in a cohort of chronically treated patients undergoing non-emergent coronary artery surgery. The purposes of the study were to:

1 determine the extent of the nexus between plasma and myocardial drug concentrations;
2 determine whether the concentration of perhexiline within the myocardium varies between atria and ventricle; and
3 identify demographic/chemical factors which might modulate the nexus between plasma drug concentrations and perhexiline uptake into the myocardium.

Methods

Study design

Perhexiline administration occurred in the setting of a prospective, double-blind, randomized, placebo-controlled trial of pre-operative perhexiline. This was conducted in patients undergoing isolated, first time coronary artery bypass grafting (CABG) on cardiopulmonary bypass (CPB) for multi-vessel coronary artery disease (http://www.clinicaltrials.gov/show/NCT00845364). The study was approved by the Cambridgeshire 1 Research Ethics Committee (06/Q0104/41) and the Medicines and Healthcare products Regulatory Agency (2006-003164-62), and patients were enrolled between February 2007 and April 2010. All research was performed in accordance with the Declaration of Helsinki and the UK Human Tissue Act 2004 within a research governance framework. Following written informed consent, patients were randomized to either *rac*-perhexiline maleate (Aspen Pharmacare, St Leonards, NSW, Australia) or placebo and trial medication was commenced at least 5 days prior to the planned date of surgery. A standardized loading and maintenance regime was used: 200 mg twice daily for 3 days followed by 100 mg twice daily until the morning of surgery.

Plasma analysis

Blood was drawn prior to the induction of anaesthesia, centrifuged and the plasma stored at −80°C. Concentrations of perhexiline (C_{Px}) and OH-perhexiline (C_{OHPx}) were measured in all patients by high performance liquid chromatography (HPLC) as previously described [6]. This enabled identification of poor metabolizers in the treatment group and confirmation of zero levels in controls. CYP2D6 metabolizer phenotype was determined by the plasma concentration ratio ($C_{OHPx} : C_{Px}$) and classified according to published criteria [6].

Myocardial biopsies, extraction and analysis

Atrial and ventricular biopsies were obtained during surgery and promptly snap-frozen in liquid nitrogen. The right atrial appendage was truncated during venous cannulation for CPB. Transmural Tru-Cut needle (Alle-

giance, McGaw Park, IL, USA) biopsies of the left ventricular free-wall between the left anterior descending artery and the first diagonal branch were taken whilst on CPB but before application of the aortic cross clamp.

Myocardial perhexiline concentrations were determined using techniques adapted from a published method [10] and previously validated in an animal model. In brief, right atrial biopsies were mechanically digested in 0.15 M phosphate buffer solution (pH 6.0) using a homogenizer and a tissue grinder to form a suspension (approximately 100 mg tissue in 5 ml buffer). A fine tissue grinder alone was used for the left ventricular biopsies which had far lower masses (approximately 5 mg tissue in 0.5 ml buffer). Atrial and ventricular biopsies from patients in the perhexiline arm constituted the experimental groups. Right atrial homogenate from patients in the control arm, with proven zero plasma perhexiline concentrations, was also prepared for generating standard curves and quality control (QC) samples. The precision and accuracy of the method were evaluated over a perhexiline calibration range of 0.01–2.0 mg l^{-1}. Both inter- and intra-assay coefficients of variation and bias over the calibration range were within ± 15%.

Statistical analysis

Analyses were conducted using SPSS Statistics version 17.0 (SPSS Inc., Chicago, IL, USA). Poor metabolizers of perhexiline (C_{OHPx} : C_{Px} ratio ≤ 0.3) were excluded from the primary analysis as they were unlikely to have reached steady-state in either the plasma or myocardium [6]. Data are presented as mean (SD) or median (interquartile range, IQR). The concentration of perhexiline is reported as the sum of the concentration of the two enantiomers. Spearman's rank test was used to correlate non-parametric data and Wilcoxon signed-rank test to compare paired atrial and ventricular concentrations. The relationship between variables was modelled using simple and multiple linear regression.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed with the manuscript as written.

Results

The study group consisted of 97 patients treated with perhexiline for whom paired plasma perhexiline concentrations and atrial biopsies were available. Of these, three were analyzed separately because of poor metabolizer status, making it unlikely that steady-state had been approached at the time of study. Primary analyses were therefore performed in 94 patients, who had been treated with perhexiline for a median of 8.5 days with median total perhexiline dose of 2300 mg (Table 1).

Table 1

Characteristics of patients included in the study

Variable	Perhexiline (n = 94)
Plasma perhexiline, median (IQR) (mg l^{-1})	0.24 (0.12–0.44)
Plasma OH-perhexiline, median (IQR) (mg l^{-1})	1.18 (0.96–1.52)
Metabolizer status, *n* (%)	
Intermediate	18 (19.1)
Extensive	68 (72.3)
Ultra-rapid	8 (8.5)
Length of therapy, median (IQR) (days)	8.5 (6.5–12.5)
Age, mean (SD) (years)	64.3 (8.9)
Weight, mean (SD) (kg)	84.2 (15.0)
Pre-treatment heart rate, mean (SD) (beats min^{-1})	60 (10)
Left ventricular function, *n* (%)	
Good (≥50%)	77 (82)
Moderate (30–49%)	17 (18)
Pre-ischaemia cardiac index, mean (SD) (l min^{-1} m^{-2})	2.13 (0.60)
Pre-operative medications, *n* (%)	
ACE inhibitor or ARB	60 (63.8)
Statin	87 (92.6)
β-adrenoceptor antagonist	66 (70.2)
Calcium channel antagonist	34 (36.2)
Long acting oral nitrate	32 (34.0)

ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker.

Plasma perhexiline concentration

The median plasma perhexiline concentration at the time of surgery was 0.24 mg l^{-1} (IQR 0.12–0.44) with 64% patients within the established therapeutic range of 0.15–0.60 mg l^{-1}, 28% sub-therapeutic and 9% above the range. Of the total study group, three were poor metabolizers (mean C_{Px} 1.67 mg l^{-1}), 18 were intermediate metabolizers (0.65 mg l^{-1}), 68 were extensive (0.25 mg l^{-1}) and eight were ultra-rapid metabolizers (0.05 mg l^{-1}). Excluding the poor metabolizers, there was no correlation between plasma C_{Px} and length of therapy (r_s coefficient 0.05, $P = 0.60$) (Figure 1).

Myocardial perhexiline uptake: data excluding poor metabolizers

Atrial perhexiline concentration Right atrial biopsies from all 94 patients in the primary analysis group had a mean mass of 114 mg (range 10–458) and median perhexiline concentration of 6.02 mg kg^{-1} of tissue (IQR 2.70–9.06). Atrial and plasma perhexiline concentrations were closely correlated ($r = 0.87$, $P < 0.001$, Figure 2) and the median atrial:plasma perhexiline ratio was 21.5 (IQR 18.1–27.1) with a range from 4.9–55.6. Stepwise multiple linear regression was performed using plasma perhexiline, length of therapy, age, weight, pre-anaesthesia heart rate, pre-sternotomy cardiac index, gender and creatinine clearance as potential correlates of atrial concentration. A model containing plasma perhexiline (β 0.87, $P < 0.001$), age (β 0.12, $P < 0.01$) and heart rate (β −0.09, $P < 0.05$) achieved an r value of 0.91, r^2 of 0.83 and an F ratio of 108.0 ($P < 0.001$), noting that plasma perhexiline alone has an r

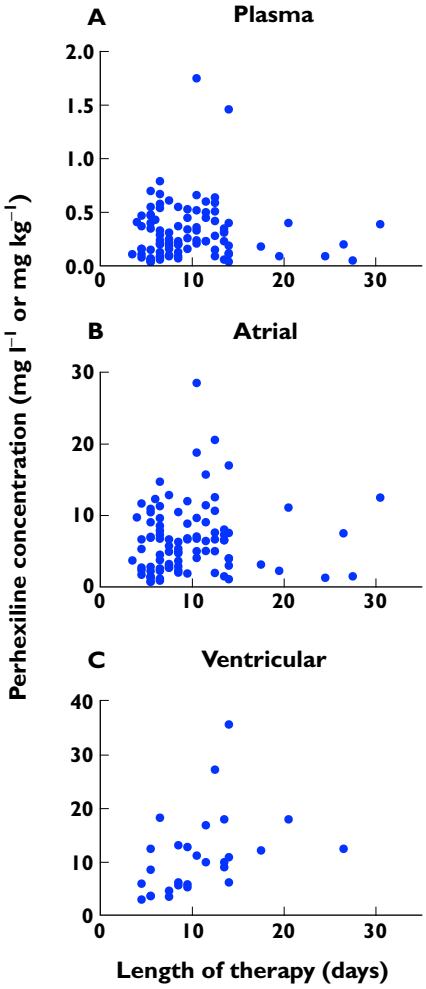

Figure 1

Correlation between length of therapy and perhexiline concentration in (A) plasma, (B) atrial and (C) ventricular myocardium

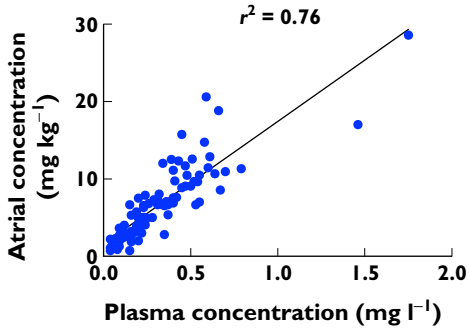

Figure 2

Linear regression demonstrating that plasma perhexiline is predictive of atrial concentration in 94 patients ($r^2 = 0.76$, $P < 0.001$)

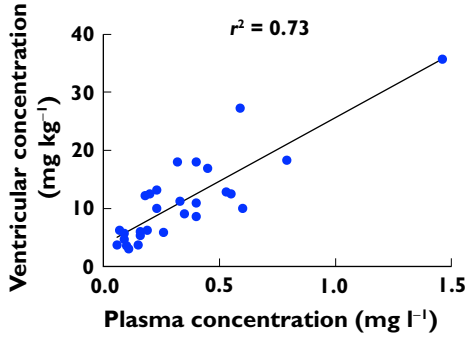

Figure 3

Linear regression demonstrating that plasma perhexiline is also predictive of ventricular concentration in 28 patients ($r^2 = 0.73$, $P < 0.001$)

value of 0.87. Length of therapy had no impact on the model suggesting that atrial perhexiline concentration was not significantly affected by the length of therapy beyond 5 days and that most patients had reached a steady-state of atrial perhexiline concentrations.

Ventricular perhexiline concentration Left ventricular biopsies were available in only 28 patients in the study group who had been treated for a median of 9.5 days (IQR 7.0–14.0) and had a median perhexiline concentration of 10.0 mg kg^{-1} (IQR 5.76–13.1). As with the atrial biopsies, there was a close correlation ($r = 0.85$, $P < 0.001$) between plasma and ventricular perhexiline concentrations (Figure 3). The median ventricular : plasma perhexiline concentration ratio was 34.9 (IQR 24.5–55.2) with a range of 16.7–89.3. The ventricular concentration was significantly higher than the atrial concentration (Z –4.4, $P < 0.01$) with a median ventricular : atrial ratio of 1.67 (IQR 1.39–2.22) and a range from 0.9–5.0. The atrial concentration was also strongly correlated with ventricular concentration ($r = 0.88$, $P < 0.001$).

However, unlike atrial concentrations, ventricular concentrations of perhexiline were directly correlated with duration of therapy ($r = 0.54$, $P < 0.001$, Figure 1). Stepwise multiple linear regression was used to identify correlates of ventricular concentration. Plasma perhexiline concentration (β 0.74, $P < 0.001$) remained the principal correlate but the model was improved by the addition of length of therapy (β 0.30, $P < 0.01$), with an overall r value of 0.92 and F ratio of 43.0 ($P < 0.001$).

Myocardial perhexiline uptake in poor metabolizers

In all, seven phenotypic CYP2D6 poor metabolizers were treated with perhexiline in the trial but atrial biopsies were only available for three of these. Mean plasma perhexiline concentration was elevated beyond the therapeutic range in all seven of the poor metabolizers (1.35 ± 0.67 mg l^{-1}). Atrial perhexiline concentrations were 31.51, 17.59 and 17.46 mg l^{-1}, respectively, in the three cases with associated biopsy data.

Discussion

The current study utilized atrial and ventricular biopsies to compare plasma and myocardial concentrations of perhexiline in patients treated with the drug for a median of 8.5 days prior to undergoing surgical coronary revascularization. The use of ongoing therapy to attain steady-state plasma and atrial perhexiline concentrations allowed the investigation of factors that may affect net myocardial uptake, which at steady-state is directly related to the myocardial : plasma concentration ratio (assuming constant myocardial clearance). Thus, the major findings of this study are:

1. in a group of patients whose plasma perhexiline concentrations were generally within the therapeutic range, plasma perhexiline concentrations were closely correlated with both atrial and ventricular drug concentrations;
2. perhexiline is concentrated in the myocardium relative to plasma, with approximately 20-fold and 35-fold myocardial : plasma ratios in the right atrium and left ventricle, respectively;
3. while on multivariate analysis plasma drug concentration is the major determinant of right atrial perhexiline concentration, there is also a direct relationship between net myocardial drug uptake and age, and a significant inverse relationship with heart rate prior to surgery and
4. net perhexiline uptake into the left ventricle appears to be more extensive than into the right atrium and also requires a greater period of time to reach steady-state.

These findings have implications both as regards the general principles of drug uptake into the heart and therapeutics with perhexiline.

The dosing regimen utilized in this study was identical to one developed over 20 years ago for the attainment of therapeutic perhexiline concentrations within 3 days [7]. While this regimen achieves its purpose in most patients, it is associated with sub-therapeutic concentrations (<0.15 mg l^{-1}) in approximately 10–15% of patients and in toxic concentrations (>0.6 mg l^{-1}) in a slightly higher proportion [11], particularly ultra-rapid and poor metabolizers of the drug, respectively. Current practice is for treatment regimens to be titrated on the basis of plasma perhexiline concentrations measured during the first week of therapy [6] although the short term treatment strategy utilized in the current trial did not permit this to occur.

The close linear relationship between plasma and myocardial perhexiline concentrations suggests that net uptake is not saturable, and is therefore relatively constant within the range of concentrations evaluated. The observation that perhexiline concentrations within the myocardium are far greater than in plasma is consistent with the lipophilicity of the drug and with previously demonstrated intracellular concentrations in hepatocytes [9]. These data also establish that the intracellular concentration of perhexiline within the human ventricle is sufficient to induce substantial inhibition of myocardial CPT-1, the postulated principal mechanism of action of the drug [8].

The finding that left ventricular perhexiline concentrations had not reached steady-state is also important, confirming that there may be substantial hysteresis between attainment of the 'therapeutic' plasma drug concentrations and steady-state therapeutic effects. However, the impact of length of therapy on ventricular perhexiline concentrations was relatively small. Hence in most cases, net myocardial uptake was at near equilibrium with plasma concentrations, as shown in Figure 1.

The impact of increasing age on net atrial perhexiline uptake, although small, was a potentially important finding, especially given the frequent use of this agent in elderly patients with angina refractory to other treatment options. We postulate that the finding reflects a diminution of the relative uptake of perhexiline into extracardiac tissues such as skeletal muscle and/or fat, perhaps due to decreased mass of these tissues in the elderly [12] or, less likely, a small age-dependent decrease in myocardial clearance. It also raises the possibility that hepatic drug uptake, the precursor to potential long term perhexiline toxicity, may also be increased in aged patients, despite apparently 'therapeutic' plasma drug concentrations.

Previous studies in animal models, isolated tissues and in man have suggested that myocardial mechanical activity, perhaps linked to coronary blood flow, increased drug uptake into the heart [2]. This has been examined extensively in the case of digoxin [13, 14], and in the short term for class I anti-arrhythmic agents [15–17]. In the current study, it is possible that the greater mechanical activity (and associated perfusion) of the left ventricle might have contributed to a greater uptake into the ventricular than atrial myocardium. However, the finding that atrial perhexiline concentrations varied inversely with heart rate is unexpected and indeed is contrary to a number of other reports, including acute drug uptake studies in humans [2]. However, it is important to note that the heart rate measurement in the patients studied was performed prior to surgery whilst on therapy. As perhexiline is a weak L-channel calcium antagonist [18], it is therefore likely that the correlation between right atrial perhexiline concentration and bradycardia resulted from the effect of increasing perhexiline concentrations on sinus node function, rather than constituting a paradoxical effect on drug uptake.

The study had a number of limitations. In particular, we were unable to evaluate the impact of regional heterogeneity of coronary perfusion in this group of patients with potentially severe myocardial ischaemia on the distribution of perhexiline within the left ventricular myocardium.

This is an important issue, as drug uptake into ischaemic myocardium is critical to the improvement of myocardial energetics which has been shown to occur during perhexiline therapy [5], and which results, at least in part, from CPT-1 inhibition [8, 19]. Furthermore, the study provided only limited information regarding myocardial drug uptake in CYP2D6 poor metabolizers, who represent about 7% of Caucasian patients. Drug uptake in such patients is likely to be in similar equilibrium with plasma perhexiline concentrations but this is difficult to prove without a specific investigation in poor metabolizers. Finally, tissue biopsy was of necessity limited to the myocardium. Measurement of perhexiline uptake into the liver, skeletal muscle and adipose tissue would have been of interest, particularly to help evaluate the basis for differential myocardial uptake in the elderly.

In conclusion, this study serves to establish a close nexus between plasma and myocardial perhexiline concentrations, albeit with other factors modulating net uptake. In doing so, the results have strengthened the basis for monitoring of plasma perhexiline concentration and perhaps for therapeutic drug monitoring in general.

Contributions

The study was developed by JDH and BCS based upon the clinical trial conceived by DP and MPF for which DP was the Principal Investigator. NED conducted the trial, recruited patients, administered the trial medication, collected the biopsies and performed the experiments in conjunction with GL and BCS. All authors were involved in drafting and reviewing the final manuscript.

Competing Interests

MPF is inventor of the method of use patents for perhexiline in heart muscle diseases. No other authors declare a conflict of interest.

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