CARDIAC METABOLISM IN HEALTH AND DISEASE

Developments in Molecular and Cellular Biochemistry

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Cardiac Metabolism in Health and Disease

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CARDIAC METABOLISM IN HEALTH AND DISEASE
S. Abdel-aleem and J.E. Lowe, guest editors

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Preface

This special issue of *Molecular and Cellular Biochemistry* contains original research papers as well as invited reviews focused in the field of cardiac metabolism and its regulation under normal and disease conditions. These papers cover many areas under intensive and rapid development such as regulation of fatty acid oxidation in the heart, role of cardiac glycogen during ischemia, role of CPT I isoenzymes, pathophysiology of diabetic cardiomyopathy, cardiac protection through regulation of energy production, role of fatty acid binding protein under normal and pathological conditions and several other important topics in this area of research. We hope that this special issue of *Molecular and Cellular Biochemistry* provides an up-to-date source of information for scientists and clinicians interested in the mechanism by which cardiac metabolism is regulated in health and disease and mechanistic relationship between disturbance in cardiac metabolism and genesis of cardiovascular diseases.

The editors would like to express their gratitude to the Editor-in-Chief, Naranjan S. Dhalla, for publishing this focused issue. The editors are also grateful to Dr. Robert W. Anderson, Chairman of Surgery Department, Duke University Medical Center, for his support and encouragement.

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Glucose and glycogen utilisation in myocardial ischemia – Changes in metabolism and consequences for the myocyte

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Abstract

Experimentally, enhanced glycolytic flux has been shown to confer many benefits to the ischemic heart, including maintenance of membrane activity, inhibition of contracture, reduced arrhythmias, and improved functional recovery. While at moderate low coronary flows, the benefits of glycolysis appear extensive, the controversy arises at very low flow rates, in the absence of flow; or when glycolytic substrate may be present in excess, such that high glucose concentrations with or without insulin overload the cell with deleterious metabolites. Under conditions of total global ischemia, glycogen is the only substrate for glycolytic flux. Glycogenolysis may only be protective until the accumulation of metabolites (lactate, H^+, NADH, sugar phosphates and Pi) outweighs the benefit of the ATP produced.

The possible deleterious effects associated with increased glycolysis cannot be ignored, and may explain some of the controversial findings reported in the literature. However, an optimal balance between the rate of ATP production and rate of accumulation of metabolites (determined by the glycolytic flux rate and the rate of coronary washout), may ensure optimal recovery. In addition, the effects of glucose utilisation must be distinguished from those of glycogen, differences which may be explained by functional compartmentation within the cell. (Moll Cell Biochem **180:** 3–26, 1998)

Key words: glycolysis, glycogenolysis, high energy phosphate stores, glycolytic flux, cardiomyocytes, myocardial ischemia

Introduction

Increased provision of glucose is usually beneficial to the ischemic myocardium, as anaerobic glycolysis may be the sole source of energy. The 'glucose hypothesis' [1] suggests that the benefits of enhanced glucose provision include increased energy production, reduced loss of K^+ ions and attenuated arrhythmias, inhibition of changes in the transmembrane action potential, altered extracellular volume, and decreased circulating free fatty acids whose intermediates may be toxic to the ischemic heart. These mechanisms result in reduced incidence of arrhythmias, reduced ischemic contracture, and improved recovery of function.

Despite many studies showing benefits of enhanced glucose utilisation [2–11], there are several important exceptions. These include (1) a trial published by the British Medical Research Council in the Lancet, which did not find a beneficial effect associated with glucose-insulin-potassium (GIK) treatment of patients with infarction [12], (2) a report showing that addition of glucose in cardioplegic solutions was detrimental to the myocardium [13], (3) a finding that reduction in glycogen levels may be beneficial to the globally ischemic rat heart [14] and (4) the benefits ascribed to preconditioning (one or more brief episodes of ischemia and reperfusion protect against a subsequent sustained ischemic episode [15]), which are associated with reduced glycogenolysis [16, 17].

These studies have led to a controversy over the therapeutic use of glucose provision, with the resultant discontinuance of GIK therapy for patients with myocardial infarction. However, the adverse findings described above may largely be attributed to incorrect dosage of glucose and impaired

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removal of metabolites, as well as lack of differentiation between glycogen and glucose utilisation. We suggest that glucose provision is beneficial at an optimal concentration, but that increased glycolysis may indeed be deleterious by increased metabolite accumulation. Removal of the metabolites by increased residual flow should result in increased recovery with higher glucose concentrations. The basis for this relationship relies on an understanding of the utilisation of glucose in ischemia. The regulation of glucose (and glycogen) metabolism is discussed briefly (for fuller discussions see refs e.g. [18–20]). The perturbations in metabolism induced by ischemia are discussed, together with the implications for the ischemic myocardium. Finally, the implications of differences in glycogen vs. glucose metabolism for the ischemic myocardium are reviewed.

Glucose uptake

The primary determinant of glucose utilisation is the rate of uptake into the cell, which is mediated by facilitated diffusion through specific glucose transporters. The rate of uptake is determined by the concentration gradient, the number of pores available in the membrane, and the affinity of the carriers. Once taken up into a cell, glucose is rapidly phosphorylated, preventing efflux and providing a constant gradient for glucose uptake.

Two types of facilitated glucose uptake mechanisms are found in animals. The Na^+-dependent co-transport of glucose, driven by the Na^+ gradient, does not occur in heart cells [21]. Up to six isoforms of the facilitative transporters, designated GLUT, are found, the distributions of which are determined by the glucose requirements of the individual tissue types [21, 22]. GLUT 1 is particularly abundant in red blood cells, and is also found in myocytes, both within the sarcolemmal and intracellular vesicle membranes. The insulin-sensitive glucose transporter, designated GLUT 4 [23], is also distributed between vesicles in the cytosolic pool, and the plasma membrane. Insulin acutely stimulates glucose transport in muscle and fat by recruiting up to about 40% of cytosolic GLUT 4 to the plasma membrane, compared to a normal level of 1%. Glucose uptake can thus be increased by 10–40 fold. GLUT 1 translocation may also be triggered by insulin [24]. A recent finding using a transgenic mouse model is that ablation of the GLUT 4 gene results in cardiac hypertrophy [25], suggesting that this mechanism of metabolic regulation is an important determinant of cell growth.

GLUT 1 has a lower affinity (about 5–10 mM [22]), but a high capacity for glucose, while GLUT 4 has a low capacity but a higher affinity (4.3 mM [26]). However, the relative numbers of transporters in the cell indicate that GLUT 4 is largely responsible for insulin-stimulated glucose uptake, and is far more efficient [26].

Insulin is a major regulatory hormone of carbohydrate metabolism in the heart [27]. Insulin promotes glucose entry and stimulates glycolysis, enhances synthesis of glycogen, fatty acids and proteins, and inhibits glycogen and fat utilisation. Insulin receptor activation promotes an intrinsic tyrosine kinase activity which leads to insulin receptor substrate 1 (IRS-1) phosphorylation. IRS-1 binds with Src homology 2 proteins (SH2-phosphotyrosine binding sites), including phosphoinositide 3-kinase (PI 3-kinase), Ras GTPase-activating protein, phospholipase C and others [28]. PI 3-kinase phosphorylates phosphoinositides, and is involved in growth factor stimulation. In addition, PI 3-kinase may mediate GLUT 4 translocation to the membrane [28] and increase glycogen synthesis. The insulin receptor in turn can be phosphorylated by protein kinase Cα [29]. The subsequent effects are not clear, but this finding suggests that insulin activity is affected by mechanisms which regulate intracellular Ca^{2+} (e.g. inositol polyphosphates [30]), and may thus be altered in ischemia.

In transgenic mice, increased expression of GLUT 1 increases glucose uptake and glycogen storage, implying that glucose transport is the rate-limiting step of glucose utilisation. Most factors which increase glucose utilisation act via increased recruitment of transporters to the membrane, including cAMP following β adrenergic stimulation [31, 32], hypoxia [33],and also possibly ischemia [34, 35]. In addition, the function of GLUT 4 transporters within the sarcolemma may be upregulated, by correct orientation within the membrane [36–39]. For example, adenosine, an important regulator of glucose utilisation, may enhance only insulin-stimulated glucose uptake [35], without any measurable change in sarcolemmal GLUT 4 density. Changes in transporter orientation may also be important in modulating glucose uptake in ischemic tissue. Cyclic guanosine monophosphate (cGMP) may also stimulate glucose transport by a direct action on the transporters [40], although the mechanism is unclear, and has been disputed [41].

The rate of glucose uptake is also determined by the metabolic requirements of the cell, dietary state, oxygen availability, hormones other than insulin (glucagon, catecholamines, thyroid), and the relative availability of the different substrates [27, 42]. In a perfused rat heart, glucose uptake increases linearly in the range of 1.25–5 mM glucose, but is saturated at concentrations above 10–12 mM [43]. Glucose uptake is increased with glycogen depletion [44], increased work rate [45] or exercise [46] in direct response to increased energy requirements. In an isolated heart, the majority of glucose taken up is oxidised, although some may go to glycogen synthesis (about 5–10% [47, 48]) and to lactate formation, depending on the availability of oxygen. In the presence of other substrates and insulin, the distribution to glycogen may increase.

Fatty acids compete with glucose as the substrate of choice [45], and limit entry of glucose when present in high concen-

trations – the 'glucose-sparing' effect [49]. *In vivo*, when blood free fatty acids are elevated, glucose is directed to the liver for storage as glycogen.

Glucose utilisation within the cell

Glucose utilisation involves several integrated major pathways, including glycolysis, glycogen synthesis/breakdown, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. Intersecting pathways include mechanisms to reduce the redox potential (the malate-aspartate and the α-glycerophosphate shuttles), anaplerotic pathways to replenish the TCA cycle, and the contribution of a number of amino acids, as well as fatty acid synthesis/breakdown. These cycles in turn are regulated by substrate balance, hormones (insulin, adrenaline, glucagon), ions (Mg^{2+}, Ca^{2+}, H^+), and the energy status of the cell (levels of high energy phosphate metabolites and the cytosolic phosphorylation potential).

Glycogen

Glycogen is the storage form of glucose, and is found mainly in large macroparticles, consisting of 10 000–30 000 glucose molecules bound with either α-1,4 (majority of bonds – straight bond) or α-1,6 linkages (branch points). The macromolecules have a molecular mass of 10^4 kDa, and constitute

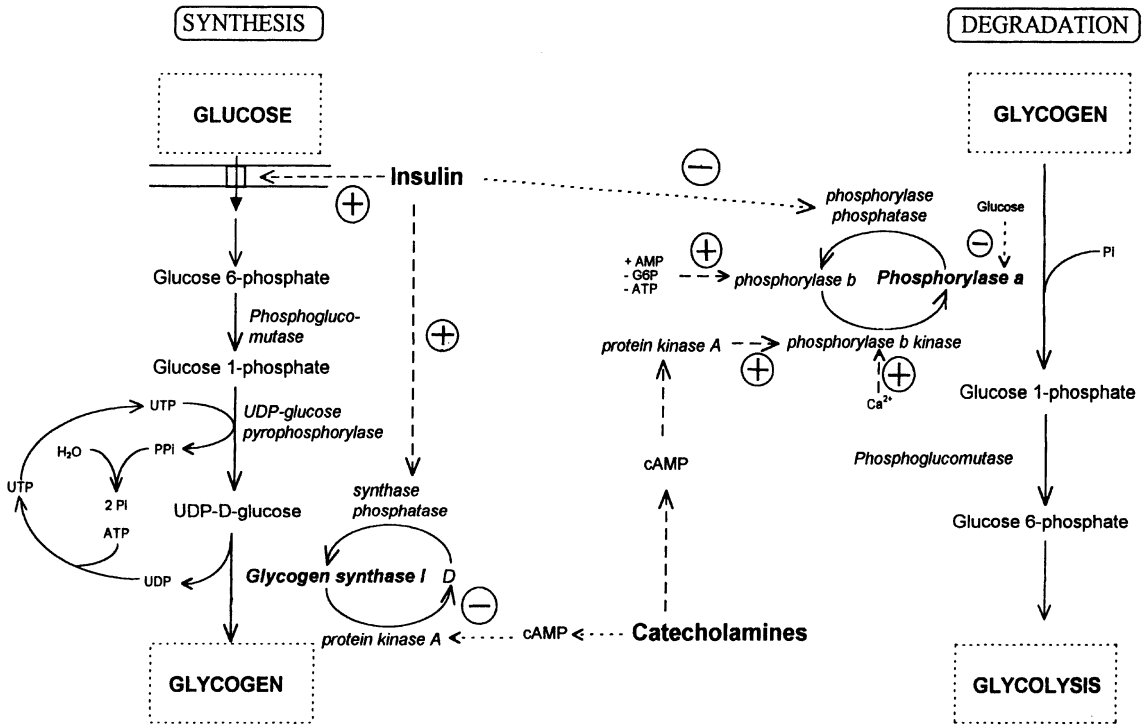

Fig. 1. Glycogen synthesis and degradation. *Glycogen synthesis* – Glycogen is synthesised from uridine diphosphate glucose (UDP-glucose), an activated form of glucose derived from glucose 1-phosphate (G1P) and uridine triphosphate (UTP). Pyrophosphate (PPi) is formed, and then hydrolysed to 2 Pi, an essentially irreversible reaction which drives glycogen synthesis. The UDP is then cleaved from the glucose by glycogen synthase and the glucose moiety is attached to the non-reducing end of a glycogen branch. Synthase phosphatase activates glycogen synthase from an inactive phosphorylated (b or D) to an active dephosphorylated form (a or I) (D – dependent on; I – independent of G6P). PKA phosphorylates and thus inhibits glycogen synthase. G6P is a potent stimulator of glycogen synthase activity (from [19, 235]). *Glycogen degradation* – Phosphorylase is the main enzyme of glycogen breakdown, cleaving glucose moieties at α-1,4 bonds from a non-reducing end of a glycogen branch, and phosphorylating these with Pi. However, phosphorylase stops cleaving when there are 4 terminal residues from the α-1,6 branch point. A transferase is required to transfer the remaining α-1,4 residues to the linear branch. The remaining α-1,6 bond is cleaved by amylo-1,6-glucosidase, which results in a glucose molecule, not G1P. Phosphorylase is upregulated by phosphorylation (from *b* – inactive to *a* – active) by a Ca^{2+}-dependent phosphorylase *b* kinase. Thus a close co-operation between contraction and glycogen breakdown exists, regulated by Ca^{2+}. This kinase is activated by cAMP-dependent protein kinase A following β-stimulation. PKA simultaneously inhibits glycogen synthesis. Phosphorylase phosphatase inhibits phosphorylase *a* by dephosphorylation [20, 51]. Inactive phosphorylase *b* can be directly stimulated by increases in AMP, and decreases in G6P and ATP. Phosphorylase *a* is inhibited by high concentrations of glucose.

1% of muscle mass. Recently, a new acid-precipitable 'form' of glycogen, or proglycogen, has been described [50]. This 400 kDa molecule has a high protein content (10 vs. 0.35% in macromolecular form) accounting for its response to acid. Proglycogen constitutes about 3–50% of total glycogen, depending on the tissue type (as much as 50% in heart muscle [19]). Proglycogen is an efficient receptor of glucose residues from UDP-glucose and may be an intermediate in the synthesis and degradation of glycogen [19]. Glycogenin, a self-glycosylating protein, thought to be the primer, or backbone of glycogen synthesis, has also been described. Glycogenin forms proglycogen and finally becomes part of the macroglycogen molecule (for in-depth discussion, see [19]).

Glycogen synthesis is stimulated by insulin, and increased glucose or glucose 6-phosphate (G6P) levels, while glycogenolysis is enhanced by cyclic adenosine monophosphate (cAMP), increased energy requirements and decreased glucose availability. There are two distinct pathways for glycogen synthesis and breakdown, each regulated by hormones which stimulate one pathway, while inhibiting the other (Fig. 1). Glycogen synthesis and utilisation was thought to follow the 'last on, first off' principle, whereby the last carbon molecule to be attached is the first to be cleaved off, but this has recently been disputed [47].

Glycogen synthase is the most important enzyme in glycogen synthesis (Fig. 1). Two forms of glycogen synthase, one acting on proglycogen and another on macromolecular glycogen, have been identified [19], possibly accounting for different rates of synthesis of the different forms of glycogen. Insulin stimulates glycogen synthesis by enhanced glycogen synthase dephosphorylation via a number of protein kinases (Fig. 1) [51]. Insulin also promotes dephosphorylation, and thus inactivation, of phosphorylase, the primary enzyme in glycogen breakdown (Fig. 1). Insulin also greatly increases glucose uptake, increasing substrate levels for glycogen synthesis. Fasting (short term) increases glycogen deposition by enhanced fatty acid oxidation, and inhibition of glycolysis in the absence of insulin [52]. Transient ischemia also activates glycogen synthase, possibly via G6P-mediated activation of phosphatase [53]. This effect appears contrary to the normal concept of ischemia-induced glycogen breakdown, but may be involved in preconditioning and in ischemia with a residual coronary flow (or hibernation, a chronic moderate reduction in coronary flow [54]).

About 9 protein kinases phosphorylate/dephosphorylate glycogen synthase [51], the most important being cAMP-dependent protein kinase A (PKA) [20]. PKA phosphorylation inhibits glycogen synthase and thus glycogen synthesis (Fig. 1) [55]. Protein kinase C and phosphorylase kinase also inhibit the enzyme. These factors in turn stimulate glycogen breakdown.

Glycogen breakdown follows a simple pathway (Fig. 1). A glucose moiety bound by an α-1,4 (straight) bond is cleaved from the large macromolecule and phosphorylated by phosphorylase *a* with Pi. This occurs without the expenditure of an ATP molecule, and prevents diffusion out of the cell. G1P is converted to G6P by phosphoglucomutase, an enzyme which favours the formation of G6P, unless G6P is in high concentrations. G6P then enters the glycolytic pathway. The complete oxidation of G1P (or G6P) yields about 37 ATP, while storage consumes slightly more than one ATP (although these values are now disputed [56]). The equilibrium favours glycogen breakdown, thus the energy yield is very efficient, at about 97%. Glycogenolysis is stimulated by hypoxia, ischemia, glucagon, and epinephrine.

The rates of breakdown of the different forms of glycogen appear to be very different [57]. Acid-extractable glycogen reflects changes in ischemia more closely, representing a subtraction of glycogen more responsive to degradation i.e. macromolecular glycogen. Glycogen in perchloric precipitate, i.e. proglycogen, remained unchanged during ischemia [57, 58].

The glycolytic pathway

The glycolytic pathway is shown in Fig. 2. For the purposes of the present discussion, glycolysis is defined as the breakdown of G6P to pyruvate. G6P is the entry point for both glucose and glycogen. Glucose is phosphorylated by hexokinase (HK) with the hydrolysis of ATP, an essentially irreversible reaction. An additional ATP is consumed, but 4 ATP are then produced if glycolysis goes through to pyruvate, together with 2 ($NADH + H^+$). The net ATP production is thus 2 ATP. If glycogen is broken down, the net production is 3 ATP, because Pi is utilised in the initial phosphorylation step, rather than ATP. The final product of glycosis is pyruvate, which can then follow a number of pathways, which determines the total amount of ATP derived from a glucose molecule. Anaeroic glycolysis implies that pyruvate is converted to lactate.

Levels of regulation of glycolysis

Glucose utilisation is regulated at several points along the glycolytic pathway as well as by factors governing glucose transport and glycogenolysis. However, the significance of regulation at each of these points is still controversial (see below). In addition, there is much controversy over whether the control of glycolysis exists at certain defined points [59–61] or regulation is distributed along the entire length of the pathway [62–64]. Recent evidence suggests that, under control conditions with sufficient substrate, the control of glycolysis rests largely at the level of glucose transport and hexokinase (58%), and that only 25% of control is mediated at steps below phosphoglucoismerase (see Fig. 2) [63] (remainder – glycogen synthesis). While these proportions may alter in the presence of insulin and other substrates, as well as ischemia, the fundamental concept is that regulation occurs

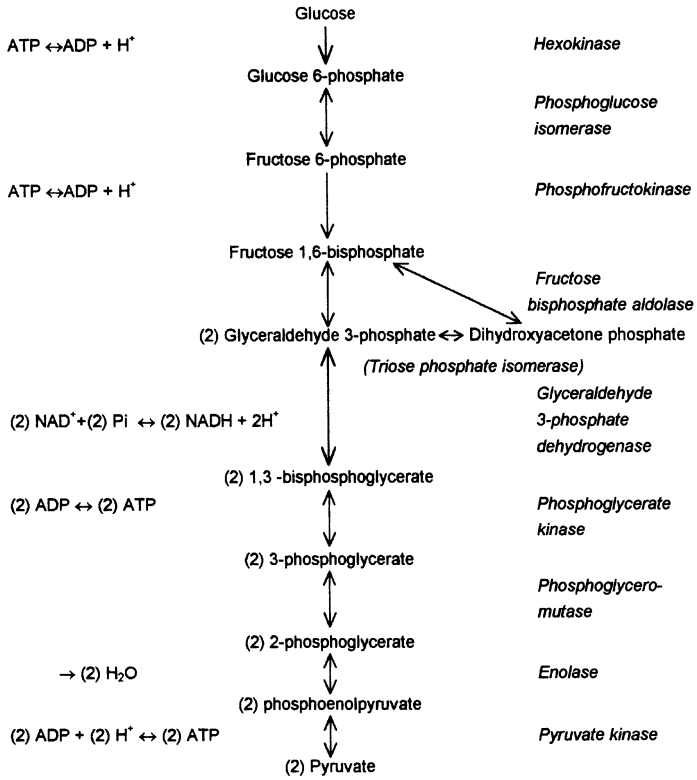

Fig. 2. The glycolytic pathway. Hexokinase phosphorylates glucose on entering the cell. G6P then undergoes a fully reversible conformational change to fructose 6-phosphate (F6P). Concentrations of G6P are generally 10 fold higher than of F6P. F6P is phosphorylated to fructose 1,6-bisphosphate (F1,6-BP) by phosphofructokinase-1 (PFK-1). Because this reaction uses the energy of ATP hydrolysis, the equilibrium greatly favours F1,6-BP. Glucose utilisation has thus consumed two ATP molecules by this stage, whereas glycogenolysis has consumed only one ATP per glucose residue, and a Pi. The above compounds are all hexose sugars. However, with 2 phosphate groups attached, the 6-C chain can be symmetrically broken down to two phosphorylated 3-C chains. Two distinct molecules are formed – glyceraldehyde 3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP). DHAP is either converted to GAP by triose phosphate isomerase, or to α-glycerophosphate (αGP) (see Fig. 3). To continue glycolysis, GAP is simultaneously oxidised and phosphorylated with Pi to 1,3-bisphosphoglycerate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). One phosphate group on each 3-C molecule is subsequently cleaved off to form ATP, when 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate (3PG) by phosphoglycerate kinase. 3PG undergoes a conformational change to 2-phosphoglycerate, which is dehydrated with the formation of an enol group, to phosphoenolpyruvate (PEP). The high phosphoryl-transfer potential of PEP allows the transfer of the remaining high energy phosphate group to ADP+H^+, with the end products of pyruvate and ATP. This reaction, catalysed by pyruvate kinase (PK), is virtually irreversible. The net ATP production from the breakdown of glucose to pyruvate is 2 ATP; from glycogen, 3 ATP are produced.

at multiple steps, and the importance of the classical points of regulation, specifically PFK and GAPDH, is reduced [63].

The concept of 'channelling' [65] implies cellular localisation of enzymes to allow efficient transfer of products of one enzyme reaction to the next [65]. The enzymes of a given pathway thus form a 'metabolon' or efficient set of linked pathways [62, 66], which suggests tight regulation of substrate utilisation. Glycogen metabolism is an example of a metabolon, with discrete particles containing both substrate and enzymes for efficient synthesis and breakdown. Glycolysis (breakdown of glucose) may also be viewed as a 'metabolon', with the enzymes functionally grouped together, in particular near the sarcolemma and sarcoplasmic reticulum (SR). These concepts imply that entry of a substrate into the pathway ensures complete breakdown under normal conditions, and thus that overall regulation at any single point (or enzyme) is unlikely. In addition, in 'normal' conditions when substrate is in excess, the point of regulation may differ greatly from that in ischemia, when reduced substrate supply may be the main limiting factor. Regulation of glycolysis in low flow ishcemia, whether by substrate supply or enzyme inhibition, is particularly controversial.

Within these limitations, the following steps are thought to be the main sites of glycolytic regulation.

Hexokinase (HK) is stimulated by increased cytosolic glucose levels, and inhibited by increased G6P. As extracellular glucose levels increase, the intracellular G6P concentration reaches a plateau, which does not change with glucose concentrations greater than 2 mM. The estimated intracellular glucose concentration at this point is about 300 μM. HK is then saturated with glucose, and any further increase in glycolysis is prevented. If HK is inhibited, the glucose can pass out of the cell again; if the extracellular concentration is high, glucose can accumulate intracellularly, and affect cell osmolarity. If G6P accumulates, glycogen can be synthesised; alternatively, increased glycogen breakdown will increase G6P levels. G6P levels and HK activity are thus major determinants of glucose utilisation in muscle in the presence of physiological concentrations of glucose and insulin i.e. adequate supply of substrate [63, 67].

Phosphofructokinase (PFK-1) is inhibited by a high ATP content, an effect enhanced by citrate (from the TCA cycle – although the mitochondial carrier for citrate may not be very active in myoctes [68]) and reversed by AMP. The reaction catalysed by this enzyme is virtually irreversible because of ATP hydrolysis, which suggests that this is an important step in the control of glycolysis [69]. The rate of glucose breakdown is thus signalled by the need for ATP as determined by the ATP/AMP ratio. The reaction catalysed by adenylate kinase (ATP + AMP ↔ ADP) amplifies the signal. If ATP falls by 15%, ADP levels increase 2 fold, with a greater than 5 fold increase in AMP. Thus a relatively small decrease in ATP levels markedly stimulates gycolysis.

PFK-1 is also strongly inhibited by an increased $[H^+]_i$ [69], an effect which may prevent excessive lactate and H^+ accumulation. However, fructose 2,6-bisphosphate (F2,6-BP) may be the most potent stimulator of PFK-1, with a feed forward effect [70, 71]. F2,6-BP is formed from F6P by hydrolysis of ATP, catalysed by an enzyme called PFK-2 (to distinguish from PFK1) [71]. This reaction is stimulated by F6P. Dephosphorylation of F2,6-BP to F6P allows continued glycolysis from F6P (Fig. 3).

In ischemia, regulation of glycolysis is thought to occur mainly at GAPDH [59] (although this is open to question – see later). However, if cytosolic glycolytic substrate is in excess, e.g. with the addition of insulin, G6P and F6P levels rise substantially [72], suggesting that under these conditions, PFK-1 may be inhibited (by a low pHi), and thus this step becomes more important in overall control of the rate of glycolytic flux.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inhibited by an accumulation of NADH and lactate [73, 74], and was thought to be the major regulatory step of glycolysis in ischemia [59–61, 73, 74]. The product of the reaction, 1,3-bisphosphoglycerate, is also a potent inhibitor of the enzyme. Breakdown products of ATP (ADP, AMP and Pi) may stimulate the enzyme. GAPDH is especially implicated as the control step in ischemia [59]. However, in hypoxia when glycolysis is stimulated, NADH levels rise substantially, which contradicts the findings in ischemia. Inhibition of glycolysis at this step in ischemia by the end products is thus open to question (see below).

Pyruvate kinase (PK) may also be regulated, with inhibition by ATP, and stimulation by fructose 1,6-bisphosphate (F1,6-BP). However, the contribution of other glycolytic enzymes, and the major role of pyruvate dehydrogenase (see below), render this regulatory step less significant [63].

Alternate fates of metabolites of glycolysis

Glucose 6-phosphate is a main branch point of carbohydrate metabolism (see Fig. 3). G6P is the precursor of glycogen synthesis, as well as the entry point of glycogen breakdown into glycolysis. G6P can also be used to restore levels of NADPH and D-ribose 5-phosphate by the pentose shunt (see Fig. 3).

Glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) are interconverted by triose phosphate isomerase. GAP can be converted directly to glycerol, and then to glycerol 3-phosphate (also called α-glycerophosphate – αGP) but the more usual reaction is the conversion of DHAP to αGP, which can then be incorporated into triglycerides (Fig. 3). αGP accumulates in ischemia as an end product of glycolysis, and is also involved in regulation of the redox potential (see below).

Pyruvate is the major end-product of glycolysis. From this point a number of options are available, dependent on the energy status of the cell (see Fig. 4). Under normal conditions, pyruvate is converted to acetyl Co A by pyruvate dehydrogenase (PDH), the irreversible step to glucose oxidation. This enzyme is regulated closely by ratios of substrate and product (see Fig. 4), and its activity can be modified by many other factors, including importantly fatty acid oxidation. This step determines whether pyruvate from glycolysis will be converted to acetyl CoA or to lactate, and thus whether the energy from glucose will be efficiently extracted. Acetyl CoA may then enter the TCA cycle by combining with oxaloacetate to form citrate, or can elongate fatty acids. Pyruvate can also be exported from the cell, together with a H^+ [75].

Under anaerobic conditions, or with extreme exercise, the TCA cycle is inhibited by accumulated NADH from inhibition of oxidative phosphorylation. Pyruvate is then converted to lactate by lactate dehydrogenase (LDH), to allow regeneration of NAD^+ and continuation of glycolysis (Fig. 4). Lactate is a 'dead end', its only possible fate is re-conversion to pyruvate, or export from the cell (also with a H^+ [75]). When sufficient

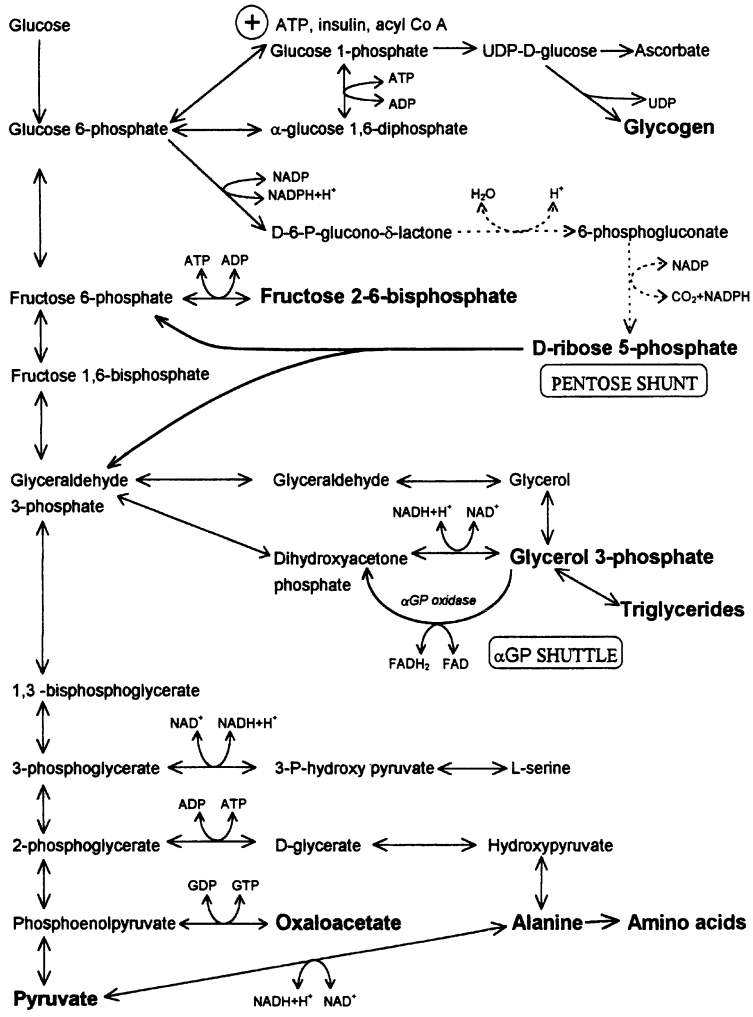

Fig. 3. Branch points from glycolysis. G6P is a junction for the formation and breakdown of glycogen, as well as the pentose shunt. In the pentose shunt, G6P is converted to D-6-P glucono-δ-lactone by glucose 6-phosphate dehydrogenase (G6PDH), which uses NADP. This compound is hydrolysed to 6-phosphogluconate which is converted to D-ribose 5-phosphate, with an additional NADP utilised. 2 molecules of NADPH are thus regenerated. D-ribose 5-phosphate can then be converted to F6P or GAP, re-entering the glycolytic pathway. The proportions which flow along the pathways are dependent on the requirement of the cell for D-ribose 5-phosphate, NADPH, or continued glycolysis. αGP shuttle – conversion of DHAP to αGP is catalysed by α-glycerophosphate dehydrogenase. αGP, readily permeable to the mitochondrial membrane, can subsequently be re-oxidised to DHAP by αGP oxidase, a transmembrane mitochondrial protein linked to FAD, with the consumption of a molecule of O_2 and production of H_2O. This system allows transport of NADH into the mitochondria against a high concentration gradient, although the cost is an ATP molecule, given that only 2 ATP are produced per $FADH_2$, rather than 3 per NADH. However, the level of αGP oxidase is low in muscle, and this mechanism may not be important. αGP is required in the formation of triglycerides, and levels increase significantly during ischemia. Other glycolytic metabolites contribute to various pathways as shown. These are not considered important in ischemia. For fates of pyruvate see Fig. 4.

oxygen is available, lactate can be oxidised to pyruvate and NADH will be regenerated. NADH can then be transported into the mitochondria, and utilised by oxidative phosphorylation (Fig. 5). If glycolysis continues in the absence of oxygen, H^+ accumulate from a reduced turnover of ATP [76] together with lactate. Most of the deleterious effects associated with enhanced glycolysis in ischemia are attributed to these products.

Pyruvate is also a major anaplerotic substrate (mechanisms which 'top up' the TCA cycle intermediates), supplying α-

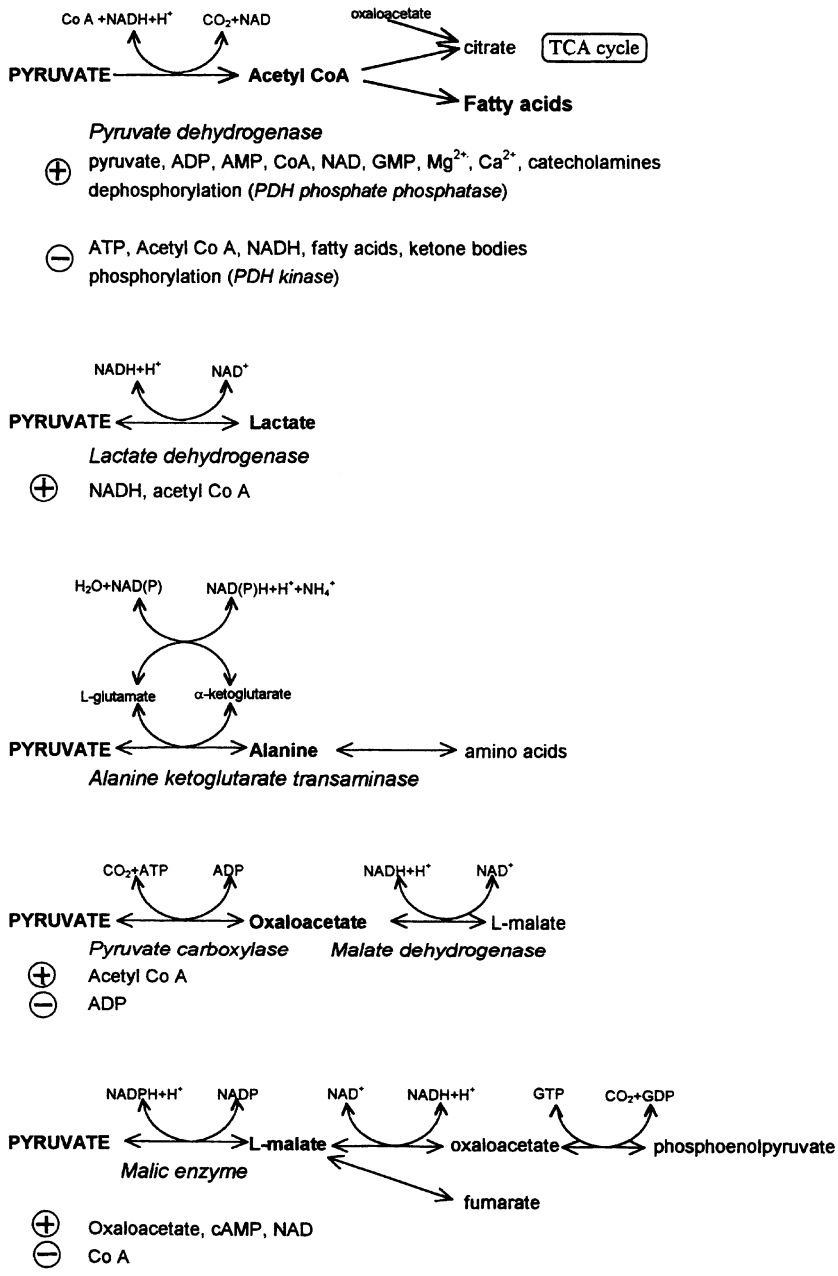

Fig. 4. Fates of pyruvate. Pyruvate is converted to acetyl Co A by pyruvate dehydrogenase (PDH) in the presence of sufficient oxygen, and reduced free fatty acid levels. PDH is a complex of three enzymes which require a number of cofactors. The enzyme complex is tightly regulated by the ratios of NADH/NAD^+, acetyl Co A/Co A, and ATP/AMP ratios (end product inhibition), as well as phosphorylation (inactivation – by PDH kinase) and dephosphorylation (activation – by PDH phosphate phosphatase). Under anaerobic conditions, pyruvate is converted to lactate to allow regeneration of NAD^+, and continuation of glycolysis. Pyruvate can also be converted to alanine, with the concomitant transamination of glutamate, and the formation of α-ketoglutarate. Pyruvate can also be converted to oxaloacetate by pyruvate carboxylase, and then to malate, or directly to malate by malate dehydrogenase. These are important anaplerotic mechanisms, and are involved in the malate-aspartate shuttle (Fig. 5).

ketoglutarate and oxaloacetate to the TCA cycle. Pyruvate is converted to oxaloacetate in the mitochondria by pyruvate carboxylase (Fig. 4). While activity of this enzyme may be low in heart muscle, some evidence for the activity of this pathway in heart tissue has been found [77].

Other anaplerotic pathways include conversion of phosphoenolpyruvate to oxaloacetate in the cytosol by pyruvate carboxylase, and the contribution of alanine (Fig. 4). Alanine is a major product of glycolysis, and accumulates during ischemia [78]. Alanine can also contribute to amino acid synthesis from glucose. The formation of alanine from pyruvate, with the concomitant transamination of glutamate to α-ketoglutarate, replenishes the latter, in an important anaplerotic mechanism.

Sequential pathways and alternate substrates

Tricarboxylic acid cycle and oxidative phosphorylation
The TCA cycle is the meeting point of substrate metabolism, whereby reducing equivalents for the subsequent generation of ATP by the respiratory chain are produced. Acetyl CoA is the entry point of most substrates into the TCA cycle, while NADH and $FADH_2$ are the end products, which enter oxidative phosphorylation for the formation of high energy phosphates. The TCA cycle is the most important generator of ATP of each substrate, such that after oxidative phosphorylation, an additional 36 ATP can be produced from glucose breakdown (as well as 2 ATP from glycolysis), and from a fatty acid such as palmitate (16 C), 129 ATP can be formed (although the exact number of ATPs produced from this process has been questioned, such that a maximum of 31 ATP from glucose and 104 ATP from palmitate can be produced [56]).

Regulation of NADH/NAD^+, glycolysis and the TCA cycle
Under conditions where acetyl Co A accumulates – when there is insufficient oxygen, or when NADH accumulates – NAD^+ must be regenerated, both to ensure continued glycolysis at the levels of PFK and GAPDH, and maintain turnover of the TCA cycle. The former is cytosolic, the latter mitochondrial.

Lactate dehydrogenase oxidises cytosolic NADH to NAD^+, converting pyruvate to lactate (Fig. 4). As mentioned above, this is a temporary mechanism to allow continued glycolysis.

αGP dehydrogenase also lowers cytosolic NADH levels with the reduction of DHAP to αGP (Fig. 3). However, the importance of this shuttle in the heart has been disputed [79], given the low activity of αGP dehydrogenase in heart muscle [80]. In addition, under normal conditions, the level of αGP is far below the Km for αGP oxidase. In ischemia, however, αGP levels increase significantly [59] and this shuttle mechanism may become important under these conditions [79, 81]. αGP forms the backbone of triacylglycerol synthesis, but the effects of an accumulation of this metabolite in ischemia have not been elucidated.

The malate–aspartate shuttle is perhaps the most important mechanism whereby the cytosolic and mitochondrial levels of NADH are regulated [79] (Fig. 5). There is an overall shift of malate into the mitochondria, and aspartate out of the organelle, although the shuttle is readily reversible. Its direction is determined by the cytosolic to mitochondrial NADH/NAD^+, such that a higher cytosolic ratio drives NADH transport into the mitochondria. This transfer does not utilise energy, but allows production of 3 ATP per NADH by oxidative phosphorylation (although this value is questioned [56]). If oxygen is limiting, NADH accumulates in the mitochondria, and then in the cytosol. This may affect redox-dependent reactions including glycolysis. The malate-aspartate shuttle can be reversed if NADH levels rise in the mitochondria, with the formation of malate. Malate is then transported out of the mitochondria, and converted to pyruvate by NADP-dependent malic enzyme, or to oxaloacetate by MDH (Figs 4 and 5).

Free fatty acids
It has long been known that free fatty acids are the major fuel of the normoxic heart, providing over 60% of the total ATP synthesised. As originally proposed, in terms of the 'glucose-fatty acid cycle' [82], an increased availability of lipid fuels such as in fasting, fat feeding or diabetes leads to decreased carbohydrate utilisation and impairment of insulin action. Possible mechanisms have been extensively documented involving inhibition of carbohydrate metabolism at at least 3 key control sites i.e. glucose entry, hexokinase/PFK and in particular PDH [61, 83, 84].

Although key regulatory sites have not been clearly pinpointed, there is considerable evidence that the interplay between fatty acids and carbohydrates is not entirely one-way i.e. under appropriate conditions, carbohydrate fuels can decrease fatty acid availability [82]. There is now considerable evidence that the carnitine palmitoyltransferase system required to transport activated fatty acids into the mitochondria is a major regulatory site of fuel utilisation in the heart [85–87], with inhibition by malonyl CoA.

Triglycerides are formed from acyl CoA and αGP, and can be synthesised during ischemia (Fig. 3). For each mol produced, 3 mol H^+ are formed, which increases the proton load in ischemia [88]. Endogenous lipids are not normally used as an energy source, given sufficient external substrate, but may be used e.g. in a perfused heart with limited external substrate [18].

Fatty acids have a higher requirement for oxygen than other substrates, but also yield a higher ATP per molecule.

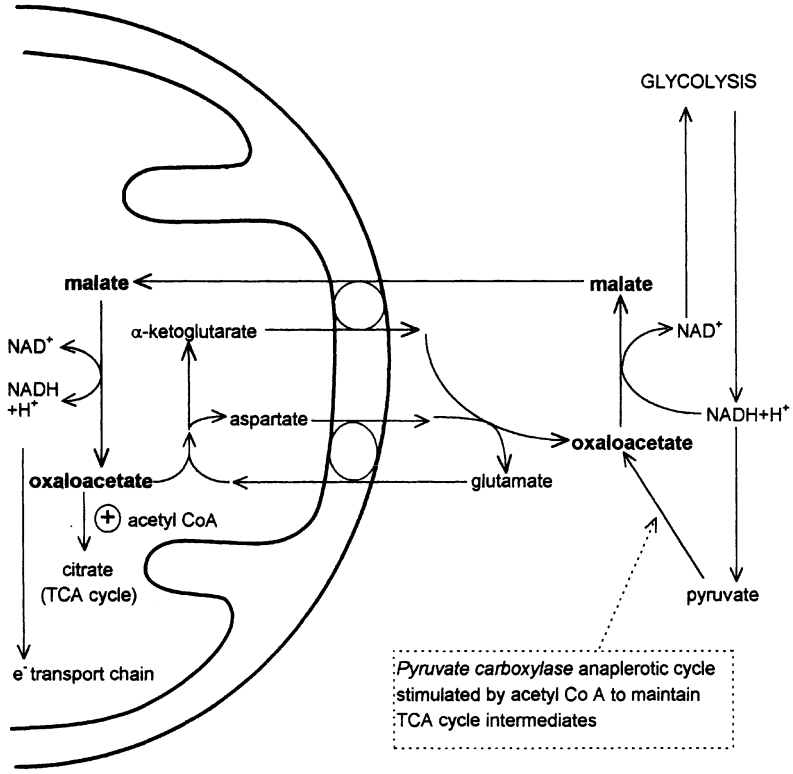

Fig. 5. The malate-aspartate shuttle. Cytosolic accumulation of NADH (e.g. with increased glycolysis) shifts the activity of malate dehydrogenase (MDH) in the direction of malate formation from oxaloacetate. Malate crosses the mitochondrial membrane in exchange for α-ketoglutarate, and is reconverted to oxaloacetate by mitochondrial MDH. NADH is thus regenerated in the mitochondria and can enter the respiratory chain. In addition, oxaloacetate replenishes the TCA cycle, combining with acetyl Co A to form citrate. Oxaloacetate can also combine with glutamate to form α-ketoglutarate and aspartate. α-ketoglutarate drives the entry of malate, as aspartate exits the cell in exchange for glutamate. In the cytosol, the α-ketoglutarate and aspartate recombine to form oxaloacetate and glutamate. There is thus an overall shift of malate into the mitochondria, and aspartate out of the organelle when cytosolic NADH increases. The whole shuttle can reverse, such that malate is transported out of the mitochondria, converted to oxaloacetate, and then to pyruvate. Pyruvate can also contribute to oxaloacetate formation via pyruvate carboxylase, or to malate by malic enzyme. These mechanisms, and the malate-aspartate shuttle, result in the anaplerotic contributions of pyruvate.

Fatty acids are therefore useful when oxygenation is high, but can be 'oxygen-wasting'. In conditions of ischemia, fatty acids and their metabolites exert a toxic effect, as well as consuming available oxygen rapidly. The breakdown products of fatty acids (acyl CoA, acyl carnitine, and lysophosphoglycerides) may be involved in many of the deleterious effects in ischemia, particularly arrhythmogenesis [18]. Fatty acids inhibit glucose oxidation; in turn, glucose provision attenuates many of the deleterious effects associated with fatty acids in ischemia. Thus the relative concentrations of fatty acids and glucose are of major importance in determining the tolerance of a heart to ischemia. However, this topic is largely beyond the scope of this review (see [27, 89–91]).

Myocardial ischemia

Glucose metabolism in ischemia

Hypoxia with maintained coronary flow stimulates glucose utilisation by reversal of the Pasteur effect, with a 20 fold increase in glycolytic flux in dogs *in vivo* [92], and a 3 fold increase in isolated rat hearts perfused with 11 mM glucose [93]. Glycolytic inhibition at the level of PFK-1 by citrate and ATP is removed [94], and glucose uptake is stimulated by increased GLUT 4 translocation [33]. If hypoxia is severe, heart function declines slowly, from insufficient ATP. If glycolysis is inhibited, developed tension dissipates more rapidly and completely [95]. Contracture may develop following ATP depletion [96, 97], an effect

counteracted by glucose provision.

In total ischemia (complete global cessation of flow and absence of oxygen), glycolysis is the sole source of ATP and may be stimulated initially following a reduction in oxygen. While ATP utilisation is reduced by mechanisms including membrane depolarisation and reduction in contractile function, the ATP demands of the myocardium rapidly exceed supply, such that net ATP decreases and Pi increases. Lactate accumulates with reduced washout, which may be deleterious. The metabolic changes occurring with ischemia thus include cessation of aerobic metabolism, onset of anaerobic glycolysis, depletion of creatine phosphate (CP), and accumulation of glycolytic products (lactate, αGP, adenine nucleotide breakdown) [98]. However, after a brief period, the glycolytic flux rate is reduced.

Differing concepts of rate-limiting steps in glycolysis in ischemia

Kübler and Spieckermann [92] found that when pO_2 becomes critical (less than 5 mmHg) in ischemic myocardium in dogs *in vivo*, glycolysis is initially stimulated by reversal of the Pasteur effect, as described above. A build-up of lactate then follows. The subsequent decline in glycolysis was attributed to the limiting effect of increased $[H^+]i$ and reduced ATP for the PFK-1 step, i.e. the conversion of F6P to F1,6-BP. The level of ATP in the tissue at this point (3.5 μmol/g wet wt) was said to be critical, with ischemic injury occurring at lower values.

This concept of glycolytic inhibition was revised by Neely and Rovetto [59, 93, 99, 100] who proposed that glycolytic flux in ischemia was inhibited at GAPDH by an accumulation of end products, specifically lactate, H^+, and NADH. This concept was based on work using an isolated working rat heart. A 60% reduction in coronary flow (from 15 ml/min to 6 ml/min) reduced oxygen consumption and accelerated glucose utilisation by 100%. Using a low flow rate of 0.6 ml/min, glucose utilisation was less than in control conditions (about 50%). After 16 min of low flow ischemia, the hearts were clamped and levels of metabolites assessed. Lactate values were high, suggesting that NADH was increased, because of the equilibrium of the LDH reaction. Increased NADH would, in turn, inhibit GAPDH, which is regulated by the $NADH/NAD^+$ ratio [74]. GAPDH inhibition was shown by an increase in DHAP levels [59] and application of the crossover theorem [101]. To date, it has been widely accepted that glycolytic enzymes are inhibited in ischemia by a build-up of glycolytic metabolites, and glycolytic flux is thus inhibited. However, further analysis of glucose uptake in severe low flow ischemia indicates that this interpretation, is not strictly correct [7, 102]; rather, glycolysis may be limited primarily by substrate supply [103] which in turn is determined by the arterial glucose concentration and the uptake ability of the membrane (i.e. insulin status, GLUT 4 availability etc.). Some modulation by enzyme inhibition is not excluded.

Extraction of glucose (calculated as absolute glucose uptake expressed as a percentage of glucose delivered (= glucose concentration * coronary flow)) from that which is available to the cell increases to about 30% as coronary flow rates fall below about 1 ml/g wet wt/min (in an isolated perfused rat heart) [102], compared to an extraction of about 1% in normal conditions. Absolute uptake does fall significantly in these conditions. *In vivo* experiments show similar findings, with a maintained glucose uptake as flow falls (normal *in vivo* coronary flows 1–2 ml/g wet wt/min; ischemia 0.07–0.15 ml/g wet wt/min) and thus an increased extraction [104]. We did not find evidence of GAP or DHAP accumulation after 15 or 30 min zero flow or low flow (0.2 ml/g wet wt/min) ischemia [72]. These findings indicate firstly, that glycolysis is not necessarily inhibited in ischemia at the level of GAPDH (although there may be some inhibition at PFK-1 as the sugar phosphates, G6P and F6P, accumulate especially with increased cytosolic substrate in the presence of insulin [72, 105] – some inhibition of GAPDH is not excluded, but its role as the rate-limiting step is questioned) but is limited by availability of substrate; secondly, that the ischemic tissue has the capacity to upregulate its ability to take up glucose, either by translocation of glucose transporters to the membrane [34], or by reorientation of the transporters within the sarcolemma [35]; and thirdly, this concept explains observations of 'mismatch' with Positron Emission Tomography (PET), using ^{18}fluorodeoxyglucose i.e. increased glucose uptake in 'hibernating' segments of the heart, which have a moderately impaired coronary flow [106–109]. Support for the concept that ischemia induces upregulation of glucose uptake comes from studies of preconditioning (short periods of ischemia and reperfusion) followed by sustained low flow ischemia, during which glucose uptake is markedly increased [110, 111].

The fate of glucose within the cell may vary with changes in coronary flow, which in turn alters oxygen availability [112]. However, in severe low flow, the availability of oxygen is greatly limited, such that the majority of ATP is derived from glycolysis [7, 11].

Role of glucose utilisation in ischemic myocardium

Specific role of glycolytic ATP

Despite a limitation on the rate of glycolytic flux in ischemia (a reduction in absolute glucose uptake), cell viability may largely be determined by the residual rate of glycolytic flux and the amount of ATP produced [113]. In conditions of metabolic stress – e.g. hypoxia, ischemia – glycolytic ATP appears to have a preferentially effective role as opposed to ATP derived from oxidative phosphorylation [114]. Much

indirect evidence suggests that ATP production within the cell is spatially compartmented [114–116], accounting for differences in the effectiveness of alternate sources of ATP. In ischemia, the processes for transport of ATP within the cell may break down. Thus provision of ATP near to the sites of utilisation becomes a crucial determinant of its effectiveness. A basal level of glycolytic activity is required to prevent irreversible injury [117], with control of cytosolic Ca^{2+} during ischemia [118] and on reperfusion [119], prevention of ischemic contracture [11, 120], inhibition of enzyme release [121], and inhibition of free radical activity [122]. Glycolytic ATP is thought to act in several ways at the membranes: blocking the ATP-dependent K^+ (K_{ATP}) channel [123], maintaining the activity of the Na^+/K^+ ATPase pumps in the sarcolemma [124], sustaining membrane integrity [114, 125], and maintaining Ca^{2+} homeostasis by the SR Ca^{2+} ATPase pumps [116]. These cellular effects modify functional responses to ischemia, with reduced diastolic tension during ischemia [2, 11, 120, 126], and improved functional recovery on reperfusion [2, 127–129].

The beneficial role of glycolytically-derived ATP therefore appears clear-cut. However, during the breakdown of glucose and glycogen, a number of metabolites accumulate which may contribute to some of the deleterious effects of ischemia. The benefit of glycogen availability in particular has been questioned in this regard, especially given the interest generated in the recently described phenomenon of preconditioning. A reduction in glycogen levels is postulated as one of the mechanisms whereby preconditioning may exert its beneficial effects [16].

Thus a crucial balance exists between rates of ATP production and rates of metabolite washout. This balance is determined by the rate of residual coronary flow and the glucose concentration, as well as the level of the endogenous glycolytic substrate, glycogen [2, 7, 11, 72, 127].

Membrane integrity

Glucose utilisation is closely involved in maintaining the integrity of the cell membrane. In isolated rat hearts made hypoxic [121, 130] or perfused with high concentrations of free fatty acids [131], enzyme release, a marker of cell damage, is attenuated by glucose provision. In normoxic isolated rat hearts, iodoacetate (glycolytic inhibitor) increases enzyme release, while cyanide (mitochondrial inhibitor) has no effect [132]. In low flow ischemia, provision of glucose rather than pyruvate, reduces LDH release, despite similar ATP levels [114].

Glucose utilisation may maintain membrane integrity in several ways. Provision of glucose limits phospholipase C degradation of membranes of isolated myocytes [125], and increased phosphorylation with increased ATP availability may also enhance the stability of the phospholipid membrane [132]. Energy is also required for membrane repair. Glucose-derived ATP may maintain the phosphatidic acid cycle, thereby preventing lysophospholipid accumulation which leads to membrane breakdown [133]. The osmotic effects of glucose provision and reduced cell swelling [98], together with reduction of contracture by glycolytic ATP, will limit cell rupture from stretching.

Membrane pump and channel activity

Glycolytic ATP appears to play a major role in ion homeostasis. A large efflux of K^+ occurs in ischemia, which depolarises the membrane and renders the cell inexcitable. This local 'cardioplegic' effect is beneficial in that the ATP demand is reduced. However, the excess extracellular K^+ can precipitate arrhythmias [134, 135]. The ATP-dependent K^+ (K_{ATP}) channel, one of the major channels involved in the efflux of K^+ in ischemia [136, 137], is blocked by ATP. However, the experimental levels to which ATP must fall to allow channel opening in excised patches [136] are well below those in ischemic tissue with a noted K^+ efflux [136]. K^+ efflux through this channel despite relatively small changes in ATP levels can be explained, firstly, by the high density of channels present such that a small change in maximal conductance can result in a large change in action potential shortening and K^+ loss [138]; secondly, by a localised fall in ATP near the membrane allowing for individual channel opening; and thirdly, by a rise in adenosine, lactate, and ADP, which relieve the inhibition by ATP [136, 139, 140]. Glucose-derived ATP, as opposed to that from oxidative phosphorylation, preferentially maintains closure of the K_{ATP} channel, suggesting that this ATP is localised near the membrane [123, 141]. Sarcolemmal-associated glycolytic enzymes may specifically maintain the ratio of ATP/ADP in the vicinity of the K_{ATP} channel [138].

Maintenance of Na^+/K^+ ATPase function may be one of the main mechanisms of protection by glycolytic ATP, as Na^+/K^+ ATPase pump inhibition by ouabain abolishes protection of ischemic hearts by glucose provision [124, 142]. Impaired Na^+/K^+ ATPase function increases osmolarity and cell swelling, as well as precipitating intracellular Ca^{2+} overload from an increased $[Na^+]i$ [142]. Glycolytic enzymes are also functionally coupled to SR Ca^{2+} transport mechanisms [116], as are glycogenolytic enzymes [143]. A preferential role for glycolytic ATP in preserving Ca^{2+} homeostasis by maintained Ca^{2+} re-uptake is thus postulated. In addition, glycolysis is central in maintaining Ca^{2+} homeostasis on reperfusion, possibly by activation of the SR Ca^{2+} ATPase pump [119], the activity of which is affected in stunned hearts [144, 145].

Arrhythmias and free fatty acids

Ventricular arrhythmias generated during ischemia or on reperfusion are complex in origin. Changes in cell membrane integrity, ionic fluxes across the membrane, increases in cytosolic Ca^{2+}, and impairment of conduction, all potentiate

arrhythmogenesis [146]. Glucose provision can combat many of these effects. The vulnerability of the dog heart to arrhythmias is reduced when glucose is administered intravenously [147]. Glucose provision also protects against arrhythmias in a model of regional ischemia in the isolated perfused rat heart [3] while reperfusion arrhythmias are attenuated compared to hearts perfused with acetate or palmitate [114].

Provision of glucose may reduce the arrhythmogenicity of ischemic tissue in several ways, including maintenance of membrane integrity, and pump and channel activity, as discussed above. Glucose provision maintains action potential duration in the isolated perfused rat heart [148], presumably by blocking the K_{ATP} channel, thereby limiting K^+ loss [123]. Glycolytic ATP also reduces cAMP accumulation, inhibits the release of noradrenaline from nerve terminals [149] and lowers LDH release, effects associated with reduced arrhythmias on reperfusion [114]. In addition, maintained glycolysis reduces cytosolic Ca^{2+} accumulation on reperfusion [119], a precipitating factor in reperfusion arrhythmias [150, 151]. Preserved Na^+/K^+ ATPase function [124, 142] would attenuate the increase in $[Na^+]i$, which in turn would reduce $[Ca^{2+}]i$, the major causative agent in arrhythmias [152]. Glucose may also have a free radical scavenging effect [153], important especially on reperfusion in maintaining membrane integrity and reducing arrhythmias [154].

A recent review highlighted the benefit of glucose and insulin in lowering the levels of circulating free fatty acids [155]. Excess free fatty acids are toxic to ischemic hearts, and lead to increased arrhythmias [155], possibly by accumulation of intracellular acylcarnitine and acyl CoA, which may promote intracellular Ca^{2+} overload [155–157]. Lysophospholipids from the breakdown of lipids are arrhythmogenic, with a detergent effect on the membranes [158]. Lipid compounds may also inhibit Ca^{2+} re-uptake mechanisms and activate Ca^{2+} channels [155–157]. Glucose provision counters many of the deleterious effects including arrhythmias associated with free fatty acids in ischemia, by reducing excess circulating free fatty acids, as well as combating the deleterious effects of free fatty acids on Ca^{2+} overload in the cell [155].

Ischemic contracture

Provision of glucose during low flow ischemia reduces ischemic contracture [11]. While there is some controversy over whether or not ischemic contracture is an index of irreversible injury [159, 160], increased contracture is generally considered to reflect increased cell damage [41, 161], and may potentiate injury, further impairing functional recovery.

The ATP level was originally thought to be a major determinant of the time to onset of contracture [161]. In general, a depletion in ATP levels reduces the time to onset; increased ATP availability delays contracture. The onset of contracture has also been linked to cessation of glycolysis, as measured by a levelling-off in the drop in intracellular pH [162]. These studies were performed in hearts exposed to total global ischemia, where the glycolytic flux rate is determined solely by the level of endogenous glycogen. With low flow ischemia (0.5 ml/g wet wt/min), a minimum rate of glycolytic ATP production of at least 2 μmol/min/g wet wt is required to prevent contracture [11]. A graded response to increased glucose concentrations was seen [11]. Therefore the onset of contracture is not associated so much with cessation of glycolysis, as with a fall in glycolytic flux rate below a threshold. Similar or larger rates of ATP production from utilisation of glycogen or non-glycolytic substrates were not effective in delaying contracture [11, 120, 126]. Other mechanisms which increase glycolysis, including glycogen loading [161, 163], inosine (increases pyruvate-to-alanine conversion, and removes glycolytic inhibition by lactate) [164], adenosine [165] and reduced circulating free fatty acids [129] delay the onset of contracture.

The exact mechanism of glycolytic ATP protection against ischemic contracture is thus as yet unclear. Contracture is triggered by the formation of rigor complexes (actin-myosin), which in experimental conditions, bond only at very low ATP concentrations (<100 μM [166]). However, total tissue ATP levels at the onset of contracture are much higher (about 3 μmol/g wet wt). Individual cells can have very low total ATP levels without contracting as long as glycolysis (from glycogen utilisation) is maintained [167]. A drop in ATP to less than 150 μM in isolated myocytes will, however, precipitate contracture [168]. These findings can be resolved by the concept of localised depletion of ATP within the cell (cf. K_{ATP} channel opening), as well as heterogeneous falls in ATP in different myocytes, resulting in dispersed foci of contracture in the muscle [169].

Glycolytic ATP may be essential for direct relaxation of the actin-myosin complexes, reversing the formation of rigor complexes, the so-called 'plasticising' effect [170]. However, some difficulties are associated with this concept, namely that glycolytic i.e. glucose-derived ATP, appears to be functionally associated with the sarcolemma and SR rather than the myofibrils [123]. Only under conditions of high glycolytic flux rates, may sufficient glycolytic ATP diffuse to the myofibrils to increase the rate of relaxation and prevent rigor formation. However, this could theoretically occur only under conditions of relatively unimpaired function i.e. a fairly high residual flow rate, and a high glucose concentration, ensuring a large intracellular ATP production [2, 127]. Glycolytic ATP may therefore be more important in maintaining ion homeostasis (as described above) and attenuating intracellular Ca^{2+} accumulation, rather than ensuring direct relaxation of the cross-bridges by binding to myosin. In addition, glucose utilisation attenuates the

deleterious effects of long-chain acyl carnitine (LCAC) on contracture [171], possibly by counteracting LCAC-induced disruption of Ca^{2+} homeostasis [155, 171]. While Eberli *et al.* [172] propose that Ca^{2+} is not involved in ischemic diastolic dysfunction, in a low flow model with maintained developed pressure, these results do not translate directly to the ischemic heart with severely impaired or no flow, and no developed pressure.

There is also evidence to suggest that pre-ischemic glycogen levels (and thus the rate of glycogenolysis during ischemia) primarily determine the time to onset of contracture (glycogen depletion with acetate [7] or preconditioning [105, 173, 174] hasten contracture; glycogen loading [7] delays contracture), while glucose-derived ATP attenuates overall contracture. The effects of the different glycolytic substrates on contracture may be attributed to compartmentation within the cell.

Mechanical function on reperfusion

Recovery of mechanical function is determined by the degree of ischemic injury, and the conditions on reperfusion [175]. At each point, substrate provision can be altered, affecting eventual recovery. While sufficient levels of ATP are essential for complete restoration of function, there is no direct correlation i e. no 'critical' level of ATP. Increased glucose provision during low flow ischemia increases the functional recovery of hearts [2, 114, 127, 129], mainly by reducing diastolic pressure on reperfusion, a consequence of reduced contracture during ischemia and improved relaxation on reperfusion [170]. Optimal recovery on reperfusion requires the presence of several compounds, one of which should be glucose [8, 176–178]. Glycolysis in early reperfusion appears to be essential in preventing energetic and contractile collapse, and specifically to facilitate Ca^{2+} homeostasis [119, 128]. Excess free radicals, one of the possible causes of stunning and arrhythmias on reperfusion, inhibit glycolysis, and thus further impair Ca^{2+} homeostasis [179]. Inhibition of glycolysis, despite the presence of pyruvate and oxygen, depresses functional recovery severely, with a persistent Ca^{2+} overload [119, 128].

Glycolytic ATP may be required to reduce $[Na^+]i$ by maintaining the activity of the Na^+/K^+ ATPase, thereby resulting in reduced Ca^{2+} overload via the Na^+/Ca^{2+} exchange which predisposes to reperfusion injury [128]. Na^+/K^+ ATPase inhibition during ischemia removes the protective effect of glucose on functional recovery [124, 142]. ATP derived from glycolysis is also functionally associated with the sarcoplasmic reticulum [116], thereby providing a means of restoring Ca^{2+} overload on reperfusion by enhanced SR Ca^{2+} ATPase function.

Palmitate is the preferred substrate on reperfusion, accounting for over 90% of ATP from exogenous substrates. High levels of fatty acids on reperfusion limit glucose utilisation by competitive inhibition [180], primarily by inhibition of glucose oxidation and not of glycolysis [181]. Inhibition of palmitate oxidation with increased glucose oxidation improves functional recovery [177], possibly because stimulation of glucose oxidation speeds the initial rate of return to total oxidative metabolism. A faster repletion of high energy phosphates is then sustained by fatty acid or pyruvate oxidation [182]. Secondly, a reduced intracellular acidosis from improved coupling of glucose oxidation with glycolysis may be beneficial by reducing intracellular Ca^{2+} overload [181, 182]. Increased rates of glycolysis and glucose oxidation with improved coupling of these two pathways, together with reduced fatty acid oxidation, may enhance recovery.

Preconditioning

Glycolytic flux is stimulated in preconditioned hearts subjected to low flow ischemia, and may exert its positive effects by this mechanism [110]. However, additional evidence to support this hypothesis is lacking, given that preconditioning does not seem to be effective when a sustained low residual flow [111, 183] or hypoxia [184] is used as the 'test', despite an increase in glucose uptake. This effect may be attributed either to an excess glycolytic flux in the presence of preconditioning (following increased translocation of GLUT 4 transporters to the membrane, resulting in stimulated uptake at the onset of the 'test' period), with excess metabolite accumulation; or it may be that preconditioning is ineffective when glycolysis is maintained. Preconditioning (with total global ischemia, the usual model investigated) is associated with a reduced tissue glycogen level prior to sustained ischemia. If, however, glycolysis is maintained during low flow ischemia, opening of the K_{ATP} channels [185] and release of adenosine [186], two of the major proposed triggers of preconditioning, may be attenuated. This phenomenon adds to the controversy over the roles of glycogen utilisation and glycolysis in ischemia.

Deleterious effects associated with increased glycolysis

Benefits associated with depleted glycogen levels

Much evidence suggests that ATP derived from glycolysis is beneficial to the ischemic myocardium. However, accumulation of deleterious end products may partly outweigh, or overcome, the benefit. Much controversy arose from a frequently-quoted paper published in 1984 by Neely and Grotyohann [14]. Following glycogen depletion by a brief period of anoxia (10 min) prior to sustained total global ischemia, a greatly improved recovery of function was found on reperfusion. The improved recovery was correlated with

a reduced lactate content at the end of ischemia. It was hypothesised that increased glycolysis is associated with a detrimental effect due to an increase in lactate and other end products. The concept then arose that provision of glucose should be detrimental.

Several other studies have linked beneficial effects to reduced glycogen levels. For example, 2 h perfusion with pyruvate depleted tissue glycogen by 40–50%, and resulted in a significant improvement in functional recovery after 25 min total global ischemia. This result was attributed to reduced $[H^+]i$ accumulation despite lower ATP levels during ischemia [187]. Glycogen-depleted hearts did show a reduced time to onset of contracture although peak contracture was similar to control glucose-perfused hearts. In addition, glycogen reduction by hypoxic perfusion, followed by 25 min total global ischemia, reduced lactate accumulation and improved functional recovery [188].

The possible benefits of glycogen depletion may also apply to preconditioning, as the brief episode(s) of ischemia deplete tissue glycogen prior to the sustained ischemic period. An increased duration of intervening perfusion was correlated with repletion of glycogen stores, but loss of recovery [189]. A more complex study found that recovery of function was improved in preconditioned and pyruvate glycogen-depleted hearts, but only if glucose was not provided to the latter. Glycogen depletion *per se* was not sufficient to explain the beneficial effects of preconditioning, but interventions which limited glycolysis and thus H^+ accumulation were beneficial [190]. We have, however, found no correlation between pre-ischemic glycogen levels (modified by acetate or glucose+insulin perfusion) and protection with preconditioning [105].

Proposed beneficial mechanisms of glycogen depletion

Protons

While intracellular pH always falls in ischemic hearts, the extent of the drop can be modified by changes in the amount of glycogen in the tissue at the onset of ischemia, such that glycogen-depleted hearts show reduced acidosis [191]. Increased $[H^+]i$ increases $[Na^+]i$, and in turn increases $[Ca^{2+}]i$ via the Na^+/H^+ and Na^+/Ca^{2+} exchange mechanisms [152]. Glycogen depletion is associated with reduced $[Na^+]i$ accumulation, linked to reduced H^+ accumulation [192].

Recovery of function is improved, with reduced cytosolic Ca^{2+} overload. However, while some reports have correlated intracellular pH with ischemic injury [193], others have dissociated these effects [191, 194]. The relationship between increased acidosis and increased ischemic injury is not clear.

Lactate

The cellular role of lactate in ischemic damage is unclear. Intracellular lactate accumulation is thought to be deleterious, both directly, and by inhibiting GAPDH following NADH accumulation [14, 73, 74], thereby inhibiting glycolysis. Increased extracellular lactate may be deleterious by inhibiting the $lactate^-/H^+$ co-transporter and increasing H^+ accumulation [195]. Thus a Na^+/H^+ exchange inhibitor on reperfusion reversed the deleterious effects of pre-ischemic lactate perfusion on functional recovery, presumably by reducing final Ca^{2+} overload [195]. However, Cross *et al.* found no change in intracellular pH with increased extracellular lactate [194], contrary to the previous findings [195]. In this model of low flow ischemia (0.5 ml/g wet wt/min) the effects of lactate/H^+ transport inhibition may not be as marked because of the maintained washout. The deleterious effects of lactate were attributed to the inhibition of glycolysis by NADH accumulation, which could account for the attenuated fall in pHi. Lactate may also activate K_{ATP} channels [196], thereby shortening the action potential and predisposing to arrhythmias. However, in a myocyte model of anoxia and reoxygenation, Geisbuhler *et al.* [197] reported no deleterious effect of high concentrations of lactate (10 and 50 mM) on anoxic myocytes. Damage was found, however, if pH was lowered. In addition, alterations in pre-ischemic glycogen do not result in a correlation between end-ischemic tissue lactate and functional recovery [198, 199].

Sugar phosphates

Glycolytic inhibition increases diastolic dysfunction in normally perfused hearts [200], attributed to increased sugar phosphates which may impair Ca^{2+} homeostasis. Phosphomonoester resonance, which measures phosphorylated compounds including G6P, F6P, α-GP and AMP, increases during ischemia and has been correlated with reduced recovery [201, 202]. However, glycolytic ATP is linked to Ca^{2+} homeostasis by functional coupling with SR Ca^{2+} ATPase reuptake [116]. A decreased cytosolic Ca^{2+} should thus reduce diastolic impairment. Therefore the relative rates of sugar phosphate accumulation vs. ATP production may be crucial in determining the efficacy of glucose provision. Increased G6P production would also lower ATP levels, as this step (from glucose) consumes an ATP. If glycolysis were inhibited at points just below this reaction, this step would be detrimental as no replacement ATP would be provided [3]. While much of the focus of the deleterious effects of increased glycolysis has been on lactate and protons, more evidence is now available to suggest that sugar phosphate accumulation may be an important factor [105, 190, 200, 201]. We found that with the addition of insulin to hearts perfused with 11 mM glucose, the levels of G6P and F6P rose considerably, and recovery of function was not improved [105, 111]. These factors may account for any detrimental effects of a high glucose concentration, and for some of the controversy surrounding the roles of glucose and glycogen utilisation in ischemia.

Evidence contradicting benefit of glycogen depletion

Taegtmeyer *et al.* [198, 199] have tried to replicate the findings of Neely and Grotyohann [14], with little success. A period of anoxia or substrate-free perfusion prior to 30 min total global ischemia reduced tissue glycogen, but did not improve recovery of function on reperfusion; rather, function was significantly impaired [198]. Interestingly, the anoxic hearts did not show a reduction in tissue lactate despite a 60% decrease in glycogen levels. This lactate could be accounted for by the residual glucose remaining in the tissue following cessation of flow. In substrate-free hearts with no residual glucose, lactate levels decreased substantially. A later publication by the same group [199] found similar results with a shorter period of ischemia (15 min). There was no correlation of lactate build-up with functional recovery, but a depletion of glycogen was detrimental. Postischemic glycogen availability and functional recovery were well correlated. We have also found no reduction in tissue lactate in preconditioned hearts with depleted glycogen, attributed to utilisation of the residual glucose at the onset of ischemia [105].

In addition, several reports show that glycogen loading can benefit hearts exposed to ischemia. Preoperative administration of GIK increased tissue glycogen levels, which were associated with a lower incidence of postoperative hypotension, reduced arrhythmias and fewer complications [203]. Glycogen loading with insulin greatly improves the tolerance of isolated rabbit hearts to ischemia, while glycogen depletion by epinephrine infusion severely impairs ischemic tolerance [204]. Fasting, which increases glycogen content, also increases the resistance of hearts to an ischemic episode [52], with improved recovery of function, reduced membrane damage and reduced loss of adenine nucleotides. Increased glycogen levels following palmitate addition to the perfusate of isolated rabbit hearts significantly improved recovery of function [205] while glycogen loading and lactate perfusion mimicked the protective effects of preconditioning, improving tolerance to ischemia [206].

A recent paper purports to explain the controversy over glycogen utilisation in ischemia suggesting that while glycogen is being utilised, the ATP provided supplies necessary energy [207]. Reperfusion at this relatively early stage results in good recoveries. However, once the glycogen has been used up (in the absence of glucose, in an ischemic model of relatively 'high' coronary flow, 0.5 ml/min/g wet wt), the accumulation of intracellular metabolites outweighs the advantages conveyed by ATP, with impaired functional recovery. A Na^+/H^+ exchange inhibitor on reperfusion after extended ischemia improves functional recovery, suggesting that the $[H^+]i$ was high following glycogen depletion [207]. However, at a lower flow rate, when the rates of ATP demand and metabolite accumulation are very different, the findings might be different.

Glucose vs. glycogen utilisation in ischemia

Major concerns with studies purporting to show deleterious effects of glycolysis are (1) glycogen is confused with glucose as the source of glycolytic ATP and (2) most studies have been done using models of total global ischemia, where glycolytic flux is limited by substrate and may also be inhibited by metabolite build-up. Glucose is therefore not present throughout the ischemic period. If glycogen loading were detrimental, increased glucose utilisation would not necessarily be detrimental; alternatively, if glycogen depletion is beneficial, glucose should not necessarily be removed. In addition, many of the early studies using brief periods of anoxia to deplete glycogen, with subsequent beneficial effects, can now be said to mimic preconditioning [208–211] which may act by different pathways excluding glycolytic involvement. We have attempted to correlate changes induced by preconditioning with changes in glycolysis (both glucose and glycogen) and find no significant relationship [105, 111]. Studies implicating a deleterious effect of glucose must thus be considered carefully in terms of the experimental model, and the source of glycolytic substrate. Thus the residual flow rate and glucose concentration, as well as the functional compartmentation of ATP production within the cell, are major determinants of the response to glycolysis.

Role of residual coronary flow and glucose concentration

The rate of residual coronary flow, together with an optimal glucose concentration, are crucial determinants of the extent of ischemic injury, and the recovery of function [7]. Provision of glucose in rat models of low flow ischemia (0.5 ml/min/g wet wt or 0.06 ml/min/g wet wt) maintains diastolic function, and improves recovery of mechanical function on reperfusion [2, 127]. With a low flow of 0.4–0.5 ml/g wet wt/min, the rate of glycolysis during ischemia is directly related to the degree of ischemic injury i.e. contracture, and functional recovery [129].

At a low residual flow rate (0.2 ml/g wet wt/min), a high glucose concentration (22 mM) with or without insulin may be deleterious [7] with an excess accumulation of end products [7, 105]. A higher coronary flow removes many of the deleterious effects associated with a high glucose concentration [2, 7, 127]. However, in the absence of glucose, even if an alternate substrate such as acetate is present, function is impaired with a higher residual flow [7, 11]. In addition, it must be noted that at no flow rate is the absence of glucose better than the presence of glucose, despite accumulation of glycolytic end products. Thus an optimal glucose concentration (11 mM in the absence of insulin) [7] with the highest possible residual flow rate results in optimal recovery. If insulin is present, a lower concentration of glucose (about 5 mM) is optimal (unpublished data), in accordance with physiological values in blood.

Compartmentation of ATP

Cellular compartmentation

Many cell components are compartmented – e.g. intracellular Ca^{2+} is highly compartmented in the myocyte, distributed among the SR, the Ca^{2+}-binding proteins and the cytosol. The adenine nucleotides including ATP are also strictly compartmented, which becomes especially evident in ischemia [212]. An extensive review also recounted the evidence for the compartmentation of glycolysis, with 'microenvironments' within the cell [213]. A high degree of functional organisation of the glycolytic enzymes, particularly in muscle cells, would allow for efficient 'channelling' of the substrate through its catabolic pathway [65]. Evidence of glycolytic vs. oxidative ATP compartmentation has been found in different cell types e.g. liver [214], vascular smooth muscle [215], and myometrium [216]. Glucose and glycogen metabolism also form two functionally exclusive compartments in the cytoplasm of vascular smooth muscle [217, 218]. This arrangement maximises the efficiency and response time of energy transduction within the smooth muscle cell. In the myocyte, the distances are greater because the cell is much larger, and the demands on energy are also increased with the much greater contractile component of the cell. Thus compartmentation within the myocyte is plausible given the requirement for sustained efficiency of contraction.

Two pools of ATP have been identified in hepatocytes – one cytosolic, and one near the membrane [214], on the basis of ATP utilisation by membrane (Na^+/K^+ ATPase) and cytosolic (ATP-sulfurylase) enzymes. Differences in the mechanisms resulting in ATP depletion indicate that the different elements are not exposed to the same ATP level. Intracellular inhomogeneities of ATP can be attributed to different rates of ATP-producing and ATP-consuming sites, such that ATP produced in large amounts from the mitochondria supply the cytosolic elements. These elements reduce the amount of ATP at the membrane because of rapid utilisation of ATP diffusing from the mitochondria, such that at further distances the ATP is depleted. Thus mitochondrial ATP utilisation by the membrane components appears to be limited by diffusion and steepness of gradients within the cell. This may certainly apply to the large, metabolically active myocyte, although this has not been shown directly.

While compartmentation is difficult to prove (ATP is a ubiquitous compound), this concept explains many observations and has been invoked by several authors to explain observations in the heart [11, 114, 123, 141, 219]. Oxidative phosphorylation and glycogen breakdown (which supplies substrate for the oxidative pathway) may supply ATP largely for contraction, while glycolysis (glucose) may be required for sarcolemmal pump function and membrane integrity [141]. A localisation of energy-producing pathways near energy-consuming processes thereby ensures efficient supply and utilisation of energy. When ATP supply is plentiful, the different systems may overlap. However, if energy supply is limited, the source of energy becomes crucial. In ischemia, the different effects of glycogen-derived, glucose-derived and oxidative ATP on contracture and on functional recovery may be explained by these differences.

Location of enzymes within the cell – basis for compartmentation theory

Oxidative phosphorylation occurs in the mitochondria, which are localised near the myofibrils [220], the primary energy consumers in the myocyte. Inhibition of oxidative phosphorylation has a major depressant effect on tension [95, 123, 221], an effect slowed down by the presence of glucose. Conversely, inhibition of glycolysis with maintained oxidative phosphorylation has a much lesser effect on reduction in tension [123]. These findings and others [10] suggest that ATP from oxidative phosphorylation is used preferentially by the contractile apparatus.

The ATP produced in the mitochondria needs to be transported to the sites of utilisation, a process mediated by the creatine kinase shuttle [222, 223], which maintains a local ratio of ATP/ADP around the myofibrils [224–226], and sustains contraction. In ischemia, CP levels fall and mitochondrial activity is inhibited. ATP becomes trapped in the mitochondria [212], which in turn become major sites of ATP utilisation as the mitochondrial ATP synthase reverses, and becomes an ATPase [227] (this mechanism is not thought to be important in the rat [228]). ATP availability to the myofibrils is thus reduced.

Utilisation of glucose may be localised within the cell. In vascular smooth muscle, an unexpectedly high proportion of glucose is converted to lactate under fully oxygenated conditions. This may be due to a preferential utilisation of glycolytic ATP by the Na^+/K^+ ATPase membrane pump in these cells [115, 215]. Following Na^+/K^+ ATPase inhibition by ouabain, glycolysis (in terms of lactate production) is reduced, with no change in force development. When Na^+/K^+ ATPase activity is stimulated, lactate production is increased [215]. While this mechanism requires a feedback regulation of ATP utilisation on glycolytic flux which is difficult to understand, the evidence for the association of glycolytic enzymes with the membrane is fairly convincing.

The enzymes and substrates of glycolysis were initially thought to be freely dispersed in the aqueous medium of the cytoplasm [213]. However, subsequent studies have found that the glycolytic enzymes are at specific locations within the cell [213]. The glycolytic enzymes, GAPDH and PK, bind to sarcolemma and SR membranes, possibly to charged phospholipids which are more prevalent in the former

membranes and thus show greater binding affinity for the enzymes [229]. Further evidence for physical compartmentation comes from excised inside-out patches of sarcolemmal membrane (with no cytosol present). Addition of substrates for the ATP-producing steps of glycolysis (phophoglycerokinase and pyruvate kinase) block the K_{ATP} channel [123], which can only occur by a localised production of ATP. This finding substantiates the concept that key glycolytic enzymes are located in or near the sarcolemma. In addition, if glycolysis is inhibited in myocytes with maintained oxidative phosphorylation, a large efflux of K^+ is found, with little change in tension [123]. However, if oxidative phosphorylation is inhibited, tension is depressed, but K^+ efflux is much lower. The enzymes of the glycolytic cascade also appear to be associated with the plasma membrane in smooth muscle, allowing isolated vesicles to produce ATP and maintain Ca^{2+} pump function [230], an effect which can be blocked by iodoacetate. Glycolytic enzymes are also functionally coupled to the SR, maintaining Ca^{2+} homeostasis [116], which in turn greatly modifies contraction.

While glycolytic ATP therefore appears to be closely involved with regulating K^+ efflux and Ca^{2+} homeostasis, glycolytic ATP is not necessarily distributed to the contractile apparatus. The mechanisms of ATP transport may be limited in conditions of metabolic impairment such as hypoxia and ischemia. Glycolytic enzymes are not found near myofibrils in any great quantity [213], suggesting that glycolytic ATP is not synthesised in these regions, and thus is of less importance than oxidatively derived ATP for the contractile apparatus.

Glycogen particles are located along the mitochondrial columns between myofibrils, and are also present in the perinuclear sarcoplasm and the SR including the sub-sarcolemmal cisterns [220]. The enzymes for glycogen breakdown (phosphorylases etc.) are also found near the SR in cardiac muscle [143], and form 'metabolons' for the efficient metabolism of glycogen [62].

The substrate supply from glycogen utilisation is coordinated with mitochondrial substrate utilisation whereas glucose utilisation correlates with Na^+/K^+ ATPase activity in smooth muscle cells [215]. Glycogenolysis may be linked to the contractile and cytosolic elements in vascular smooth muscles, rather than membrane functions [230]. This may explain the role of glycogen utilisation as a major determinant of the onset of contracture [7, 105, 161].

Conclusions

The ischemic myocardium can upregulate its ability to extract glucose from that made available to the tissue, which is contrary to previous conceptions that glycolysis is inhibited in ischemia. Thus provision is made to enhance the numerous protective effects of glucose in the ischemic cell. However, excess glycolysis can be detrimental. A balance between substrate supply and ATP production, and metabolite accumulation, must be maintained. In terms of clinical applications, despite the original report showing that administration of GIK had no benefit [12], many subsequent studies argue for a re-investigation of this therapy [231–234], with a great potential for protection in patients with myocardial infarction.

Glucose seems to exert a beneficial effect mainly at the membranes, regulating intracellular ion homeostasis, while glycogen-derived ATP is present mainly at the myofibrils, and possibly at the SR, thereby attenuating ischemic contracture. The functional distinction between these two glycolytic substrates and the compartmentation within the cell clarifies many controversial issues regarding the benefit or detriment associated with increased glycolysis during ischemia.

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Differential regulation in the heart of mitochondrial carnitine palmitoyltransferase-I muscle and liver isoforms

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Abstract

Carnitine palmitoyltransferase-I (CPT-I) plays a crucial role in regulating cardiac fatty acid oxidation which provides the primary source of energy for cardiac muscle contraction. CPT-I catalyzes the transfer of long chain fatty acids into mitochondria and is recognized as the primary rate controlling step in fatty acid oxidation. Molecular cloning techniques have demonstrated that two CPT-I isoforms exist as genes encoding the 'muscle' and 'liver' enzymes. Regulation of fatty acid oxidation rates depends on both short-term regulation of enzyme activity and long-term regulation of enzyme synthesis. Most early investigations into metabolic control of fatty acid oxidation at the CPT-I step concentrated on the hepatic enzyme which can be inhibited by malonyl-CoA and can undergo dramatic amplification or reduction of its sensitivity to inhibition by malonyl-CoA. The muscle CPT-I is inherently more sensitive to malonyl-CoA inhibition but has not been found to undergo any alteration of its sensitivity. Short-term control of activity of muscle CPT-I is apparently regulated by malonyl-CoA concentration in response to fuel supply (glucose, lactate, pyruvate and ketone bodies). The liver isoform is the only CPT-I enzyme present in the mitochondria of liver, kidney, brain and most other tissues while muscle CPT-I is the sole isoform expressed in skeletal muscle as well as white and brown adipocytes. The heart is unique in that it contains both muscle and liver isoforms. Liver CPT-I is highly expressed in the fetal heart, but at birth its activity begins to decline whereas the muscle isoform, which is very low at birth, becomes the predominant enzyme during postnatal development. In this paper, the differential regulation of the two CPT-I isoforms at the protein and the gene level will be discussed. (Mol Cell Biochem **180**: 27–32, 1998)

Key words: CPT isoforms, fatty acid oxidation, mitochondrial metabolism

Introduction

Mitochondrial fatty acid oxidation has long been known to be required for production of energy needed for the contraction of cardiac muscle, and extensive research has shown that the oxidation of fatty acids is the preferred energy producing pathway [1]. Evidence has been presented more recently that indicates the importance of the mitochondrial enzyme carnitine palmitoyltransferase-I (CPT-I) as well as its physiological inhibitor malonyl-CoA, an intermediate in the fatty acid biosynthetic pathway, in regulating rates of myocardial fatty acid oxidation (for review, see [1]). Although the heart is not a tissue in which there is abundant fatty acid synthesis, acetyl-CoA carboxylase is known to function as a producer of malonyl-CoA in the heart, possibly for the primary purpose of regulating CPT-I and thus fatty acid oxidation.

The carnitine palmitoyltransferase enzyme system (Fig. 1) consists of two enzymes, CPT-I and CPT-II, that are required for transport of long chain fatty acids into the mitochondrial matrix for β-oxidation [2]. Following activation of fatty acids by acyl-CoA synthetase on the outside of the mitochondrial outer membrane to produce acyl-CoA thioesters, CPT-I, also located in the outer membrane, catalyzes the transfer of acyl groups from coenzyme A to carnitine to produce acyl-carnitine. Cytoplasmic acylcarnitine is exchanged for free carnitine in the mitochondrial matrix by a specific mitochondrial antiport protein called carnitine-acylcarnitine translocase. CPT-II, located inside the mitochondrial matrix, then regenerates acyl-CoA for β-oxidation by catalyzing a reaction that is the reverse of the CPT-I reaction.

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The orientation of CPT-I in the mitochondrial outer membrane as shown in Fig. 1 is based on experiments with liver and heart mitochondria in which proteases have been used to hydrolyze mitochondrial proteins partially, resulting in an active CPT-I that is no longer sensitive to inhibition by malonyl-CoA [3]. These experiments suggest that the active site of the enzyme is located within the protective outer membrane barrier, but that a portion of the enzyme to which malonyl-CoA binds projects into the cytosol. Comparison of the amino acid sequences of CPT-I [4] and CPT-II [5] indicates that CPT-I possesses an additional sequence on the N-terminal end that is not found in CPT-II. The deduced protein sequence of CPT-I also suggests that amino acids 103–122 form a membrane-spanning domain [4]. Partial proteolysis of hepatic CPT-I in intact mitochondria has indicated that most drugs that inhibit CPT-I bind to the protein domain located between the mitochondrial outer and inner membranes, but malonyl-CoA binds to the protein domain that extends into the cytosol outside the mitochondrial membrane [6–8]. Proteolysis of CPT-I can be prevented by preincubating mitochondria with malonyl-CoA [9]. Proteolytic removal of the cytoplasmic domain of the hepatic CPT-I can completely eliminate malonyl-CoA binding to the enzyme as well as removing inhibition, but proteolysis does not appear to be the mechanism by which malonyl-CoA sensitivity is altered in insulin-dependent diabetes [10]. The malonyl-CoA binding site on the cytosolic domain of CPT-I is highly specific for the two carbonyl groups of malonyl-CoA; acetyl-CoA inhibits the enzyme, but does so by binding at the active site not the malonyl-CoA site [7–8].

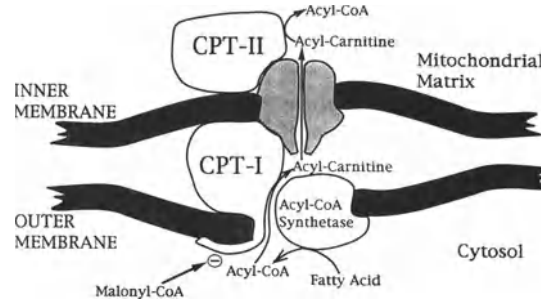

Fig. 1. Fatty acid oxidation pathway in mitochondria. This figure illustrates the pathway of long-chain fatty acid oxidation in mitochondria including locations of the enzymes involved. The dark-shaded unlabeled protein in this figure is carnitine–acylcarnitine translocase. Orientation and membrane location of the enzymes is based partly on studies of partial proteolytic digestion of intact mitochondria [3, 6–8].

Metabolic control at the CPT-I step in the fatty acid oxidation pathway

Studies of the hepatic fatty acid oxidation pathway

The earliest studies of the role of CPT-I in regulating fatty acid oxidation concentrated on rat liver where dramatic changes in rates of fatty acid oxidation occur during the feeding–fasting cycle. A major factor in the control of hepatic mitochondrial fatty acid oxidation is the inhibition of CPT-I by malonyl-CoA [2], an important intermediate in the fatty acid synthetic pathway. Shortly after it was discovered that malonyl-CoA was an inhibitor of hepatic CPT-I, we discovered that the fatty acid oxidation pathway in mitochondria from fed rats was extremely sensitive to inhibition by malonyl-CoA in contrast to fasting rats where inhibitory sensitivity was greatly diminished [11]. This regulatory mechanism has become well established by several laboratories that have demonstrated dramatic alterations in hepatic CPT-I during the feeding–fasting cycle [12–14]. These differences in inhibition of CPT-I by malonyl-CoA are caused by changes of 10 fold or more in K_i values of hepatic CPT-I for malonyl-CoA that occur with no change in K_m for the substrates [15]. The ability of malonyl-CoA to inhibit CPT-I is practically eliminated in the ketotic diabetic state which results in a change in K_i for malonyl-CoA that can be reversed by insulin [16]. Diabetes also modestly increases the activity of the hepatic CPT-I, and insulin administered to streptozotocin-diabetic rats or BB-Wistar diabetic rats reverses these effects [16]. Insulin therefore acts in a coordinated manner to inhibit release of fatty acids from the adipose tissue and to inhibit oxidation of fatty acids in the liver. Extensive research into the mechanism by which insulin changes the sensitivity of CPT-I has failed to produce a precise mechanism, but it has been found that the inhibitory sensitivity of the hepatic CPT-I can be greatly altered by membrane phospholipids, possibly by changing membrane fluidity [17–19]. The phospholipid cardiolipin is the most effective at decreasing malonyl-CoA sensitivity and also produces increased CPT-I activity [17]. This finding may have important implications for the fatty acid oxidation pathway since CPT-II, acyl-CoA synthetase and carnitine-acylcarnitine translocase are also activated by cardiolipin. The rat CPT-II has been produced in bacterial and yeast expression systems free of other CPT enzymes [20–22]. These CPT-II enzymes are not inhibited by malonyl-CoA [20–21] and cannot be made inhibitable using cardiolipin [20]; however, the bacterially expressed enzyme is activated by cardiolipin [20], and by detergents [22].

Regulation of fatty acid oxidation in the heart

While CPT-I in the liver has been widely recognized to regulate fatty acid oxidation in response to a variety of dietary and hormonal influences by altering its sensitivity to inhibition by malonyl-CoA, CPT-I in the adult heart has not been extensively studied. Before it was known that a different

isoform of CPT-I was expressed in the adult heart, we proposed that CPT-I in the adult cardiac muscle was controlled by a different mechanism than the hepatic enzyme because we found that the cardiac CPT-I did not alter its sensitivity to inhibition by malonyl-CoA during fasting [23]. We also discovered that CPT-I of the adult heart did not demonstrate a malonyl-CoA-induced shift to sigmoid kinetics as the liver CPT-I did and that cardiac CPT-I was characterized by partial inhibition unless mitochondrial outer membranes were purified [24]. The partial inhibition seen with most heart mitochondrial preparations can be distinguished from the physiologically reduced sensitivity seen in the liver by using kinetic analyses [23]. These differing kinetic characteristics in various preparations of heart mitochondria have complicated the study of CPT-I in the heart and led to erroneous conclusions that the heart CPT-I was identical to that expressed in liver. It has also been found that the adult cardiac CPT-I was much more sensitive to malonyl-CoA inhibition than the liver enzyme and had somewhat different acyl-CoA specificity [24]. Regulation of fatty acid oxidation in the adult heart apparently responds to large changes in malonyl-CoA concentrations produced by changes in fuel supply (glucose, fatty acids, lactate/pyruvate and ketone bodies) [1]. It is not yet known whether this may also be true of the heart during the perinatal period.

Identification of two tissue-specific isoforms of CPT-I

Cardiac muscle expresses two isoforms of CPT-I

Based on studies with inhibitors of the CPT-I enzyme and by cDNA cloning, two isoforms of CPT-I have been identified [4, 25–27]. The muscle CPT-I isoform (M-CPT-I) was recently discovered during differential screening of cDNA libraries from brown and white adipose tissue [25]. Northern analyses of RNA from tissues and isolated cells have led to the discovery that this new isoform was found in skeletal and cardiac muscle [25, 27] as well as both white and brown adipocytes [28]. The non-adipocytes of brown adipose tissue contain an abundance of the liver isoform [28]. The cDNAs for L-CPT-I and CPT-II have been cloned from rat and human libraries [4, 29], and the cDNAs for rat and human M-CPT-I have been cloned [25–27]. There is a difference of only one amino acid in the size of the liver and muscle CPT-I isoforms, but there are numerous differences in the amino acid sequence. Figure 2 illustrates that these isoforms are not the product of alternate splicing of mRNA but are derived from different genes. The first 18 N-terminal amino acids are identical in the two isoforms, but their nucleotide sequences demonstrate the use of alternate codons in both rat and human species. Similar, though less striking, results are found throughout the protein coding sequences. The greatest differences appear to occur near the membrane spanning region of each molecule. The 5′ and 3′ flanking sequences of the two cDNAs are different in size and sequence homology. The liver isoform (L-CPT-I) is expressed in the liver and heart, but not in skeletal muscle [27, 30]. Both liver and muscle isoforms exist in cardiac muscle cells since both L-CPT-I and M-CPT-I proteins [30] and mRNAs [31] are found in isolated cardiac myocytes. During the perinatal developmental period, a switching of isoforms occurs in the heart as L-CPT-I decreases very slowly from birth until weaning and decreases more rapidly after weaning on a high carbohydrate diet [32]. Conversely, M-CPT-I increases from birth to weaning and remains highly expressed thereafter [32]. There is considerable evidence for the presence of additional CPT

rL-CPT-I

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MetAlaGluAlaHisGlnAlaValAlaPheGlnPheThrValThe ProAspGlyIleAspLeuArgLeuSer-
ATGGCAGAGGCTCACCAAGCTGTGGCCTTCCAGTTCACCGTCACCCCCGATGGCATTGACCTCCGCCTGAGC
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hL-CPT-I

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 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
--------A-------------------T--------G-----T--G--C--G--------G--G------
```

rM-CPT-I

```
 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  * Val * Phe *  *  *
-----G--A--A-----G--A--A--T-----------T--G-----A--C--GG-C---T----G--T--T
```

hM-CPT-I

```
 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  * Val * Phe *  *  *
-----G--A--------G--C----------------G--G-----A--C--GG-C---T----G--C--T
```

Fig. 2. N-Terminal sequences of currently known CPT-I proteins and cDNAs. Deduced amino acid sequences of the N-terminal regions of rat and human CPT-Is and their respective nucleotide sequences are compared. A dash (–) indicates a nucleotide identical with rL-CPT-I, and an asterisk (*) indicates an amino acid identical with rL-CPT-I. Sequences for rL-CPT-I are from [4], for hL-CPT-I are from [29], for rM-CPT-I are from [25] and for hM-CPT-I are from [27].

isoforms in liver peroxisomes and microsomes based on cDNA cloning, protein purification and antibody studies [23, 33–34], but the presence of these isoforms has not been demonstrated in the heart.

Regulation of the L-CPT-I and M-CPT-I genes

Developmental regulation of the L-CPT-I gene in the liver
L-CPT-I mRNA and protein levels are very low prior to birth, but L-CPT-I mRNA and enzyme activity in the liver increase 5 fold in the first day of life [35]. The activity of L-CPT-I remains high throughout the suckling period [35]. The enzyme activity and mRNA abundance continue to be elevated if the rats are weaned to a high fat diet, but weaning to a high carbohydrate diet markedly suppresses L-CPT-I mRNA levels [35]. The CPT-II gene is expressed in the liver early in fetal life and remains constant throughout. Dietary manipulations do not affect the abundance of CPT-II [35]. These observations suggest that developmental expression of CPT-I is regulated by changes in the glucagon/insulin ratio that occur at birth and by the abundance of long-chain fatty acids. Using the nuclear run on assay, it was found that the rate of transcription of the L-CPT-I gene was elevated in the livers of rats on high fat diets and decreased in the livers of rats on high carbohydrate diets [36]. Insulin inhibits transcription of the L-CPT-I gene in isolated hepatocytes, and cAMP stimulates transcription [36]. In isolated hepatocytes the peroxisome proliferator, clofibrate, and fatty acids stimulate L-CPT-I gene expression. The transcriptional effects of clofibrate are mediated through the peroxisomal proliferator receptor (PPAR) [37]. Recently it has been shown that long chain fatty acids can also stimulate transcription via PPAR [37]. Insulin inhibits fatty acid induction of the L-CPT-I gene but does not affect the induction by clofibrate [36]. While long-chain fatty acids and clofibrate appear to utilize the same receptor, this differential response to insulin suggests they do not function in an identical manner [36].

Hormonal regulation of L-CPT-I expression in the liver
Only a few direct studies have been conducted on the regulation of L-CPT-I mRNA levels by hormones. In our studies, it was found that L-CPT-I activity, sensitivity to malonyl-CoA and mRNA levels were altered by thyroid hormone [38]. In hyperthyroid rats, there was a 5 fold increase in L-CPT-I mRNA abundance in the liver while L-CPT-I mRNA levels decreased by a factor of 5 in hypothyroidism [38]. L-CPT-I activity was increased in hyperthyroid rat liver while it was decreased in the hypothyroid animal [38]. T_3 has profound effects on development and metabolism. The actions of T_3 are mediated through nuclear T_3 receptors (TR) of which there are two isoforms – α and β [39]. The TRs are part of a family of related nuclear receptors encompassing the retinoic acid (RAR), vitamin D (VDR) and peroxisomal proliferator receptors (PPAR) [40]. The TR binds to a loosely conserved AGGTCA motif. The TR forms high affinity heterodimers with members of the retinoid X receptor (RXR) family [40]. RXRs appear to be expressed in all tissues and also form heterodimers with RAR, VDR and PPARs [40]. Non-steroid hormone receptors may have an important role in regulation of CPT-I transcription by modulating the transcriptional stimulation by long chain fatty acids, T_3 and peroxisomal proliferators. The role of these receptors in CPT expression will be addressed as the promoters of the L-CPT-I and M-CPT-I genes are analyzed.

We recently examined L-CPT-I enzyme activities and mRNA levels in livers of normal, fasting and insulin-dependent diabetic rats. L-CPT-I activity increased 2 fold during fasting and 3 fold with diabetes. The K_i of the enzyme for inhibition by malonyl-CoA was increased (increased K_i for malonyl-CoA = decreased sensitivity to inhibition) 20 fold by fasting and 50 fold in diabetes [41]. L-CPT-I mRNA abundance was increased 7 fold by fasting and 14 fold in the diabetic state [41]. To determine if insulin directly affected L-CPT-I mRNA levels, experiments were conducted in H4IIE rat hepatoma cells. H4IIE cells were treated with either dexamethasone or dexamethasone plus 100 nM insulin. Insulin depressed L-CPT-I mRNA abundance by a factor of 10 over 8 h in culture. Actinomycin D was added to H4IIE cells to assess the half-life of the CPT-I mRNA. Following the inhibition of transcription by the addition of actinomycin D, a half-life of 3.5 h was determined. The rate of L-CPT-I mRNA disappearance was the same in cells treated with actinomycin D or actinomycin D plus insulin, suggesting that insulin did not destabilize L-CPT-I mRNA [41].

Regulation of the expression of L-CPT-I gene in the heart
Very little has been published on the L-CPT-I genes in tissues other than liver, but two papers describing expression in the small intestine and the heart are notable [42–43]. L-CPT-I and CPT-II are expressed in the intestine, where they follow the pattern shown in the liver in which the L-CPT-I is expressed in response to fatty acid delivery to mucosal cells by way of the mother's milk and CPT-II remains unchanged throughout development [42]. L-CPT-I and CPT-II are also believed to be expressed to some extent in smooth muscle cells of the intestine [42]. CPT-I (probably L-CPT-I (see ref. [32])) and CPT-II activities have been reported to increase (paradoxically) in neonatal rat cardiac myocytes in response to insulin, and CPT-II mRNA was also found to increase [43]. These responses to insulin are exactly opposite to those seen in liver and are thought to be cell-growth related [43]. These observations raise the possibility that mRNA abundance of the L-CPT-I isoform may be differentially regulated by hormones or diet in a tissue-specific manner. Precedents do exist for this type of differential action of hormones in

different tissues. As an example, the expression of the PEPCK gene is stimulated by glucocorticoids in liver, kidney and small intestine, but depressed by glucocorticoids in white adipose tissue [44]. The differential tissue-specific regulation of L-CPT-I expression will be an exciting new area of study.

CPT-I genes and proteins remain a fascinating model for developmental regulation, hormonal regulation and substrate control of fatty acid metabolism in the heart. Many questions remain regarding the regulation of the CPT-I system. Some of these issues include determining the mechanism by which malonyl-CoA differentially regulates the CPT-I isoforms and defining the factors controlling the hormonal regulation of CPT-I gene expression. The recent cloning of the CPT-I cDNAs and genes has provided new avenues for addressing these questions.

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Carnitine deficiency-induced cardiomyopathy

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Abstract

The results of clinical and animal studies suggest that a short term period of moderate secondary carnitine deficiency, in and of itself, does not have a major effect on the cardiac contractile function, although substrate oxidation may be altered. However, with longer durations of carnitine deficiency, alterations occur within the heart that may result in impaired contractile performance, particularly at high workloads. At this point, the mechanisms responsible for the cardiac depression are uncertain. We hypothesize that the alterations in substrate metabolism produced by the carnitine deficient state results in inadequate ATP production under high workload conditions which result in impaired cardiac contractile performance. Carnitine deficiency may also induce a number of changes in gene expression of key enzymes required for normal cardiac contractile function and metabolism. (Mol Cell Biochem **180**: 33–41, 1998)

Key words: mitochondrial metabolism, long chain fatty acids, acyl carnitine derivitives

Introduction

L-Carnitine (β-hydroxy-γ-trimethylaminobutyric acid) is an essential component in the transport of long chain fatty acids into mitochondria, where they undergo β-oxidation [1] (Fig. 1). Carnitine serves two roles in this process. First, through the enzyme carnitine palmityl transferase (CPT-I), carnitine reacts with long chain fatty acids to make long chain acyl carnitine esters. The second role of carnitine is to shuttle free carnitine and long chain acyl carnitine across the inner mitochondrial membrane. Inside the mitochondrial matrix, long chain acyl carnitine is converted back to free carnitine and long chain fatty acids via CPT-II. Since, the normal heart obtains approximately 60% of its total energy production from fatty acid oxidation [2], this function of carnitine is thought to be of major importance. Another role of carnitine is to aid in the removal of excess acyl groups from mitochondria [3]. Via this mechanism, carnitine may also be important in the regulation of glucose oxidation through the mitochondrial enzyme carnitine acetyl transferase [4, 5]. By buffering fluctuations in the acetyl CoA to free CoA ratio, carnitine may indirectly regulate the activity of pyruvate dehydrogenase. Because of these functions of carnitine and since a number of case reports have shown that some patients with carnitine deficiency will exhibit cardiomyopathy [7–9], it is believed that adequate levels of carnitine are required for normal energy metabolism and contractile function of the heart [6]. However, not all patients with carnitine deficiency have cardiac alterations. The explanation for the fact that some carnitine deficient patients do exhibit cardiac alterations while others do not is uncertain, but may be related to the degree of carnitine deficiency and how cardiac performance was assessed. The purpose of this review is to examine the clinical and experimental evidence that carnitine deficiency adversely affects cardiac metabolism and contractile function.

Metabolic consequence of carnitine deficiency

Although it is assumed that inadequate myocardial carnitine content will impair the ability of the heart to oxidize fatty acids, there is little direct evidence to support this hypothesis. It has been suggested that carnitine levels are not in excess but rather at appropriate levels for normal substrate metabolism. This concept has previously been investigated by Long *et al.* [10]. They found that the concentrations of carnitine required for maximal fatty acid oxidation in tissue homogenates were not constant in various tissues. Rather, the amount of carnitine required for maximal rates of beta-oxidation paralleled the endogenous content of this compound in various tissues. Tissues with low levels of carnitine exhibited low rates of fatty acid oxidation and those with high

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levels of fatty acid contained higher levels of carnitine. However, studies by Bieber *et al.* [11] on palmitoyl transferase indicate that the K_m for carnitine is much lower than the *in vivo* concentration in the heart. Van Hinsbergh *et al.* [12] showed that maximal stimulation of fatty acid oxidation by isolated rat skeletal muscle mitochondria was obtained with 0.4 mM carnitine. This concentration corresponds to a cytosolic carnitine content of 1.2 mmole/g tissue, which is much lower than normal muscle levels. Increasing carnitine concentration had no further effect on fatty acid oxidation. If tissue carnitine levels are well above the *in vivo* K_m, it is possible that a mild to moderate deficiency of tissue carnitine may have no effect on fatty acid oxidation.

It has now been shown that there are at least two isoforms of CPT I each with unique physiologic and kinetic properties [13–15]. The liver contains predominately the L-isoform which has a lower K_m of approximately 30 mM. This K_m corresponds to the relatively low carnitine content found in the liver (Table 1). In skeletal muscle, the predominant form is M-CPT I which has a high K_m, 500 mM. The content of carnitine in skeletal muscle is much higher than liver. The heart which contains both L- and M-CPT I isoforms has an intermediate K_m, but the tissue levels of carnitine are even higher than that found in skeletal muscle. The higher levels of carnitine in the heart may be required for other functions

Table 1. Carnitine content, CPT I K_m and the predominant isoforms of CPT I in tissues

Tissue	Carnitine content (μmoles/g)	CPT-I: K_m (μM)	CPT-I isoforms
Liver	301	30	L-CPT I
Skeletal muscle	859	500	M-CPT I
Heart	1289	200	M-CPT I and L-CPT I

such as modulating glucose oxidation. An interesting issue that has not yet been addressed is whether carnitine deficiency will alter the expression of the CPT I isoforms, particularly in the heart where both the L- and M-isoforms are present.

It is expected that glucose oxidation would be increased in a carnitine deficient heart in order to compensate for the decrease in fatty acid oxidation. However, it is uncertain whether the increase in glucose oxidation would completely compensate for the decrease in fatty acid oxidation, such that total ATP production by the heart would be maintained. Another mechanism that may account for the deleterious effects of carnitine deficiency is that the impaired fatty acid transport into mitochondria may lead to the cytosolic accumulation of lipid intermediates such as long chain acyl carnitine and acyl CoA, which have been shown to inhibit a number of key enzymes. For example, long chain acyl CoA has been

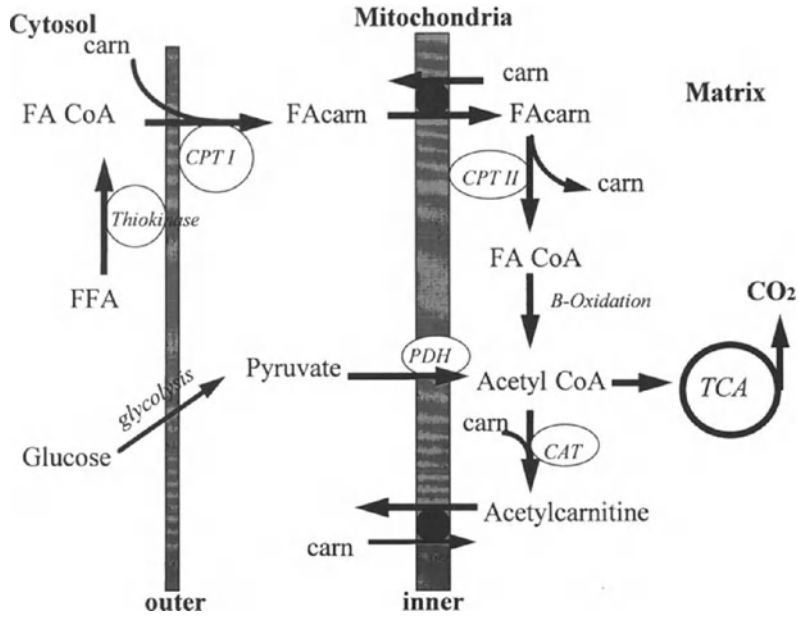

Fig. 1. Role of carnitine in fatty acid and glucose oxidation in the heart. FFA – free fatty acids; carn – carnitine; FA – fatty acyl; CPT – carnitine palmitoyl transferase-I; CAT – carnitine acetyltransferase; PDH – pyruvate dehydrogenase.

shown to inhibit the adenine nucleotide translocator of mitochondria, the suggested rate-limiting step in oxidative phosphorylation [16]. Long chain acyl carnitine reportedly inhibits sarcolemmal Na^+, K^+-ATPase [17].

Carnitine deficiency and the resulting alteration in substrate metabolism may also be harmful because of changes in the gene expression of key enzymes required for normal cardiac contractile function and metabolism. A number of studies have shown that gene expression is regulated by specific nutrients. These studies have shown that specific nutritional states can alter gene transcription, mRNA processing, mRNA stability and mRNA translation of specific genes [18]. For example, it has been suggested that enhanced lipid utilization and impaired glucose oxidation of the diabetic heart is responsible for the shift in myosin isoform composition from V_1 towards the slower V_3 isoform [19]. Rupp *et al.* [20, 21], showed that the activity of sarcoplasmic reticulum ATPase may be depressed by intermittent fasting of rats. Sucrose supplementation prevented this depression. It has also been demonstrated that the substrates utilized by tissues will affect the genes coding for glucose and fatty acid metabolism [22]. For example, Gulick *et al.* showed that administration of a CPT I inhibitor to mice or rats resulted in an upregulation of medium-chain acyl-CoA dehydrogenase an enzyme in the β-oxidative pathway [23]. There is also evidence for a functional coupling between cardiac performance and altered gene expression of enzymes associated with energy metabolism. During cardiac hypertrophy and in the failing heart, fatty acid utilization is decreased and glucose utilization is increased [24]. Sack *et al.* showed expression of genes encoding cardiac fatty acid oxidation were coordinately repressed in the failing heart [25].

Clinical carnitine deficiency syndromes

Deficiencies of carnitine are classified as either primary or secondary [6, 26–30]. Primary carnitine deficiencies may arise from a genetic disorder resulting in one or more of the following: (1) a defect in carnitine synthesis; (2) abnormal renal handling of carnitine; (3) alterations in cellular mechanisms for carnitine transport affecting uptake and/or release of carnitine from tissues; (4) excessive degradation of carnitine; or (5) defective intestinal absorption of carnitine. A number of case reports have established that primary carnitine deficiency is associated with cardiomyopathy [31–33]. This cardiomyopathy is not present at birth, but usually takes 3–4 years to develop [34]. Since, L-carnitine therapy has been shown to prevent and reverse this cardiac dysfunction [7, 8, 35–37], the term symptomatic carnitine deficiency has been used to describe this condition [34]. This form of carnitine deficiency is relatively severe with muscle and plasma carnitine being reported as low as 10% of normal values. The cardiac depression is also severe and will result in death unless carnitine supplementation is initiated. However, this condition is relatively rare [33].

Secondary carnitine deficiencies are much more common and arise from a large number of genetic diseases that are associated with defects in acyl CoA metabolism [26–28, 38]. These include genetically-induced metabolic errors, such as short-, medium- [39], or long chain acyl CoA dehydrogenase deficiencies [40], isovaleric acidemia [41, 42], propionic acidemia [43, 44], methylmalonic aciduria, hydroxymethylglutaryl-CoA lyase deficiency [45], glutaryl-CoA dehydrogenase deficiency, and beta-ketothiolase deficiency. Carnitine deficiency can also be found in patients with carnitine/acylcarnitine translocase deficiency [34]. In these cases of secondary carnitine deficiency, tissue carnitine levels are reduced to about 25–50% of normal. It has been suggested that the build up of excess organic acids leads to the formation of acylcarnitine derivatives which are excreted from the tissues and body faster than carnitine can be replaced by dietary sources or synthesis [40, 46]. However, this hypothesis is controversial since not all patients with secondary carnitine deficiencies have abnormal acylcarnitine excretion, particularly those with medium chain acyl CoA dehydrogenase deficiency. In these patients, the impaired renal conservation of free carnitine may be involved [39]. Whether carnitine supplementation will be effective in all of these conditions is unclear at this point, since there is some controversy in the literature [34]. In patients with long chain- or short chain acyl-CoA dehydrogenase deficiency [26, 27] a depression in cardiac contractile performance has been found. In the other cases, no alteration in cardiac performance has been noticed, but it should be pointed out that few studies actually examine contractile performance of the heart, and those that do only assess cardiac contractile performance at rest.

Carnitine deficiency can also be acquired as the result of liver disease, renal disease (Fanconi syndrome, renal tubular acidosis), premature birth, and dietary insufficiency (chronic TPN, malabsorption, soybean based infant formula) [47–51]. Tissue and plasma carnitine levels are also affected by Reye's syndrome, diabetes mellitus, infections and heart failure [52–55]. Many of these examples of secondary carnitine deficiency are often associated with cardiomyopathy [33, 55].

Secondary carnitine deficiency may also arise as the result of drug therapy such as valproate acid and pivampicillin [56–62]. These patients typically also have plasma and tissue total carnitine concentrations that are 10–50% of normal and an increased ratio of plasma acylcarnitine to total carnitine. Pivampicillin is an antibiotic which is used in several countries for the treatment of infections of the urinary and respiratory tracts. The pivalic acid moiety is used in this drug preparation to promote absorption of ampicillin. Pivalic acid is liberated during absorption, esterified with carnitine, and

consequently lost in the urine as the pivaloylcarnitine ester. In humans, both acute and long-term treatment with pivalic acid has been shown to impair ketone-body production [60, 62]. Whether these conditions in humans will induce alterations in myocardial performance is uncertain, but recent evidence in animals treated with pivalate suggest that cardiac depression may result (see below).

The reported incidence of secondary carnitine deficiency is increasing as more and more physicians become aware that this condition is associated with a variety of diseases. Those physicians who screened for carnitine deficiency found the number of cases to be surprisingly large. For example, Winter and colleagues [63] have found 643 patients with diverse conditions associated with carnitine deficiency. The medical conditions included 109 cases of metabolic disease, 128 cases of valproic acid therapy, 77 newborn and premature infants, 92 cases of dietary deficiency, 10 cases of renal tubular dysfunction, 5 cases of malabsorption and 4 patients being treated by the oncology service. The etiology of the carnitine deficiency in the other 218 patients was uncertain.

While the reported incidence of secondary carnitine deficiency is rising, the biological and medical consequences of this disease are uncertain. The symptoms associated with carnitine deficiency include myopathy, lipid accumulation, hypotonia, cardiomyopathy, encephalopathy, hypoglycemia and metabolic acidosis. However, not every patient with carnitine deficiency exhibits all of these symptoms. Because of this broad range of symptoms associated with carnitine deficiency, it has been difficult to distinguish whether the symptoms are due to carnitine deficiency or to the underlying genetic metabolic disorder.

Based upon these clinical observations it appears that there is a clear and strong association between primary carnitine deficiency syndrome and the development of cardiomyopathy. However, the effects of secondary carnitine deficiency on the heart are less certain. The carnitine deficiency in these patients is moderate and does not usually result in severe cardiomyopathy. In addition, in patients with secondary carnitine deficiency, it is difficult to differentiate between the effects of carnitine deficiency and those which are due to the primary metabolic disorder. Thus, it has been difficult to demonstrate a cause and effect relationship between the secondary carnitine deficiencies and cardiac alterations. Another problem stems from the lack of comprehensive cardiac assessments in most patients with secondary carnitine deficiency. When cardiac performance was assessed, it was measured only under resting conditions. It is conceivable that with secondary carnitine deficiency that if cardiac performance is impaired, it may only be manifested at high workloads where the metabolic needs of the heart are increased. A carnitine deficient heart may not be able to meet the increased metabolic demands during exercise or increased exertion.

Animal models of carnitine deficiency

Part of the difficulty in determining the metabolic and functional consequences of carnitine deficiency stems from the lack of adequate experimental models of carnitine deficiency. Deficiencies of carnitine have been induced in rats by dietary depletion, intraperitoneal injection of D-carnitine [64], uremia, and administration of oral sodium pivalate [65, 66]. Only a few of these studies have addressed the cardiovascular consequences of carnitine deficiency in these models.

In experimental animals, it has been shown that carnitine levels are depressed in the diabetic [55], pressure and volume overloaded [67] and Syrian hamster cardiomyopathic [68] heart. Cardiac contractile function is depressed in all of these hearts, however, the role of the carnitine deficiency in this cardiac depression is uncertain. Nonetheless, carnitine therapy has been demonstrated to be beneficial in all three models. Carnitine deficiency has also been induced in rats by intraperitoneal injection of D-carnitine, the inactive isomer, resulting in depressed cardiac contractile function [68]. This model of carnitine deficiency was shown to induce an enhancement of enzymes associated with fatty acid metabolism in the liver [69]. El-Alaoui-Talibi *et al.* [67] showed, in volume overloaded rat hearts, that myocardial carnitine content was decreased by 30%. In this case, the deficiency of myocardial carnitine was associated with similar decreases in fatty acid oxidation of isolated perfused hearts. However, when mitochondrial respiration was measured in carnitine deficient hearts, respiratory rates remained depressed when palmitate and carnitine were used as substrates. These findings suggest that simply a lack of carnitine in the carnitine-acylcarnitine translocase system may not be the primary defect, but rather a defect in the enzymes associated with the fatty acid oxidative pathway may also be involved. Keene *et al.* [70] reported that in an 18 month old male boxer dog exhibited dilated cardiomyopathy, elevated plasma carnitine, but severely low myocardial carnitine levels. L-carnitine supplementation produced a marked improvement in cardiac performance. In the mouse with juvenile visceral steatosis (JVS mouse), it has been recently shown that this animal model will develop systemic carnitine deficiency which results in cardiac hypertrophy [71] and a number of mitochondrial abnormalities [72]. There is also an increased expression of the CPT I gene in these animals, presumably as a compensation for the deficiency of carnitine [73]. Interestingly, the increase in CPT I mRNA was thought to be primarily the L- isoform which has a lower K_m [13–15]. Carnitine therapy to these animals was shown to prevent the cardiac hypertrophy and decrease CPT I mRNA levels to normal.

There have only been a few studies on animal models of moderate secondary carnitine deficiency. Heinonen and

Takala [74] demonstrated that serum, skeletal muscle and liver carnitine contents were reduced by 50% after 7 weeks of oral feeding of carnitine-free parenteral nutrition solutions in rats. Presumably, in this model of carnitine deficiency, the endogenous synthesis of carnitine is insufficient for maintenance of normal tissue carnitine levels. The reduced tissue carnitine concentration had no effects on *in vivo* fatty acid oxidation, exercise capacity or nitrogen balance. Bianchi and Davis were able to induce a secondary carnitine deficiency in young rats by oral administration of sodium pivalate and these animals exhibited impaired ketogenesis.

We have used the pivalate-induced model of secondary carnitine deficiency to examine the long term effects on myocardial metabolism and contractile function [76–79]. In this model, sodium pivalate (20 mM) was added to the drinking water of young male rats. After 11–12 weeks, we showed that the pivalate treatment significantly decreased total myocardial carnitine levels by 50–60% [76]. Despite this deficiency of myocardial carnitine, there were no significant differences in left ventricular pressure, rate of pressure development or aortic flow as compared to control hearts. ^{14}C-Glucose oxidation rates of isolated perfused working hearts were elevated in carnitine deficient hearts, presumably, because fatty acid oxidation was depressed in these hearts. Since cardiac performance was not depressed the increase in glucose oxidation was able to compensate such that overall ATP production was not significantly altered.

We also measured the activity of some of the enzymes involved in glucose and fatty acid metabolism [76]. The activity of citrate synthase was not affected by carnitine deficiency. However, the activity of hexokinase was increased and 3-hydroxyacyl CoA dehydrogenase (HAD) was decreased. These findings indicate that a 50–60% myocardial deficiency of L-carnitine for 11–12 weeks does not adversely affect myocardial function but does alter myocardial substrate utilization. There was a reduction in HAD, an enzyme found in the β-oxidative pathway, while the activity of hexokinase, an enzyme found in glycolysis, was elevated. Consistent with the data on contractile performance, the myosin isoenzyme distribution was not altered. We also found that the ability of 11 week carnitine deficient hearts to recover from a period of ischemia and reperfusion was also not altered relative to control hearts (Fig. 2).

Since 11–12 weeks of carnitine deficiency did not apparently alter cardiac contractile function, but changed the oxidative rate of glucose and alter the activities of enzymes associated with glucose and fatty acid oxidation, we next determined whether a longer duration of carnitine deficiency would affect myocardial performance. To test this hypothesis, rats were treated for a 24–26 week period [77].

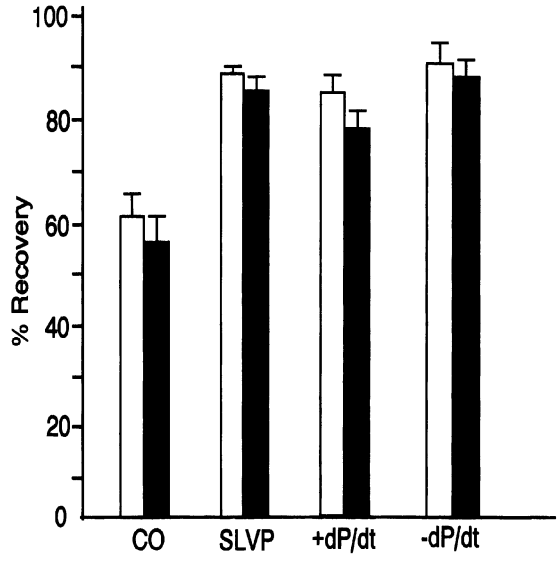

Fig. 2. Effects of 12 weeks carnitine deficiency on the ability of hearts to recover from a 60 min period of low flow ischemia followed by 30 min of reperfusion. CO – cardiac output; SLVP – systolic left ventricular pressure; + and – DP/dt – rate of pressure development and relaxation. All values are mean ± S.E.M. for n = 7–12.

This duration of sodium pivalate treatment also resulted in a similar reduction in total myocardial carnitine content compared to control hearts. In this case, the reduction in myocardial carnitine content was associated with a depression in cardiac performance and decreased rate of fatty acid oxidation. However, the magnitude of these alterations were dependent upon the level of external work. Mechanical performance parameters, cardiac output, and positive- and negative- dP/dt in response to increases in left atrial filling pressures, were depressed in sodium pivalate hearts compared to control hearts. Another series of hearts were used to ascertain whether carnitine depletion would alter myocardial substrate use. At low work load conditions, there were no differences in mechanical function and fatty acid oxidation between normal and carnitine deficient hearts. However, at high work loads, palmitate oxidation, as determined by production of ^{14}C-CO_2 from the oxidation of [U-^{14}C]-palmitate, was decreased in carnitine deficient hearts as compared to control hearts (Table 2). Glucose oxidation rates were increased compared to control hearts. Under a high workload condition, overall myocardial steady state ATP production from these substrates was depressed in the carnitine deficient hearts. Thus a 50% reduction in myocardial carnitine content for a period of 24 weeks significantly depressed mechanical function and palmitate oxidation at high workloads.

Table 2. Effects of pivalate-induced secondary carnitine deficiency on myocardial glucose and palmitate oxidation by isolated perfused hearts

Group	Glucose oxidation (nmoles/g dry wt/min)	Palmitate oxidation (nmoles/g dry wt/min)
High workload		
Control	315 ± 83	882 ± 87
Carnitine deficient	666 ± 175	544 ± 37*
Low workload		
Control	ND	526 ± 69*
Carnitine deficient	ND	404 ± 47*

All values are mean ± S.E.M. *$p < 0.05$ significantly different from control; ND: not determined.

These results raise an interesting question. How does a heart with a 50% decrease in carnitine content maintain normal rates of fatty acid oxidation at a low workload and only exhibits a 40% decrease in fatty acid oxidation at a higher workload? One possibility is that the heart compensated for the loss of L-carnitine by increasing the expression of the L-isoform of CPT I. Since this isoform has a lower K_m, the heart may be able to maintain fatty acid oxidation. Interestingly, an increase in the L-isoform of CPT I was found in the JVS carnitine deficient mouse [73].

In another study, we examined whether acute addition of carnitine to the perfusion medium of hearts isolated from 24 week sodium pivalate-treated rats would restore both cardiac contractile performance and fatty acid oxidation [79]. Isolated working rat hearts from control and sodium pivalate-treated animals were perfused with 11 mM glucose and 0.4 mM [U-^{14}C]-palmitate at 15 cm H_2O left atrial filling pressure and 100 cm H_2O aortic afterload for a 60 min period. Carnitine, when used, was added to the perfusate at a concentration of 5 mM. Carnitine deficiency was associated with a depression in aortic flow and steady state rates of palmitate oxidation as compared to control hearts. Acute treatment with carnitine increased total myocardial carnitine content in both control and sodium pivalate-treated hearts. In the carnitine deficient hearts, aortic flow was significantly increased by L-carnitine administration, but had no significant effect in the control hearts.

By restoring total carnitine content in pivalate-treated hearts, we were expecting to observe a concomitant increase in palmitate oxidation. In contrast, the addition of carnitine to the perfusion medium resulted in no significant changes in palmitate oxidation. If anything, the mean rate of palmitate oxidation was depressed by the addition of carnitine to the perfusion medium. A similar effect was also observed in control carnitine-treated hearts. It has been suggested that carnitine may remove the inhibition on the pyruvate dehydrogenase complex by decreasing the intramitochondrial acetyl CoA/CoA ratio via the enzyme carnitine acetyl transferase [5], (Fig. 3). As a result, glucose oxidation was subsequently enhanced. Furthermore, it

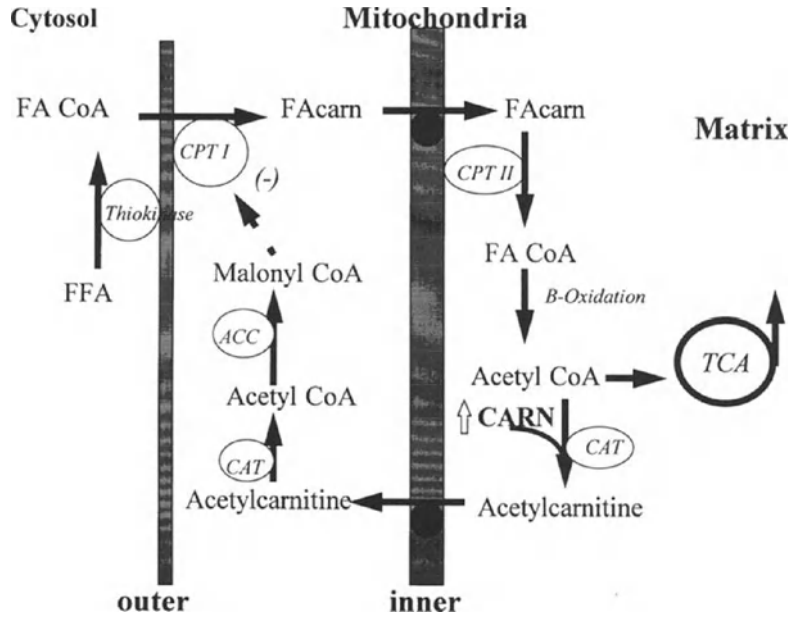

Fig. 3. Mechanism for carnitine inhibition of fatty acid oxidation. FFA–free fatty acids; carn–carnitine; FA–fatty acyl; CPT–carnitine palmitoyl transferase-I; CAT–carnitine acetyltransferase; PDH–pyruvate dehydrogenase; ACC–acetyl CoA carboxylase .

has been hypothesized that the increased production of acetylcarnitine led to elevated levels of malonyl CoA which, in turn, inhibited palmitoyl carnitine transferase, and, therefore, prevented fatty acid oxidation from being increased. Fatty acid oxidation may not have been stimulated by carnitine because the activities of the enzymes associated with β-oxidation are decreased, as shown in the previous study. Thus, the beneficial effects of carnitine, in this case, may have been due to an acute stimulation of glucose oxidation rather than a correction of the metabolic and functional alterations associated with carnitine deficiency. To test this hypothesis, these experiments were repeated on another set of rats, only this time glucose oxidation was measured. Carnitine addition to the perfusion medium stimulated glucose oxidation in both control and carnitine deficient hearts. These findings suggest that the improved cardiac contractile performance in carnitine supplemented hearts may be due to an acute stimulation of glucose oxidation.

The cardiac depression found in this animal model of secondary carnitine deficiency provides good evidence that myocardial contractile function is depressed with time. Whether a similar depression in cardiac contractile performance will be seen in patients with secondary carnitine deficiency remains uncertain. Holme *et al.* [80] showed that 1–3 years of pivalate treatment to children produced a 5–10% reduction in plasma carnitine with no evidence of cardiac alterations. However, in follow up studies of these patients, it was found that many of the patients complained of tiredness and irritability, symptoms that improved with carnitine administration. Impaired ketogenesis was also found in these patients. It is important to note that cardiac performance was assessed only under resting conditions and not in response to a stress such as exercise. As suggested from our experimental studies, the cardiac contractile depression associated with moderate secondary carnitine deficiency may only occur at high workloads. Recently there has been a clinical study indicating that the carnitine deficiency induced by pivalate treatment induces alterations in fatty acid metabolism and cardiac structure in humans [81]. In this study, 7 healthy volunteers were given pivmecillinam for 7–8 weeks and then followed for 15 months. Total muscle carnitine was reduced by 46%. The median diastolic interventricular septum and median thickness decreased by 14% and the left ventricular mass by 10%. Fifteen months after treatment with L-carnitine, these parameters had increased but not to pretreatment values. Thus extended pivalic acid treatment caused carnitine deficiency and changes in cardiac structure.

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Fatty acid-binding proteins in the heart

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Abstract

Long-chain fatty acids are important fuel molecules for the heart, their oxidation in mitochondria providing the bulk of energy required for cardiac functioning. The low solubility of fatty acids in aqueous solutions impairs their cellular transport. However, cardiac tissue contains several proteins capable of binding fatty acids non-covalently. These fatty acid-binding proteins (FABPs) are thought to facilitate both cellular uptake and intracellular transport of fatty acids. The majority of fatty acids taken up by the heart seems to pass the sarcolemma through a carrier-mediated translocation mechanism consisting of one or more membrane-associated FABPs. Intracellular transport of fatty acids towards sites of metabolic conversion is most likely accomplished by cytoplasmic FABPs. In this review, the roles of membrane-associated and cytoplasmic FABPs in cardiac fatty acid metabolism under (patho)physiological circumstances are discussed. (Mol Cell Biochem **180**: 43–51, 1998)

Key words: myocardium, fatty acid metabolism, fatty acid uptake, fatty acid-binding protein

Introduction

To fuel its continuous contractions, heart muscle is able to oxidize a variety of substrates including fatty acids, glucose, pyruvate, lactate, ketone bodies, and several amino acids. Under normal workload conditions the energy requirement of the heart is primarily met by oxidation of long-chain fatty acids (FAs). Cardiomyocytes have limited capacity for FA storage in triacylglycerols and, therefore, depend on a continuous supply of FAs from the circulation [1].

FAs are obtained from the diet, *de novo* synthesized by the liver or released from adipose tissue. In the intestine, dietary FAs are esterified with glycerol and incorporated into chylomicrons which are subsequently secreted into the bloodstream. In the liver, newly synthesized FAs or FAs released by hepatic lipolysis of chylomicrons, are incorporated into the triacylglycerol core of very-low density lipoproteins (VLDLs). Unesterified FAs, released by lipolytic processes in adipose tissue, are bound to albumin which has several high-affinity binding sites for FAs. The low solubility of FAs in aqueous environments necessitates the presence of transport proteins (e.g. albumin) or transport vehicles (e.g. VLDLs) to allow their supply to tissues in sufficient quantities. FAs transported in the esterified form are available after lipolytic action of lipoprotein lipase (LPL) on the triacylglycerol core of these transport vehicles. LPL is an enzyme associated with the glycocalyx on the luminal surface of heart capillary endothelium [1].

Cardiac muscle is very efficient in extracting FAs from the vascular fluid, 50–70% of circulating FA can be taken up during a single capillary passage [1]. Similar extraction ratios are observed for lactate, whereas only a relatively minor proportion of glucose is taken up at first passage [1]. Current insight suggests that a number of (non-enzymatic) proteins participate in the uptake of FAs (see Table 1). Among these proteins are membrane-associated and cytoplasmic fatty acid-binding proteins (FABPs).

In this review, the current knowledge on the functions of FABPs in myocardial FA uptake and metabolism is discussed. Because cytoplasmic heart-type FABP has been studied most extensively, the role of this protein in cardiac FA metabolism, under both normal and pathophysiological conditions, will be considered in more detail.

Uptake of fatty acids by the heart

A schematic representation of myocardial FA uptake is shown in Fig. 1. Transendothelial FA translocation is poorly understood and might involve albumin-binding proteins

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Table 1. Non-enzymatic proteins presumably involved in myocardial FA uptake and metabolism

	Molecular mass (kDa)	(Putative) Localization	(Putative) Function	References
Extracellular				
Albumin	68	circulation, interstitium	plasma FA transporter	[1]
Plasma membrane-associated				
ABP	18, 31, 60	endothelium, cardiomyocyte	plasmalemmal albumin receptor	[2, 10]
$FABP_{pm}$	43	endothelium, cardiomyocyte	plasmalemmal FA transport	[4, 13]
FAR	60	cardiomyocyte	sarcolemmal FA transport	[18]
FAT	88	cardiomyocyte (transmembrane)	sarcolemmal FA transport	[19, 20, 21]
FATP	63	cardiomyocyte (transmembrane)	sarcolemmal FA transport	[23]
Intracellular				
E-FABP	15	vascular endothelium	intracellular FA transporter	[13]
H-FABP	14.5	vascular endothelium, cardiomyocyte	intracellular FA transporter	[13, 57]
ACBP	10	cardiomyocyte	intracellular acyl-CoA transporter	[44]

Abbreviations: ABP – albumin-binding protein; $FABP_{pm}$ – plasmalemmal fatty acid-binding protein; FAR – fatty acid receptor; FAT – fatty acid translocase; FATP – fatty acid-transport protein; FABP – fatty acid-binding protein; ACBP – acyl-CoA binding protein.

(ABPs) which have been identified on the surface of capillary endothelial cells [2]. ABPs might facilitate transendocytosis of albumin-FA complexes and/or enhance the dissociation of FAs from albumin. The movement of albumin-FA complexes through endothelial clefts is impeded by the size of albumin and by tethering of glycocalyx lining endothelial clefts [3].

Lateral diffusion of FAs within the plasmalemmal leaflets may relocate FAs from the luminal side to the abluminal side of the endothelium. Alternatively, FAs may be translocated across the endothelial plasmamembrane by a protein-mediated mechanism or via movement of FAs from the outer to the inner membrane leaflet (flip-flop). Protein-mediated FA uptake might involve plasmalemmal FABP ($FABP_{pm}$), a peripheral membrane protein which has been implicated in the transfer of FAs across the endothelial cell layer [4]. Passive diffusion of FAs via flip-flop was shown to be rapid in model membranes [5], but it is not known how well this proposed FA translocation mechanism translates to biological membranes [6, 7].

Once internalized, cytoplasmic FABPs could be involved in shuttling FAs from the luminal to the abluminal plasmamembrane of the endothelial cell. Two types of FABP have

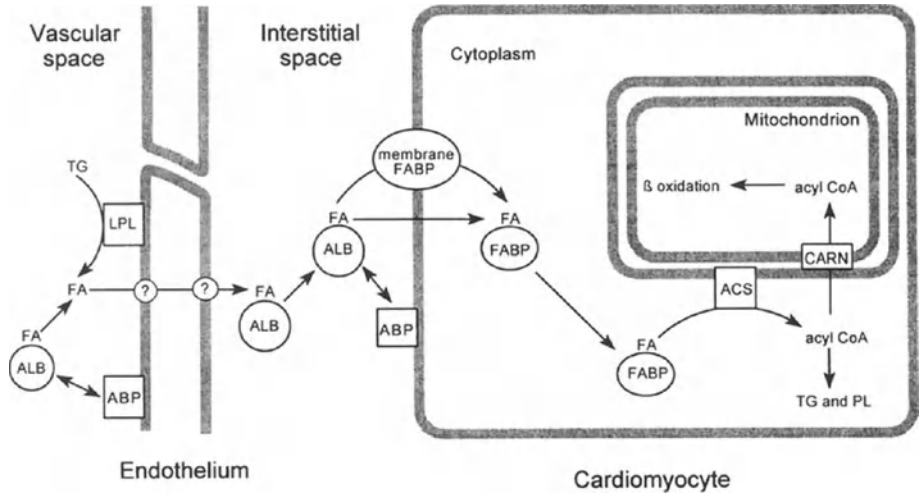

Fig. 1. Model for myocardial FA uptake and intracellular FA metabolism. Abbreviations: ALB – albumin; ABP – albumin binding protein; ACS – acyl-CoA synthetase; CARN – carnitine shuttle; FA – long-chain fatty acid; FABP – fatty acid-binding protein; LPL – lipoprotein lipase; PL – phospholipid; TG – triacylglycerol. Question marks denote unresolved mechanisms for FA translocation. Modified from reference [21], with permission.

been identified in endothelial cells, epidermal-type FABP (E-FABP) and heart-type FABP (H-FABP). Expression levels of these proteins are relatively low and theoretical considerations indicate that in these cells an intracellular FA carrier is dispensable [1, 8]. Though the physiological relevance of E-FABP/H-FABP in transcytoplasmic FA transport in endothelial cells is questioned, the high-affinity binding of arachidonic acid and certain eicosanoids observed for E-FABP [9] suggest a possible involvement of this protein in the arachidonic acid cascade.

In the interstitial space, albumin is presumed to desorb FAs from the abluminal plasmamembrane and deliver the FAs to ABPs on the surface of cardiomyocytes [10]. The existence of ABPs is supported by observations that increasing albumin concentrations, at a fixed albumin:palmitate ratio, resulted in saturable uptake of palmitate by isolated rat cardiomyocytes [11]. The molecular mechanism of FA transport across the sarcolemma is still a matter of debate. Traditionally, it was assumed that FAs traverse the phospholipid bilayer by a simple, non-saturable diffusion mechanism [5, 12]. However, evidence is accumulating that myocardial FA uptake involves mainly a carrier-mediated, saturable uptake system [11, 12]. In resting rat cardiomyocytes this protein-mediated component accounts for 80% of the palmitate uptake [11]. Several membrane proteins thought to be involved in cellular FA uptake have been identified in the heart [7, 13, 14]. These so-called membrane-associated FABPs will be discussed in the following section.

After translocation across the sarcolemma, FAs are bound to H-FABP and delivered to sites of metabolic conversion. Prior to further metabolism FAs are activated by esterification with CoA, a reaction catalyzed by membrane-associated acyl-CoA synthetases (ACS). FAs destined for oxidation are activated by ACS on the outer mitochondrial membrane. The carnitine shuttle promotes subsequent entry of the acyl-moiety of acyl-CoA esters into the mitochondrial matrix where β oxidation takes place. In the heart, peroxisomal β oxidation forms a quantitatively minor pathway for FA degradation [1]. An endoplasmic reticulum-bound ACS activates FAs destined for triacylglycerol or phospholipid synthesis. The various functions proposed for H-FABP in myocardial FA metabolism will be discussed later.

Membrane-associated FABPs

Early indications for the involvement of membrane-associated proteins in FA uptake included the observation of saturable uptake kinetics and inhibition of uptake by treating cells with trypsin or phloretin (an aspecific inhibitor of membrane transport proteins). Subsequently, several candidate FA transporters were identified by diverse methods such as labelling of proteins with (photo)reactive FA analogues, expression cloning and affinity chromatography [7, 13, 14]. Most of the proteins thus found were initially isolated from adipose tissue or liver. Those proteins with known expression in the heart are listed in Table 1.

Plasmalemmal FABP ($FABP_{pm}$), a 43 kDa peripheral membrane protein with possible identity to the mitochondrial isoform of aspartate aminotransferase (mtAAT) [15], has been identified in numerous cell types including hepatocytes, adipocytes and cardiomyocytes. Antibodies directed against this protein diminished oleate uptake by isolated rat cardiomyocytes with 40% but had no effect on glucose uptake [16]. Moreover, 3T3 fibroblasts transfected with mtAAT cDNA displayed saturable uptake of FA which was several-fold higher than uptake in mock-transfected cells [17].

A 60 kDa protein isolated from rat heart sarcolemmal membranes was shown to bind palmitate with high affinity (K_d 1 μM) [18]. Unfortunately, no functional data on this protein, which was tentatively called FA receptor (FAR), are available.

Incubation of rat adipocytes with a reactive FA derivative resulted in labelling of an 88 kDa membrane protein and a concomitant 75% decrease in FA uptake [19]. A cDNA encoding this protein, which was designated Fatty Acid Translocase (FAT), showed high homology with mouse and human CD36 [19]. Expression of FAT cDNA in Ob17PY fibroblasts resulted in enhancement of FA uptake [20]. Northern blot analysis [21, 22] and immunochemistry [Van Nieuwenhoven, personal communication] revealed FAT expression in rat cardiomyocytes. Moreover, developmental regulation of FAT expression correlated well with the increase in cardiac FA oxidation capacity during development [21, 22].

A murine 3T3-L1 adipocyte cDNA library was expressed in COS7 cells to identify cDNAs which could augment uptake of a fluorescent FA analogue [23]. Using this functional approach a cDNA encoding a 63 kDa integral membrane protein named Fatty Acid-Transport Protein (FATP) was identified. Northern blot analysis revealed FATP expression in various tissues with high FA fluxes, including the heart [23].

As indicated above, the involvement of membrane-associated FABPs in cellular FA uptake is supported by several lines of evidence of which the transfection experiments are most convincing. However in spite of this, little is known about tissue contents of the various proteins, the contribution of each to myocardial FA uptake and whether the proteins interact and/or cooperate. It will be interesting to learn if an interaction occurs between membrane-associated FABPs and proteins that facilitate the final part of the myocardial FA uptake chain, the cytoplasmic FABPs. *In vitro* experiments with CD36 (FAT) and FABP isolated from bovine mammary gland, suggest that such an interaction indeed occurs [24].

Cytoplasmic FABPs

Following their initial discovery in the early 70s [25], cytoplasmic FABPs are now known to comprise a family of proteins showing high-affinity binding of lipophilic ligands [13, 26, 27]. This so-called lipid binding protein (LBP) family belongs to a larger family of ligand binding proteins with similar protein architecture, the calycins [28]. Other protein families belonging to this superfamily are the lipocalins, a group of small extracellular transport proteins having diverse functions, and the avidins, a group of biotin binding proteins [28].

In mammals, the LBP family currently consists of one bile acid-binding protein, two retinol-binding proteins, two retinoic acid-binding proteins (CRABPs) and eight 'true' FABPs [13]. LBPs consist of 126–137 amino acids (MW 14–16 kDa) and, in the mouse, their homology ranges between 14–66%. Although LBPs were named after the initial source of isolation, this does not necessarily mean that expression is restricted to that particular tissue.

In cardiac tissue, two types of FABP have been identified (Table 1), heart-type FABP (H-FABP) and epidermal-type FABP (E-FABP) [13, 29]. The presence of FABP in myocardium and other tissues was already established in 1972 [25]. Subsequently, FABP was isolated from rat heart and its FA binding characteristics were determined [30]. Only in 1984 it became apparent that cytoplasmic FABPs from heart (H-FABP) and liver (L-FABP) were related, but distinct proteins [31, 32]; reviewed in [33].

Structure of H-FABP and binding characteristics

H-FABP, which appears to be identical to mammary-derived growth inhibitor (MDGI) [34] and is also known as FABP3 or muscle-FABP, consists of 132 amino acid residues and has a molecular mass of 14.5 kDa. It has been fully characterized in cattle, man, rat, and mouse. Interestingly, apparently homologous FABPs have been identified in flight muscle of desert and migratory locusts [35] and in skeletal muscle of the antarctic icefish [36]. In bovine heart, two H-FABP isoforms (Asp98 and Asn98) are encoded by distinct mRNAs [37] which might result from transcription of different alleles.

H-FABP is post-translationally modified by acetylation of its N-terminus and is a stable protein with a half-life of 2½ days, as measured in cultured neonatal cardiomyocytes [38]. A minute amount of the H-FABP pool in isolated rat cardiomyocytes was found to be phosphorylated on Tyr19 upon stimulation with near-physiological insulin levels [39]. The physiological significance of this finding is unknown, although it was speculated that phosphorylated H-FABP might modulate the insulin signal transduction pathway in the presence of FAs [39].

Crystal structures have been determined for bovine H-FABP at intermediate resolution and for recombinant human H-FABP complexed with various saturated and (poly)unsaturated fatty acids at high resolution (1.4–2.1 Ångstroms) [40, 41]. The protein structure consists of 10 antiparallel β strands (β_A-β_J) and two short α-helices arranged into two more or less orthogonal β sheets (Fig. 2). The FA is bound in an internal binding cavity where its negatively charged carboxylate group forms a hydrogen-bond network with Arg106 (via a water molecule), Arg126 and Tyr128. Mutation of any of these residues is likely to affect FA binding affinity, e.g. an Arg126Gln substitution completely abolishes FA binding activity [42]. A detailed description of the various structural aspects of H-FABP and other LBPs can be found in reference [40].

Several methods have been employed in the past to determine kinetic parameters of FA/H-FABP interaction [13]. Recent methodological improvements revealed equilibrium dissociation constants in the nanomolar range and a binding stoichiometry of one mole FA per mole of protein [43]. Both saturated and (poly)unsaturated FAs are bound to H-FABP with high-affinity (K_d 2–60 nM). As apparent from the interactions between the FA and residues in the binding cavity [40], the binding of FAs to H-FABP is pH-dependent. In the neutral and basic pH range, FAs are bound whereas they dissociate at pH values below 4.5, the

Fig. 2. Ribbon diagram showing the tertiary structure of a cytoplasmic FABP with bound fatty acid (balls and sticks). The β strands are numbered A-J. The structure shown is for intestinal-type FABP. The structure of heart-type FABP is similar with the difference that in H-FABP the fatty acid is bound in a U-shaped conformation. Reproduced from reference [59], with permission.

pK of free FA. Acyl-CoA esters, acyl-carnitine esters and long-chain fatty alcohols do not bind to H-FABP. Acyl-CoA esters are bound with high affinity by acyl-CoA binding protein (ACBP), a protein expressed in most – if not all – cell types [44].

Regulation of H-FABP expression

The gene encoding H-FABP consists of four exons and has been mapped to human chromosome 1p32–35 [27, 45]. Apart from the expressed H-FABP gene on chromosome 4, several intronless pseudogenes located on different chromosomes have been identified in the mouse [46]. The human H-FABP/MDGI gene is a candidate tumor suppressor gene, its locus lying in a region commonly found to be lost in sporadic breast cancers [45]. However, a detailed genetic analysis suggested that mutations in the H-FABP/MDGI coding region are uncommon in breast tumorigenesis [45]. These studies revealed a rare polymorphism in the H-FABP gene resulting in a Lys54 to Arg54 transition.

Because H-FABP is a stable protein and the factors influencing H-FABP levels generally result in mild and gradual changes, expression seems to be regulated primarily at the transcriptional level. Several putative transcription factor binding sites have been identified in the promoter/enhancer regions of the mouse H-FABP gene [46], but no experimental evidence is available about involvement of these sites and transcription factors binding to them in controlling H-FABP levels. Typical mRNA destabilizing elements, such as those commonly found in the 3′ untranslated regions of growth factor mRNAs [47], are not present in H-FABP mRNAs.

H-FABP is abundantly expressed in the cytoplasm of cardiomyocytes. In rat heart, H-FABP accounts for 3% of the cytoplasmic proteins (0.74 mg/g wet weight) [48]. Using immunochemistry, small amounts of H-FABP were also detected inside the nucleus and in the mitochondrial matrix [13]. Moreover, H-FABP was found to be associated with myofibrils. Because H-FABP lacks nuclear localization signals and it is generally assumed that small proteins can pass nuclear pores unrestricted, the presence of H-FABP in the nucleus probably reflects equilibration with the cytoplasmic fraction.

Besides cardiomyocytes, the presence of H-FABP has been demonstrated in other heart cells albeit in less abundance [49]. Heart capillary endothelium, cultured endothelial cells from bovine aortic tissue [38], cardiac fibroblasts and vascular smooth muscle cells [50] contain low levels of H-FABP. Outside the heart, relatively high-level expression of H-FABP has been detected in red skeletal muscles, renal cortex, testis, and brain [13, 51].

Testosterone-treatment, endurance training [52], circadian rhythm [31], nutritional state and pharmacological intervention [53] have been shown to influence the myocardial H-FABP content. Moreover, H-FABP expression is altered in certain pathophysiological conditions. In general, metabolic situations that tend to increase fatty acid oxidation result in elevated H-FABP levels. Interestingly, FAs stimulate H-FABP and FAT expression in isolated neonatal rat cardiomyocytes (De Vries, personal communication). The regulation of H-FABP expression in heart muscle in relation to proposed functions is discussed in the ensuing sections.

Functions of cytoplasmic FABPs in the heart

Throughout prenatal development, H-FABP is present at constant levels in rat heart [51]. Around birth there is a marked increase in H-FABP protein and mRNA levels which reach a maximum after one month [22, 51]. A parallel increase in oxidative capacity of heart tissue is found during development. When comparing H-FABP content and oxidative capacity in different skeletal muscle types similar observations were made [48]. Both the levels of H-FABP and oxidative capacity increased in the order: fast-twitch white muscle << slow-twitch red muscle < heart muscle. Likewise, H-FABP concentrations correlated with palmitate oxidation measured in homogenates of various heart cell types such as cardiomyocytes, endothelial cells and fibroblasts [49].

Together these findings suggest that cellular H-FABP levels are directly linked to the tissues' capacity for FA oxidation. Therefore, the major postulated functions pertain to a role for H-FABP in FA metabolism. H-FABP is thought to (1) stimulate the cellular uptake of FAs and (2) enlarge the cytoplasmic FA-flux. Protection against detergent-like effects of FAs [13] and modulation of signal transduction pathways [54] have been proposed as additional functions of H-FABP.

Enhancement of cellular FA uptake

By intracellular binding of FAs and, thus, maintaining a gradient between the extracellular and intracellular compartments, H-FABP might increase the influx of FAs into the cell. Proof for such a function was obtained for liver-type FABP; expression of its cDNA in transfected L-fibroblasts resulted in elevated FA uptake [55]. Similar observations were made for other LBPs [13]. However, H-FABP cDNA expression in COS7 cells did not affect FA uptake [23]. The latter observation does not exclude a role for H-FABP in cardiac FA uptake, as simultaneous expression of membrane-associated FABPs may be a prerequisite for H-FABP in order to stimulate FA uptake.

Enhancement of cytoplasmic diffusion

In contrast to short-chain FAs, the physiologically important long-chain FAs (C_{16} and C_{18}) are virtually insoluble in aqueous environments. While it seems obvious that H-FABP

increases the solubility of FAs and, accordingly, enhances the intracellular FA flux, experimental proof for this function *in vivo* is scarce.

In vitro, H-FABP was shown to transfer FAs between a variety of model membranes [13]. Moreover, when added to a cytosolic preparation lacking endogenous FABP, H-FABP enhanced the diffusion of oleate in this system several fold [56]. Inside the cardiomyocyte the majority of FAs are associated with phospholipid bilayers [57]. Theoretical calculations indicated that H-FABP raises aqueous phase FA levels from about 3 nM to 3 μM, thereby potentially stimulating intracellular FA-fluxes several fold [13, 57].

The mechanism by which FA are transferred from H-FABP to synthetic phospholipid membranes is believed to proceed through collisional transfer [58]. This process requires an interaction between FABP and the acceptor membrane. Using a fluorescence resonance energy transfer assay, it was shown that FA transfer from H-FABP to model membranes depends on the composition and structure of membrane phospholipids, and the ionic strength [58]. Acidic phospholipid bilayers were most efficient in accepting FAs from H-FABP, suggesting an interaction between positively charged residues on the surface of H-FABP and the negatively charged phospholipids in the membrane. In H-FABP the portal region, which is formed by the two α-helices (Fig. 2) and probably forms a flexible structure which caps the internal binding cavity [40, 59], contains several positively charged residues. Mutation of these residues, e.g. Lys22 to Ile22, did not affect FA binding affinity but did result in diminished FA transfer from H-FABP to model phospholipid membranes [60].

These latter findings suggest that positively charged residues in the portal region are involved in the collisional transfer of FAs between H-FABP and membranes. Interestingly, the outer mitochondrial membrane of cardiomyocytes contains large amounts of negatively charged phospholipids (e.g. cardiolipin) which could possibly interact with these positively charged residues. Moreover, in the vicinity of actively respirating (H^+ pumping) mitochondria the FA binding affinity of H-FABP, which is pH dependent, could be lowered. These speculations imply a possible mechanism for enhanced transfer of FAs from cytoplasmic H-FABP to mitochondrial membranes.

Extracellular functions proposed for H-FABP

Several extracellular functions have been postulated for H-FABP (MDGI). This protein is thought to be involved in growth-regulation and differentiation of mammary epithelial cells [61, 62]. Recently it was shown that MDGI is in fact a mixture of adipocyte-FABP and H-FABP, but its anti-proliferative activity resides apparently in H-FABP [34]. For exerting its extracellular function, MDGI, which is synthesized by lactating mammary epithelial cells, is probably co-secreted with other constituents of milk fat droplets.

A possible involvement of H-FABP in development of cardiac hypertrophy has been reported [63]. In neonatal cardiomyocytes, low extracellular concentrations of H-FABP were found to induce expression of proto-oncogenes and cause an increase in cellular surface area. As H-FABP normally has an intracellular localization, the physiological relevance of these findings remains to be established.

Pathophysiological disturbances of myocardial FA metabolism

In the healthy heart oxidation of FAs supplies up to 70% of the overall energy requirement [1]. Under certain conditions, such as fasting and diabetes, the contribution of FA oxidation to total ATP production may even exceed 90% [1, 64]. A number of pathophysiological conditions which affect the workload of the heart, such as chronic hypertension and hypertrophy, cause alterations in myocardial FA metabolism [1, 64]. A possible involvement of H-FABP in metabolic adaptation under (patho)physiological conditions is discussed.

Diabetes and fasting

Circulating FA levels are elevated in diabetes mellitus and during fasting. In diabetic hearts, the uptake and utilization of glucose is impaired and the heart becomes almost entirely dependent on FA oxidation to fulfil its energy needs. The increased FA utilization by the diabetic heart may be accompanied by enhanced uptake and/or intracellular transport of FAs. Both, FAT [14] and H-FABP mRNA [65] contents were found to be slightly increased in hearts from diabetic rats. In red skeletal muscle of rat, the mechanism by which H-FABP mRNA levels are raised appear to be on the transcriptional level in fasted animals whereas an increased mRNA stability is implied in the streptozotocin-induced diabetic animals [66]. The close relationship between FABP content and oxidative capacity is strengthened by the observation that both parameters decrease in liver and adipose tissue of diabetic animals [13].

Hypoxia and myocardial ischemia

It was shown that in rats exposed for 3 weeks to hypoxic conditions the myocardial FABP content had decreased by 20% [53]. Moreover, hearts from these rats had somewhat reduced capacity to oxidize FAs [53]. Apparently, H-FABP is a determinant of FA oxidation fluxes or FA oxidation capacity influences H-FABP content.

The reduced oxygen supply in ischemic heart severely impairs mitochondrial oxidation of FAs [1]. Under ischemic conditions, residual FAs extracted from the extracellular compartment are mainly incorporated into triacylglycerols [1]. Moreover, accumulation of acyl-CoA and acyl-carnitine

esters is readily (within 5 min) observed in low-flow ischemic heart. Intracellular FA levels are significantly raised 20–45 min following onset of ischemia [1]. Due to their amphiphilic nature, FAs can have detergent-like effects on cell membranes and influence the activity of proteins. For instance, FAs were shown to be weak uncouplers of mitochondria [67] and alter the activity of several enzymes and ion channels [13]. In the ischemic heart, tissue accumulation of FAs and their derivatives has been associated with arrhythmias, increased myocardial infarct size and depressed myocardial contractility [1]. Under ischemic conditions, H-FABP may be crucial to sequester accumulating FAs and thus prevent tissue damage.

Prolonged ischemic periods or severe coronary occlusion may result in myocardial infarction. Following myocardial infarction or skeletal muscle injury, H-FABP is released into the bloodstream by damaged myocytes and is rapidly cleared from the blood by renal filtration. The development of rapid and sensitive assays for quantification of H-FABP in plasma, makes this protein an ideal marker for diagnosis of acute myocardial infarction. The ratio of blood myoglobin/H-FABP levels allows discrimination between skeletal muscle damage and acute myocardial infarction [68].

Hypertensive and hypertrophied heart

The heart muscle adapts to prolonged periods of enhanced workload, e.g. chronic hypertension, by structural remodelling. The enlargement of ventricular size, which is observed in hypertrophied heart, forms a serious risk for development of congestive heart failure. Among the metabolic changes observed in hypertrophied heart, a decrease in FA oxidation is evident [64]. It is thought that the lowered FA oxidation capacity, which still accounts for 55% of the ATP produced, results from a decreased myocardial carnitine content. Oxidation of short-chain FAs, which do not rely on carnitine for entering the mitochondrial matrix, is not impaired in the hypertrophied heart [64]. Alternatively, a reduced H-FABP content may account for the lowered FA oxidation rate. In spontaneously hypertensive rats, which develop mild ventricular hypertrophy, H-FABP levels tended to be lower than in normotensive rats [69].

Future prospects

Research in the field of cellular FA uptake is rapidly progressing as evidenced by the identification of several putative FA transporters in a short time-span. The growing number of proteins apparently involved in FA uptake raises several questions concerning their structural organization. Perhaps these proteins are assembled into a multimeric FA transport complex in which individual proteins participate in different steps of the translocation process, e.g. binding of FAs, transmembrane movement of FAs and transfer of FAs to cytoplasmic FABPs. Post-translational modification (e.g. tyrosine phosphorylation) of membrane-associated FABPs could modulate the FA translocation process. Hence, future research is required to learn about the functions of, and interactions between membrane-associated FABPs. Moreover, the regulation of their expression/activity under diverse (patho)physiological conditions needs to be established. Use of sophisticated techniques, such as gene replacement (knock-in) and gene disruption (knock-out), which allows study of protein function in living animals, could help in resolving these issues.

The latter approach was already used to study the function of cytoplasmic FABPs. Recently, mice lacking both A-FABP alleles were shown to be developmentally and metabolically normal [70]. Interestingly, in adipose tissue of these mice an elevated expression of E-FABP was observed which probably compensated for the deficiency in A-FABP [70]. Moreover, double mutant mice lacking both CRABPs were found to be essentially normal [71]. Presumably, alternative cellular binding sites exist for retinoic acid, a vitamin A derivative which is essential for embryonic development.

The eventual development of mice lacking membrane-associated FABPs and/or cytoplasmic H-FABP, could probably resolve several unanswered questions on the roles of these proteins in myocardial FA uptake and metabolism, and in cardiac pathophysiology.

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Metabolic disturbances in diabetic cardiomyopathy

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Abstract

It has been established that diabetes results in a cardiomyopathy, and increasing evidence suggests that an altered substrate supply and utilization by cardiac myocytes could be the primary injury in the pathogenesis of this specific heart muscle disease. For example, in diabetes, glucose utilization is insignificant, and energy production is shifted almost exclusively towards β-oxidation of free fatty acids (FFA). FFA's are supplied to cardiac cells from two sources: lipolysis of endogenous cardiac triglyceride (TG) stores, or from exogenous sources in the blood (as free acid bound to albumin or as TG in lipoproteins). The approximate contribution of FFA from exogenous or endogenous sources towards β-oxidation in the diabetic heart is unknown. In an insulin-deficient state, adipose tissue lipolysis is enhanced, resulting in an elevated circulating FFA. In addition, hydrolysis of the augmented myocardial TG stores could also lead to high tissue FFA. Whatever the source of FFA, their increased utilization may have deleterious effects on myocardial function and includes the abnormally high oxygen requirement during FFA metabolism, the intracellular accumulation of potentially toxic intermediates of FFA, a FFA-induced inhibition of glucose oxidation, and severe morphological changes. Therapies that target these metabolic aberrations in the heart during the early stages of diabetes could potentially delay or impede the progression of more permanent sequelae that could ensue from otherwise uncontrolled derangements in cardiac metabolism. (Mol Cell Biochem **180**: 53–57, 1998)

Key words: diabetes, cardiomyopathy, lipids, lipoprotein lipase, calcium

Introduction

Clinical and epidemiological reports have confirmed that cardiovascular disease is the major cause of death in diabetic patients [1, 2]. The mechanisms behind the high incidence of diabetic cardiac dysfunction remain relatively obscure and may involve atherosclerosis or a combination of microangiopathy, macroangiopathy, autonomic neuropathy and other factors which produce structural, functional and biochemical alterations in the heart [3]. However, cardiac problems associated with diabetes are not always implicated with the above factors [4, 5]. Thus, a specific cardiomyopathy may be a significant causal factor in the enhanced mortality and morbidity of diabetes. Chemically-induced diabetes also results in cardiac abnormalities in animal models [6–8]. In most animal studies, depressed contractility was not accompanied by alterations in oxygenation, coronary flow or the presence of major vessel disease. Thus, similar to the human condition, there may be factors unrelated to vascular disease which could contribute to the pathogenesis of diabetic cardiomyopathy in rodent models.

Etiology of diabetic cardiomyopathy

The etiology of diabetic cardiomyopathy is complex and a number of factors have been implicated in the pathogenic process. In the acute diabetic heart, metabolic derangements in both fuel supply and utilization by heart tissue could serve as the biochemical lesion initiating disease [9]. Over a chronic period, a number of subsequent vascular and cardiac compications develop, and involve an abnormal vascular sensitivity and reactivity to various ligands, depressed autonomic function, increased stiffness of the ventricular wall, and abnormalities of various proteins that control ion movements, particularly intracellular calcium [10].

Lipid metabolism during diabetes

Plasma triglycerides

Hypertriglyceridemia (Fig. 1B) characteristic of insulin dependent diabetes mellitus results from an increased

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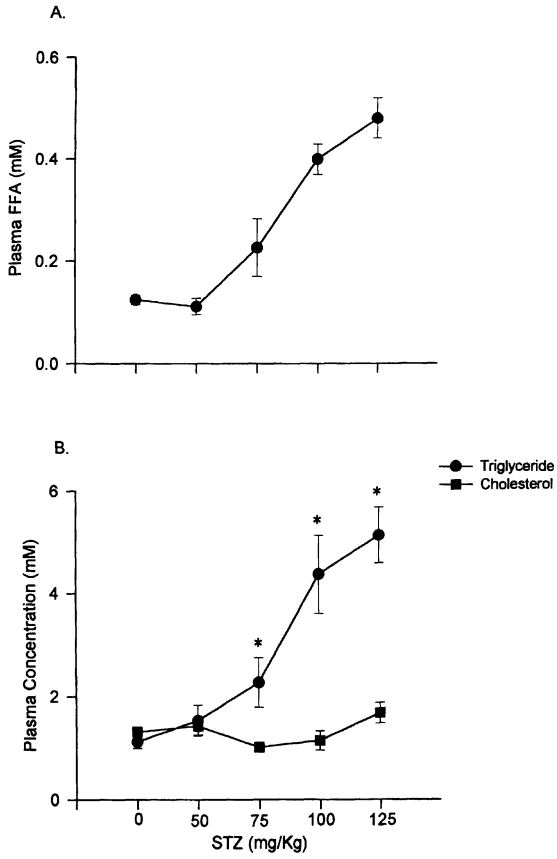

Fig. 1. Plasma free fatty acids (FFA), triglycerides and cholesterol in Wistar rats made diabetic with increasing doses of streptozotocin (STZ, i.v. 0.9% saline). After 4 days, blood samples were obtained from the tail vein prior to sacrifice. Data is mean ± S.E.M. *Significantly different from basal, $p < 0.05$.

secretion of very low density lipoprotein triglyceride (VLDL-TG) from the liver [11], and a reduced clearance of TG-rich lipoprotein as a result of depressed lipoprotein lipase (LPL) activity [12] and/or compositional changes in circulating lipoproteins [13]. Human and animals studies have attempted to correlate myocardial changes during diabetes with changes in plasma lipids. In the Framingham and WHO studies, serum triglyceride levels were a strong risk factor for cardiovascular disease among diabetics [14, 15]. Furthermore, in streptozotocin (STZ)-diabetic rats, treatments that lowered plasma lipids such as L-carnitine [16] and hydralazine [17] effectively prevented cardiac dysfunction. More recently however. several agents such as clofibrate, prazosin and enalapril, which produced triglyceride-lowering effects in STZ-diabetic rats were ineffective in preserving myocardial function [12]. Furthermore, despite the lack of hypertriglyceridemia in Wistar Kyoto diabetic rats, heart function was severely reduced in these animals [18]. Taken together, these findings suggest that in the diabetic rat, circulating triglyceride levels may not be an important determinant of cardiomyopathy.

Plasma free fatty acids (FFA)

In the heart, the source of cellular energy in the form of ATP is obtained via the oxidation of various substrates including FFA, glucose, lactate and ketone bodies, with FFA being the principal substrate utilized by the heart. Indeed in rats, the heart muscle accounts for a disproportionately large consumption of FFA with respect to body weight. The heart has a limited potential to synthesize FFA. Hence, fatty acids are supplied to cardiac cells from several sources: through lipolysis of endogenous cardiac TG stores, or from exogenous sources in the blood (as free acid bound to albumin or as TG in lipoproteins). During insulin deficiency, there is a marked increase in adipose tissue lipolysis with a subsequent outflow of FFA, which become greatly elevated in diabetic plasma (Fig. 1A). When the rate of FFA uptake exceeds the rate of disposal, myocardial triglyceride content is increased, as has been observed in perfused hearts [19] and in cardiac myocytes [20] obtained from diabetic rats. Subsequent hydrolysis of this expanded intracellular store of triglyceride could eventually lead to high tissue FFA levels in the diabetic heart [21, 21]. Circulating and cellular FFA levels if sufficiently large, may have adverse electrophysiological, biochemical and mechanical effects on the heart (for review see [9]).

Lipoprotein lipase

LPL is the enzyme that catalyzes the breakdown of the TG component of lipoproteins. Vascular endothelial-bound LPL determines the rate of plasma TG clearance and partially regulates FFA supply to the tissues; hence, it is also called 'functional' LPL [22]. Since endothelial cells cannot synthesize LPL, the enzyme is synthesized by the parenchymal cells of a variety of extrahepatic tissues, including adipose, heart, skeletal muscle, brain, and ovary. In the adult heart, LPL is synthesized and processed in myocytes and is translocated onto heparan sulfate proteoglycan (HSPGs) binding sites on the luminal surface of endothelial cells [23] where it actively metabolizes lipoproteins. Chylomicrons and VLDL bind transiently to endothelium binding lipolysis sites where functional endothelial LPL hydrolyzes the TG core to FFA and 2-monoacylglycerol [24], which are then transported into the heart for numerous metabolic and structural tasks.

The approximate contribution of FFA from exogenous or endogenous sources towards β-oxidation in the diabetic heart is not known. During diabetes, enhanced adipose tissue lipolysis together with an increased hydrolysis of the augmented intracellular cardiac TG store could lead to high circulating and tissue FFA levels. This serves to guarantee

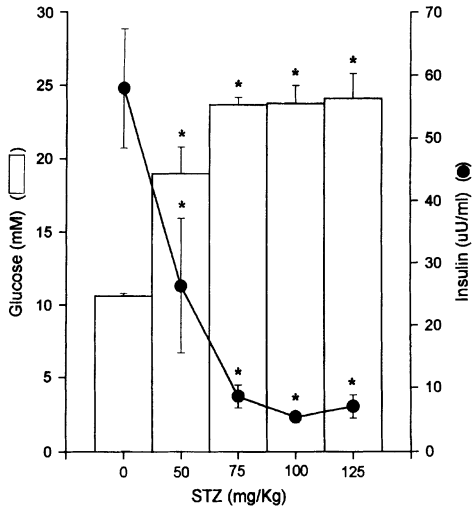

Fig. 2. Plasma insulin and glucose levels in Wistar rats made diabetic with increasing doses of STZ. After 4 days, blood samples were obtained from the tail vein prior to sacrifice. Data is mean ± S.E.M. *Significantly different from basal, $p < 0.05$.

FFA supply to the diabetic heart to compensate for the diminished contribution of glucose as an energy source. Considering these mechanisms that enhance cardiac FFA levels, the relative contribution of cardiac LPL activity to the delivery of FFA to the diabetic heart is unknown. An additional caveat is that available information on the influence of diabetes on heart LPL is inconclusive. Thus LPL immunoreactive protein or activity has been reported to be unchanged [25, 26], increased [27, 28] or decreased [29–31] in the diabetic rat heart. In part, this variability between different studies could be due to the diversity in the rat strains used, the dosage of streptozotocin (STZ) used to induce diabetes (resulting in diabetes of varying intensities, Fig. 2), and the duration of the diabetic state. In addition, many of the above investigations utilized procedures which did not distinguish between functional (i.e. heparin-releasable component localized on capillary endothelial cells that is implicated in the hydrolysis of circulating TG) and cellular (i.e. non-heparin-releasable pool that represents a storage form of the functional enzyme) pools of cardiac LPL as cellular LPL activity or protein levels have largely been obtained using whole heart homogenates.

To examine the contribution of the severity and duration of diabetes on heparin-releasable cardiac LPL activity, we induced diabetes in Wistar rats with a high (100 mg/kg; D100) or moderate (55 mg/kg; D55) dose of STZ and examined LPL activity at various times after diabetes induction. Severe diabetes in D100 rats resulted in a reduction in heparin-releasable LPL activity at one week of diabetes. However, in D55 rat hearts, peak heparin-releasable LPL activity was higher than control animals at 2 and 12 weeks after STZ injection (unpublished observations). The increased enzyme activity could lead to an accelerated hydrolysis of lipoprotein-TG, providing an additional source of FFA which could have deleterious effects in the diabetic heart. However, as compositional changes in circulating lipoproteins have also been reported during diabetes, making them poorer substrates for the enzyme [32], the role of an enhanced cardiac LPL in FFA supply to the diabetic heart is still unclear. The pathological role of an abnormally high LPL activity could also involve the modulation of LDL flux through the vascular tissue [33, 34]. As circulating LDL-cholesterol is elevated in diabetic rats [35], and can alter Ca^{2+} homeostasis in cardiomyoctes [36]. This could provide an additional mechanism for the development of cardiomyopathy in this model.

Carbohydrate metabolism during diabetes

Intracellular glucose disposal occurs through several major pathways. Nonoxidative glucose disposal primarily reflects the conversion to glycogen [37], whereas the oxidative pathway involves either the complete oxidation of glucose-derived carbon atoms to carbon dioxide or the conversion to fatty acids in lipogenic tissues. Whereas glycolysis, or the breakdown of glucose or glycogen to pyruvate provides a limited amount of ATP, it is the subsequent entry of pyruvate into the mitochondria and its oxidation that provides the majority of energy obtained from glucose. Insulin affects all areas of carbohydrate metabolism chiefly by controlling the transport of glucose. In insulin-responsive tissues (i.e. muscle, fat and heart), it has been shown that insulin can induce a rapid reversible translocation of glucose transporter proteins from a latent intracellular pool to the plasma membrane and a possible enhancement in the intrinsic activity of the transporters [38, 39]. Activation of glucose transport by insulin is followed by intracellular processes which are also further enhanced by insulin:glycogen synthesis, glycolysis and glucose oxidation. Hence, in the hypoinsulinemic condition, there is a significant reduction in the basal myocardial glucose utilization, as observed in isolated diabetic cardiomyocytes [40]. The major restriction to glucose utilization in the diabetic heart is the slow rate of glucose transport across the sarcolemmal membrane into the myocardium, which probably results from the cellular depletion of glucose transporters (GLUT 4) [41, 42]. The impaired glucose oxidation in the diabetic heart can also result from a decreased rate of phosphorylation of glucose which can subsequently limit the entry of glucose into the cell. The reduced phosphorylation has been proposed to result from the increased metabolism of FFA. An excessive FFA oxidation is at least partly responsible for the insulin resistance and depression of cardiac glucose oxidation, a notion introduced by the classic studies of Randle *et al.* [43]. They

suggested that an increased availability of FFA can stimulate the TCA cycle and increase citrate levels. The citrate formed inhibits phosphofructokinase, thereby reducing the rate of glycolysis which leads to a decrease in glucose uptake and oxidation. Furthermore, the reduction in substrate flow through the glycolytic pathway results in an eventual buildup in the tissue levels of glucose-6-phosphate which activates glycogen synthase and inhibits phosphorylase. These changes in enzyme activity appear to account for glycogen accumulation as the small amount of glucose that is transported is diverted toward glycogen production [44]. Another explanation for the reduced oxidation of glucose by the diabetic heart is that elevated FFA oxidation increases the acetyl CoA to CoA ratio which activates the pyruvate dehydrogenase kinase to phosphorylate and inactivate the pyruvate dehydrogenase complex (PDH) [45]. The end result is a diminished oxidation of pyruvate. Inhibition of glucose oxidation in the diabetic heart could also be due to a direct alteration in PDH activity [46].

Summary

The incidence of mortality from cardiovascular diseases is higher in diabetic patients. The cause of this accelerated cardiovascular disease is multifactorial and although atherosclerotic cardiovascular disease in association with well-defined risk factors has an influence on morbidity and mortality in diabetics, myocardial cell dysfunction independent of vascular defects have also been defined. We postulate that these adverse cardiac effects could presumably result as a consequence of the following sequence of events. Major abnormalities in myocardial lipid and carbohydrate metabolism occur as a result of insulin deficiency. These changes are closely linked to the accumulation of various acyl carnitine and coenzyme derivatives. Over time, chronic abnormalities such as reduced myosin ATPase activity, decreased ability of the sarcoplasmic reticulum to take up calcium as well as depression of other membrane enzymes such as Na^+-K^+ ATPase and Ca^{2+}-ATPase leads to changes in calcium homeostasis and eventually to cardiac dysfunction. From the point of view of pharmacological intervention, during the initial stages, acute disturbances in both the glucose and FFA oxidative pathways may provide the initial biochemical lesion from which further events ensue. Thus therapies which target these metabolic aberrations in the heart during the early stages of diabetes, in effect, can potentially delay or impede the progression of more permanent sequelae which could ensue from otherwise uncontrolled derangements in cardiac metabolism. Given the supportive data so far, the complex relationship between glucose and triglyceride breakdown and utilization requires more detailed examination. However, there is little dispute that a more rigorous attempt should be made to decrease the abnormally high rates of FFA oxidation and overcome the fatty acid inhibition of myocardial glucose utilization.

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Diabetes mellitus and cardiac function

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Abstract

Cardiovascular complications are the most common causes of morbidity and mortality in diabetic patients. Coronary atherosclerosis is enhanced in diabetics, whereas myocardial infarction represents 20% of deaths of diabetic subjects. Furthermore, re-infarction and heart failure are more common in the diabetics. Diabetic cardiomyopathy is characterized by an early diastolic dysfunction and a later systolic one, with intracellular retention of calcium and sodium and loss of potassium. In addition, diabetes mellitus accelerates the development of left ventricular hypertrophy in hypertensive patients and increases cardiovascular mortality and morbidity. Treating the cardiovascular problems in diabetics must be undertaken with caution. Special consideration must be given with respect to the ionic and metabolic changes associated with diabetes. For example, although ACE inhibitors and calcium channel blockers are suitable agents, potassium channel openers cause myocardial preconditioning and decrease the infarct size in animal models, but they inhibit the insulin release after glucose administration in healthy subjects. Furthermore, potassium channel blockers abolish myocardial preconditioning and increase infarct size in animal models, but they protect the heart from the fatal arrhytmias induced by ischemia and reperfusion which may be important in diabetes. For example, diabetic peripheral neuropathy usually presents with silent ischemia and infarction. Mechanistically, parasympathetic cardiac nerve dysfunction, expressed as increased resting heart rate and decreased respiratory variation in heart rate, is more frequent than the sympathetic cardiac nerve dysfunction expressed as a decrease in the heart rate rise during standing. (Mol Cell Biochem **180**: 59–64, 1998)

Key words: heart function, metabolic changes in the heart, diabetic cardiomyopathy

Introduction

Diabetes mellitus (DM) is a generalized metabolic disorder characterized by certain abnormalities in carbohydrate, fat, electrolyte and protein metabolism which ultimately lead to several acute and chronic complications. DM is classically classified into Insulin Dependent (IDDM) or (type I) and Non-Insulin dependent (NIDDM) or (type II). Predictors of diverse complications of DM could be listed as follows: the duration of the disease, previous use of insulin (negative correlation), glycemia, alcoholism, smoking habit, and intake of legumes (beans). Peripheral neuropathy, amputations, renal impairment, albuminuria, myocardial infarction, cataract, and *amaurosis fugax* are strongly associated with the duration of diabetes rather than with the age of the patient or the age at the diagnosis. In contrast, hypertension, and impotence are associated more with the age of the patient [1].

The purpose of this review is to summarize the cardiac complications associated with DM and to indicate means of cardiac protection. Cardiovascular complications are the most common causes of morbidity and mortality in the diabetic patients. The acceleration of atherogenesis occurs in all types of DM and culminates in such fatal complications as myocardial infarction, stroke and gangrene. Subjects with NIDDM exhibit 3–4 times higher rate of cardiovascular mortality than non-diabetic persons. Various hypothesis have been introduced to explain the relationship between DM and the cardiac diseases, for example, elevation of blood pressure, changes in lipid metabolism, hypo-insulinemia, abnormal hemostasis, and abnormal kidney function [2]. In this review, we will discuss three aspects of cardiac complications in the diabetic patients. These aspects are coronary atherosclerosis, diabetic cardiomyopathy and autonomic neuropathy.

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Coronary atherosclerosis

Many studies demonstrate that the prevalence of coronary artery disease (CAD) is increased among diabetic patients. CAD is the most common cause of death in type II DM, but it also contributes to mortality in type I diabetic patients. The incidence of CAD increases in female patients [3]. Myocardial infarction is the cause of death in about 20% of diabetic patients, with an increased incidence of complications such as congestive heart failure, cardiogenic shock, arrhythmias, and ventricular rupture. Letho *et al.* [6] reported that the infarct size in patient hearts, measured as maximal levels of serum cardiac enzymes and QRS score, are not significantly different between diabetic and nondiabetic patients. However, post-infarction mortality rate is higher in the diabetic patients because diabetic subjects have increased liability of complications as re-infarction and heart failure. Silent myocardial infarction is a common feature of CAD in diabetics as an indicator of neuropathy [4]. In diabetic patients, the heart shows a decrease in the maximal coronary vasodilator response to papaverine and adenosine therapy. This is accompanied by attenuation of the decreased coronary vascular resistance in response to the increasing myocardial metabolic demands as in the use of inotropic stimulation or rapid atrial pacing. These findings suggest structural and functional abnormalities in the coronary microcirculation in diabetic patients as they are not related to differences in drug therapy, resting hemodynamic or an incidence of hypertension [5].

Ionic hemostasis and ventricular arrhythmias

The ion shift in the form of intra-cellular sodium and calcium retention and potassium loss occurs in the ventricular myocyte in diabetic animals. This ion shift is pronounced when the heart is subjected to ischemia/reperfusion increasing the risk of ventricular tachycardia and fibrillation. These fatal ventricular arrhythmias are accentuated by K_{ATP} channel openers and blocked by K_{ATP} channel blockers. Glibenclamide is a K_{ATP} channel blocker commonly used as an oral hypoglycemic drug in NIDDM. This drug decreases the fatal ventricular arrhythmias mediated by ischemia reperfusion but not the spontaneous arrhythmias [7]. In the ischemic rat heart, the pH rise during reperfusion continues even with the blockade of Na^+/H^+ exchange by, amilioride. Recovery of pH occurs more rapidly in diabetic hearts receiving HEPES buffered solution than in those receiving bicarbonate buffered solution suggesting that the bicarbonate dependent mechanism of pH regulation may be depressed in diabetes [8]. Reduction of the risk of CAD in diabetics could be achieved mainly by the control of obesity, correction of hypertension, elimination of cigarette smoking and improvement of LDL/HDL cholesterol level. Tight metabolic control and insulin infusion in early post-infarction do not have immediate beneficial effect. However, the long term mortality which is associated with re-infarction and heart failure is decreased with metabolic management [6].

Coronary artery bypass graft (CABG) is usually considered in the management of diabetic patients with CAD. Diabetic CAD patients usually present with an angiographically triple vessel disease with no predominant single vessel affected [9]. The complications during and after CABG operation increase in the diabetic patients, amd especially include sternotomy complications, renal insufficiency, and cerebral stroke [10].

Diabetic cardiomyopathy

While it had been thought that atherosclerotic vascular disease was responsible for all the adverse effects of DM on the heart, recent studies support the notion that one of the major adverse complications of DM is the development of diabetic cardiomyopathy. Diabetic cardiomyopathy is characterized by early diastolic dysfunction and late systolic impairment. Contributing to the development of the cardiomyopathy is a shift of myosin isoenzyme content in favor of the least active V_3 form. The main ionic defect in diabetic cardiomyopathic cell is a defect in the regulation of calcium hemostasis during transport of calcium by the sarcolemma and sarcoplasmic reticulum. Calcium pumps are minimally affected by non-insulin dependent diabetes. Significant impairment occurs in sarcolemmal sodium-calcium exchanger activity. This defect limits the ability of the diabetic heart to extrude calcium, contributing to an elevation in intracellular calcium. The decrease in Na^+/K^+ ATPase activity increases intracellular calcium retention secondary to increased sodium; in addition, calcium influx via the calcium channel is stimulated. Although the molecular mechanisms underlying these abnormalities are presently unknown, the possibility that they may be related to aberrations in glucose and lipid metabolism are considered. Evidence suggests that classical theories of glucose toxicity, such as excessive polyol production or glycosylation appear to be insignificant factors in heart. Also defects in lipid metabolism leading to the accumulation of toxic lipid amphiphiles or tri-acylglycerol appear insignificant. Rather, the major defects seem to involve changes in membrane structure, such as phosphatidyl-ethanolamine N-methylation and protein phosphorylation which can be attributed to the state of insulin resistance [11].

To study the abnormalities in the myocardium energy metabolism in the diabetic animals, by using ^{13}C-NMR spectroscopy, glucose metabolism in the isolated diabetic perfused rat heart was studied. In the control hearts, the labelled form (^{13}C)glucose was incorporated into lactate and

glutamate through the glycolysis and the Kreb's cycle respectively. Lactate and glutamate were not formed in the diabetic hearts. Addition of insulin (0.05 unit/ml) to the buffer of the diabetic heart resulted in the appearance of (3–13 C) lactate, whereas addition of insulin and dichloroacetate (2 mM) resulted in the appearance of glutamate indicating glucose entry into glycolysis and tricarboxylic acid cycle respectively [12]. Stimulation of glucose oxidation by L-carnitine improves mechanical recovery of ischemic hearts from non-diabetic rats perfused with high levels of fatty acids. It also increases glucose oxidation and improves mechanical function following ischemia in diabetic rat hearts. Thus, carnitine improves recovery of function of ischemic non diabetic rats by stimulating glucose oxidation during reperfusion whereas it is beneficial in diabetic rat hearts by stimulating both glycolysis during ischemia and glucose oxidation during reperfusion [13]. The diastolic dysfunction in the perfused hearts of the streptozatocin-diabetic rats is related to the amount of interstitial glycogen deposition in the sub-endocardium, largely attributable to diminished degradation, which increases the left ventricular stiffness [14]. In a rat model of DM, streptozotocin (55–65 mg/kg body weight) causes hyperlipidemia and hypothyroidism and decreased myocardial sensitivity to isoproterenol and beta adrenoreceptor stimulants [15].

To study the abnormalities of fatty acid metabolism in the myocardium of diabetic patients, Shinmura *et al.* [16], studied ^{123}I-beta-methyl-iodophenyl-pentadecanoic acid (BMIPP) myocardial scintigraphy in 15 diabetic patients. They excluded patients with CAD according to patient history, EKG, treadmill exercise testing, echocardiography, and resting ^{201}T1 scintigraphy and also excluded patients with markedly impaired left ventricular systolic function (FS<30G/c). BMIPP uptake scores as the ratio of Heart/mediastinum (H/M) and liver/mediastinum (L/M) at 20 min after injection. These scores were analyzed and compared with clinical profile, serum parameters, and LV parameters obtained from echocardiography and study of the sympathetic nerve function by ^{123}I-metaiodobenzyl-guanidine (MIBG). Five of the 15 patients showed abnormal BMIPP uptake, 2 patients showed decreased uptake in the inferior segments, while 3 showed a diffuse decrease in BMIPP uptake. Body mass index, fasting blood sugar, and left ventricular end diastolic dimension (LVEDD) were higher with impaired ^{123}I-MIBG uptake in these 5 patients group with abnormal BMIPP findings. These results suggest that diabetic patients without CAD show abnormal BMIPP imaging when their general glucose utility and ^{123}I-MIBG uptake are severely impaired (progression of insulin resistance and sympathetic nerve involvement). The authors of this study concluded that BMIPP scintigraphy may be useful in investigating the pathogenesis and subclinical abnormality of diabetic heart [16].

The addition of ascorbic acid to the diet of the diabetic rats caused decrease in the hyperlipidemia with the correlation of polydipsia, hyperphagia and myocardial dysfunction, however, the decreased body weight gain, hypoinsulinemia, and hyperglycemia were not affected [17].

Impaired myocardial function was observed after 8 weeks following treatment with streptozotocin in a rat model of DM. Salt loading with 0.9% saline solution improved the impaired myocardial function with significant reduction in hyperphagia and hyperlipidemia and early decrease in the blood glucose level with no significant change in blood pressure or plasma insulin level [18]. The hypothesized atherogenic role of endogenous insulin is based on a series of epidemiological studies. Several large scale prospective studies showed that hyperinsulinemia, while fasting or after glucose stimulation, constitutes a risk factor for fatal myocardial infarction as excessive endogenous insulin precipitates hypertension and subsequent myocardial infarction [19].

Pathological studies of diabetic cardiomyopathy have shown myocardial hypertrophy and interstitial fibrosis. Considerable controversy exists regarding the presence and significance of small vessel disease involving intramural coronary arteries, arterioles and capillaries. Capillary microaneurysms have been demonstrated in the diabetic heart, earlier studies demonstrated cardiomyopathy without hypertension and post-mortem studies revealed cellular hypertrophy and interstitial and myco-cytolytic necrosis with replacement fibrosis [20]. In the experimental animals, the presence of hypertension with diabetes changes its relatively benign course. In rats, inducing hypertension by renal artery stenosis after the induction of diabetes by streptozocin, causes a severe and often fatal cardiomyopathy with congestive heart failure and marked abnormalities in myocardial function. Pathological changes demonstrate micro-vascular abnormalities with focal myo-cellular necrosis and interstitial fibrosis [21].

Hemodynamic studies in patients with congestive heart failure have shown evidence of either congestive or restrictive cardiomyopathy. Non-invasive studies have permitted evaluation of ventricular function in large groups of diabetic patients. Systolic time intervals (STIs) are frequently normal, reflecting decreased contractility and/or a reduction in the preload. Patients with micro-angiopathy are particularly, likely to have abnormal STIs [22]. The diastolic function of the left ventricle is early impaired in diabetic patients while the systolic function, especially if the subject is normotensive, is less commonly affected. In a study including 157 young type I asymptomatic subjects (mean age is 26.6 years), by the use of M-mode echocardiography, the diastolic dysfunction (slope of anterior mitral leaflet in early diastole, isovolumic relaxation time, and left atrium emptying index) was twice as common as the systolic dysfunction (Fractional shortening, mean velocity of circumference fiber shortening and stroke

index) [23]. The most typical feature of diabetic cardiomyopathy is the abnormal filling pattern of the left ventricle, suggesting reduced compliance or prolonged relaxation [24].

In dogs, when DM is induced, the heart shows decreased left ventricular compliance and increased interstitial connective tissue. In experimentally induced diabetes in rats, isolated left ventricular papillary muscle demonstrated marked slowing and prolonged contraction with delayed relaxation, despite the fact that developed tension was maintained and compliance was un-altered. Insulin reverses these findings. The explanation of this findings is that with the development of diabetes in rats, myosin ATPase, the rate limiting enzyme for force and shortening of contractile proteins is decreased with a shift in the isoenzyme form of myosin from the faster V_1 to the slower V_3 form, the delayed and slowed relaxation also correlates with a reduction in the calcium binding of isolated sarcoplasmic reticulum [25].

Diabetes mellitus accelerates the development of left ventricular hypertrophy in hypertensive patients. By using the Devereux criteria for recognition of left ventricular hypertrophy (LV index above 134 gm/m^2 in men and above 110 gm/m^2 in women), echocardiographic studies showed the increased inter-ventricular septum and posterior wall thickness than in non-diabetic hypertensive patients. Therefore, left ventricular mass index was greater in patients with hypertension and DM than those without DM, this increases the incidence of cardiovascular mortality and morbidity in patients with hypertension with diabetes mellitus [26].

Drug interaction in diabetes and cardiovascular disorders

Treating hypertension in diabetic patients with drugs must be taken cautiously. Calcium channel blockers and angiotensin converting enzyme (ACE) inhibitors are suitable agents. The ACE inhibitor Captopril prevents the increase in diastolic pressure, coronary perfusion pressure and vascular resistance and partially prevent the development of cardiac dysfunction. These observations suggest that increased release of angiotensin II plays a significant role in the change from reduced adaptability to irreversible damage. The effect of calcium blockers on glucose hemostasis, lipid metabolism and renal function support their routine use as alternations to ACE inhibitors [27].

The K_{ATP} channel openers as pinacidil causes myocardial preconditioning against myocardial infarction and decreases the infarct size in different animal models with anti-hypertensive properties [28]. Lawton *et al.*[28], described the cardioprotection of pinacidil as effective as the St. Thomas potassium cardioplegic solution in the isolated rabbit heart. Potassium openers are under investigation for the treatment of essential hypertension and angina pectoris. Pro-arrhythmic effect of potassium channel openers have been observed at high doses [29]. The effect of potassium channel openers on the pancreas is still not well established. Some investigators described the absence of a negative effect of K-channel openers on insulin secretion *in vivo* [30]. Others described the potassium channel openers' inhibitory effect on the insulin release after glucose administration in healthy patients [29]. On the other hand, the sulfonylurea derivative glibenclamide which is used in the control of type II DM, exerts its insulinotropic effect by closing the K_{ATP} channels of the pancreatic beta cells. Potassium channel blockers abolish myocardial preconditioning by their membrane depolarizing effect. However, claims that treating NIDDM with these agents increases cardiovascular mortality are not supported by sound evidence. Glibenclamide, even, protects the heart from fatal arrhythmias caused by ischemia and reperfusion, but not the spontaneous ones [30].

Autonomic neuropathy

Heart rate variability with respiration and standing are decreased in diabetic patients especially those with evidence of peripheral or autonomic neuropathy. Defects in parasympathetic innervation (expressed as an increased resting heart rate and a decreased respiratory variation in heart rate) are more frequent and occur relatively early in diabetic patients. Defects in sympathetic innervation (expressed as a decrease in the heart rate rise during standing) are less frequent and tend to occur in diabetic patients. With respect to clinical significance, a prospective study of diabetic patients with or without diabetic neuropathy revealed a markedly diminished survival in the former group, with a substantial number of sudden death. An increased tendency to ventricular arrhythmias leading to sudden death may reflect QT prolongation. A recent study revealed a high incidence of QT prolongation (at rest and especially after the exercise) in diabetic patients with autonomic neuropathy, possibly reflecting autonomic imbalance. Postural hypotension commonly causes symptoms in diabetic individuals with autonomic neuropathy especially after bed rest and use of diuretics or other anti-hypertensive medications. Silent myocardial infarction can occur with or without autonomic dysfunction, but its incidence is greater in the diabetic patient. Cardiac imaging using ^{123}I-metaiodobenzylguanine (MIBG) reflects sympathetic myocardial innervation; in diabetic patients MIBG shows sympathetic myocardial dysinnervation, and that subclinical LV dysfunction is related to the dysfunction of the adrenergic cardiac innervation [31].

The clinical assessment of cardiac involvement in diabetic autonomic neuropathy has been limited to cardiovascular reflex testing. With the recent introduction of radio-labeled catecholamines such as ^{11}C-hydroxyephedrine, the sympathetic innervation of the heart can be specifically visualized

with Positron Emission Tomographic (PET) imaging. The rest of the myocardial blood flow imaging with nitrogen 13 ammonia. In cases of autonomic neuropathy, the relative tracer retention is reduced in the apical, inferior, and lateral segments. The extent of the abnormality correlated with the severity of conventional markers of autonomic dysfunction. Absolute myocardial tracer retention index measurements show a decrease in distal compared with proximal myocardial segment in autonomic neuropathy [31]. Autonomic cardiac neuropathy affects the myocardial function, diabetic patients suffer from sub-clinical ventricular diastolic dysfunction frequently at rest and sympathetic over-activity [32]. Diabetic somatic neuropathy (by measuring the motor conduction velocities of the peroneal and tibial nerves and the sensory conduction velocity of the sural nerve) was found to be independent on the cardiosympathetic neuropathy assessed by myocardial scintigraphy in patients with non-insulin dependent DM [33].

Summary

Cardiovascular complications are the most common causes of morbidity and mortality in the diabetic patients. Coronary atherosclerosis is enhanced in diabetics, and myocardial infarction represents 20% of deaths of diabetic subjects. Re-infarction and heart failure are more common in the diabetic. Diabetic cardiomyopathy is characterized by an early diastolic dysfunction and a later systolic one, with intracellular retention of calcium and sodium and loss of potassium. DM accelerates the development of left ventricular hypertrophy in the hypertensive patients and thus, increases the cardiovascular mortality and morbidity.

Treating the cardiovascular problems in the diabetics must be done with caution, considering the ionic and metabolic changes occurring with diabetes. As a result, ACE inhibitors and calcium channel blockers are often suitable agents. Potassium channels are found in many body tissues. Potassium channel openers cause myocardial preconditioning and decrease infarct size in animal models, but they inhibit the insulin release after glucose administration in healthy subjects. Potassium channel blockers abolish myocardial preconditioning and increase the infarct size in the animal model, but they protect the heart from the fatal arrhythmias induced by ischemia and reperfusion. Potassium channel blockers are used (as oral hypoglycemic agents) to induce insulin production from the beta cells of the pancreas.

Diabetic neuropathy affects the peripheral as well as the autonomic nerves, peripheral neuropathy usually presents with silent (painless) infarction. Parasympathetic cardiac nerve dysfunction, expressed as increased resting heart rate and decreased respiratory variation in heart rate, is more frequent than the sympathetic cardiac nerve dysfunction expressed as a decrease in the heart rate rise during standing.

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Phospholipase A_2-mediated hydrolysis of cardiac phospholipids: The use of molecular and transgenic techniques

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Abstract

Under pathophysiological conditions, like myocardial ischemia and reperfusion, cardiac phospholipid homeostasis is severely disturbed, resulting in a net degradation of phospholipids and the accumulation of degradation products, such as lysophospholipids and (non-esterified) fatty acids. The derangements in phospholipid metabolism are thought to be involved in the sequence of events leading to irreversible myocardial injury. The net degradation of phospholipids as observed during myocardial ischemia may result from increased hydrolysis and/or reduced resynthesis, while during reperfusion hydrolysis is likely to prevail in this net degradation. Several studies indicate that the activation of phospholipases A_2 plays an important role in the hydrolysis of phospholipids. In this review current knowledge regarding the potential role of the different types of phospholipases A_2 in ischemia and reperfusion-induced damage is being evaluated. Furthermore, it is indicated how recent advances in molecular biological techniques could be helpful in determining whether disturbances in phospholipid metabolism indeed play a crucial role in the transition from reversible to irreversible myocardial ischemia and reperfusion-induced injury, the knowledge of which could be of great therapeutic relevance. (Mol Cell Biochem **180**: 65–73, 1998)

Key words: myocardial ischemia, ischemia-reperfusion, phospholipid metabolism

Introduction

All living cells are surrounded by membranes, consisting of phospholipids, cholesterol and proteins. The cell membrane (plasmalemma) serves as a selective barrier to create and maintain an internal environment to allow the cell to fulfil its specific functions. In addition to its function as physical barrier, the plasmalemma is also an important source of phospholipid-derived bioactive lipids, like lysophospholipids and fatty acids, such as arachidonic acid [1]. Arachidonic acid can subsequently serve as a substrate for the production of auto/paracrine factors, including prostaglandins and thromboxanes [2, 3].

The integrity of the membrane is a prerequisite for proper functioning of the cell. Therefore, the major constituents of the plasmalemma, the phospholipids, are subjected to a continuous turnover process to enable the cell to synthesize any required phospholipid and to regulate the fatty acyl composition of the phospholipids [1]. In this way, the cellular membrane adjusts its physico-chemical properties in response to changes in the extracellular environment. The turnover of phospholipids requires the liberation of fatty acids from phospholipids (deacylation), as well as the reincorporation of fatty acyl moieties into the phospholipid pool (reacylation). These phospholipid turnover cycles are also operative in cardiomyocytes [1].

During myocardial ischemia/reperfusion a net phospholipid degradation has been observed, either caused by increased activity of phospholipid hydrolyzing enzymes, such as phospholipase A_2, or caused by an impairment of the resynthesis of phospholipids [1, 4–6]. Therefore, detailed knowledge of cardiac phospholipid homeostasis

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and derangements therein under pathophysiological conditions can be of great therapeutic value, as ischemic heart diseases as a result of the occlusion of a coronary artery are still by far the most common cause of death in western industrialized countries.

In this review we will focus on cardiac phospholipase A_2-mediated phospholipid hydrolysis and its possible role in the derangements in phospholipid homeostasis as a crucial event in the transition from reversible to irreversible cell damage as a result of myocardial ischemia and reperfusion. Although not the subject of this review, it is important to note that derangements in the resynthesis of phospholipids may also be involved, especially under energy-deprived conditions like ischemia. In addition, the usefulness of sophisticated molecular biological techniques in combination with an appropriate murine heart model in the assessment of the role of phospholipases A_2 during myocardial ischemia and reperfusion will be discussed.

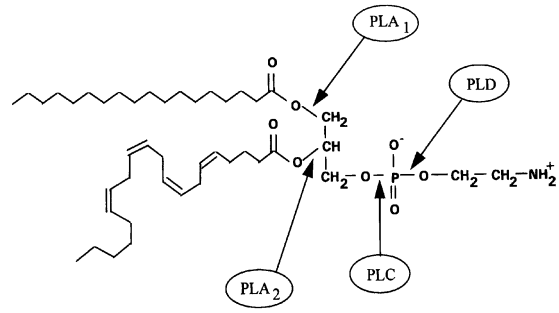

Fig. 1. Chemical structure of 1-palmitoyl, 2-arachidonoyl phoshatidylethanolamine and the cleavage site of different phospholipases. The arrows point to the covalent bond hydrolyzed by phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD).

Cardiac phospholipids

Myocardial membranes consist of a variety of phospholipid species, differing in chemical composition of the hydrophylic alcoholic headgroup, or of the hydrophobic tail that is composed of two long-chain fatty acyl residues connected to the glycerol backbone. The phospholipid subtypes are classified by their hydrophylic alcohol headgroup. The hydrophylic alcohol is attached to the third (*sn-3*) carbon atom of the glycerol moiety via an inorganic phosphate (Fig. 1). The hydrophobic part of phospholipids is formed by the fatty acyl chains bound to the first (*sn-1*) and second (*sn-2*) carbon atom of the glycerol backbone (Fig. 1). The *sn-1* and *sn-2* fatty acyl residues are generally a saturated and (poly-)unsaturated fatty acid, respectively. The number of carbon atoms of the long-chain fatty acyl residues commonly ranges from 14–24. The number of unsaturated bonds of the *sn-2* fatty acyl chain may vary between zero and six [1].

The fatty acyl residues at the *sn-1* and *sn-2* position are generally O-acyl-residues, i.e. they are connected to the glycerol backbone via an ester linkage. In membrane phospholipids, however, a certain proportion of the fatty acyl residues at the *sn-1* position is connected to the *sn-1* carbon atom of glycerol via a vinyl ether linkage (O-[1-alkenyl]-residues). These phospholipids are commonly referred to as plasmalogens. The plasmalogen content in membranes is tissue-dependent and varies between cardiac membranes of different animal species, ranging from ~5% in the rat heart to ~40% in the rabbit heart [7].

Because of their shape and amphipathic nature, phospholipids readily form lipid bilayers in an aqueous environment. In case of the sarcolemma, the hydrophilic headgroups of the inner leaflet point towards the intracellular space and those of the outer leaflet towards the extracellular space. The fatty acid tails are buried in the inner part of the bilayer.

The various phospholipid classes in the sarcolemmal bilayer are asymmetrically distributed. The outer leaflet is relatively enriched with phosphatidylcholine and sphingomyelin, while the negatively charged phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are located almost exclusively in the cytoplasmic leaflet. This phenomenon results in a more negatively charged inner leaflet as compared to the outer sarcolemmal leaflet. To compensate for differences in fluidity between the leaflets, the cholesterol/phospholipid ratio is higher in the outer leaflet of the sarcolemma [8].

Phospholipid homeostasis in the normoxic situation

Under normoxic conditions the amount of intracellular (non-esterified) fatty acids is very small, indicating that the pool size of these fatty acids is well controlled [1]. Apparently a strict balance exists between the activity of enzymes that control the liberation of fatty acyl moieties from phospholipid molecules and that of enzymes that control the rate of incorporation of fatty acyl residues into the phospholipid pool.

The cardiomyocyte is equipped with a set of enzymes required for the resynthesizing part of phospholipid turnover as well as for the hydrolytic part of the phospholipid turnover cycle. *De novo* synthesis of phospholipids takes place in the sarcoplasmic reticulum. Newly synthesized phospholipids are transported to the sarcolemma through the cytosol [1]. For *de novo* synthesis of phoshatidylcholine the condensation of 1,2-diacylglycerol and CDP-choline is achieved by the action of choline-phosphotransferase. When the sub-

strate is a lysophoshatidylcholine, the incorporation of the second fatty acyl moiety is dependent on the activity of the enzymes acyl-CoA synthetase and lysophosphatidylcholine acyltransferase (Fig. 2).

Among the enzymes involved in the hydrolytic part of the phospholipid turnover process are phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2), which hydrolyze the ester bond between the fatty acyl unit at the *sn-1* and *sn-2* carbon atom of glycerol, respectively. In case of a plasmalogen, a plasmalogen-specific type PLA_2 is present in the cardiomyocyte to remove fatty acyl chains from the *sn-2* position [9]. The vinyl ether bond at the *sn-1* position is hydrolyzed by a specific plasmalogenase [10]. Phospholipase C (PLC) breaks the covalent bond between the polar headgroup and the carbon atom at the *sn-3* position of glycerol and phospholipase D (PLD) the bond between phosphate and the alcohol unit that together form the polar headgroup (Fig. 1). The final products of phospholipase A_1 and A_2 activity are lysophospholipids and fatty acids. In turn, the remaining fatty acyl chain of lysophospholipids can be removed by lysophospholipase. It has been hypothesized that in cardiac cells the balance between the activities of lysophospholipid acyltransferase and phospholipase A_2 determines the actual level of unesterified arachidonic acid [1, 11].

Studies with radiolabelled substrates show that the cardiomyocyte is able to change the polar headgroup as well as the fatty acyl units of the membrane phospholipids [1]. Detailed knowledge about phospholipid turnover rate in cardiomyocytes, however, is still lacking. *In vitro* measurements revealed a maximal myocardial total phospholipase activity of about 3 μmol phoslolipid . h^{-1} . g^{-1} tissue in rat heart [12]. Based on the presence of approximately 30 μmol total phospholipid . g^{-1} tissue and assuming that degradation of phospholipids keeps pace with resynthesis, complete turnover of all sarcolemmal phospholipids could be accomplished in about 10 h. However, it cannot be excluded that phospholipid species in different subcellular compartments are subjected to different turnover rates [13, 14].

The heart contains at least three different types of phospholipases A_2. Recently, a member of the secretory or group II PLA_2 was cloned in our laboratory [15]. All mammalian group II PLA_2s are small molecular weight acylhydrolases of about 14 kD with a slight preference for phosphatidylserine and phosphatidylethanolamine over phosphatidylcholine, but they have no specificity for the type of fatty acyl moiety at the *sn-2* position. Group II PLA_2s have been found to be associated with membranes like the plasmalemma of guinea pig spermatozoa [16], the outer contact sites and the inner membrane of mitochondria [17], or the matrix of α-

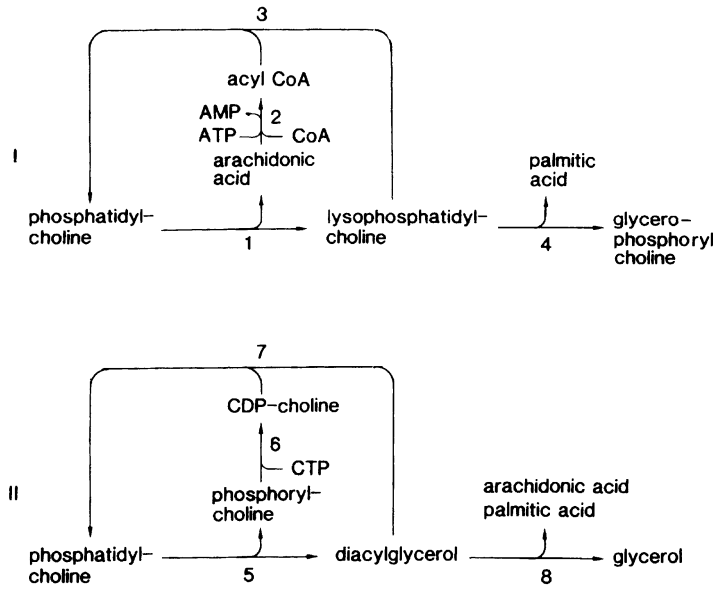

Fig. 2. Synthesis and degradation of phosphatidylcholine. *Pathway I* (upper panel) describes the deacylation-reacylation pathway for this phospholipid. In this example 1-palmitoyl, 2-arachidonoyl phosphatidylcholine is depicted. *Pathway II* (lower panel) depicts the turnover of the hydrophylic headgroup of phosphatidylcholine. Numbers refer to the enzymes that are involved in the pathways: (1) phospholipase A_2, (2) fatty acyl-CoA synthetase, (3) lysophosphatidylcholine acyltransferase, (4) lysophospholipase, (5) phospholipase C, (6) CTP:phosphocholine cytidylyltransferase, (7) phosphocholine transferase, (8) diacylglycerol + monoacylglycerol lipases. (Reprinted with permission from Van der Vusse *et al.* [42]).

granules in platelets [18]. Furthermore, group II PLA_2 can be secreted from rat platelets upon stimulation, due to the presence of a putative eukaryotic signal sequence for secretion at the N-terminal end of the enzyme [19]. Whether in cardiomyocytes group II PLA_2 is present in a membrane-associated form or can be secreted remains to be elucidated. Circumstantial evidence for a membrane-associated localization of group II PLA_2 in cardiomyocytes was provided by a cytochemical study by Kriegsmann and coworkers, in which they showed that a monoclonal antibody against bee venom group II PLA_2 bound to antigens at the level of the sarcolemma [20].

The knowledge of mechanisms involved in the regulation of group II PLA_2 activity is gradually increasing; mechanisms of short-term regulation of the enzyme activity and those involved in long-term regulation of enzyme content can be distinguished [6]. Short-term regulation is based on the requirement of (sub)millimolar calcium concentrations for maximal activity of the enzyme. This raises questions about the probability of its intracellular activity under normoxic conditions, as in the cardiomyocyte the overall intracellular calcium concentration oscillates from 0.15 μM during diastole to 2.0 μM during systole. However, Langer and colleagues [21] have demonstrated that in specific regions close to the sarcolemma calcium concentrations up to 600 μM may be reached during systole, postulating the existence of multiple compartments with different calcium concentrations within the cytosol. Accordingly, the calcium concentration in the subsarcolemmal space might be sufficient to allow intracellular activity of group II phospholipase A_2 under physiological conditions.

As far as the short-term regulation of extracellular activity is concerned evidence has been provided that group II PLA_2 activity can be modulated by the association of the enzyme via its putative C-terminal heparin-binding domain to the proteoglycans of the extracellular matrix. The association of group II PLA_2 with the sulfated polysaccharides results in changes of enzyme activity on membrane phospholipids [22]. This mechanism of regulation could be of importance for the heart, if cardiac group II PLA_2 is associated with the extracellular part of the sarcolemma, where calcium concentrations are no longer a limiting factor for the activity of the enzyme. Finally, it appears that group II PLA_2 activity can be modulated by accessory proteins, like phospholipase A_2-activating protein (PLAP) which has a stimulatory effect, or uteroglobins and annexins which are putative inhibitors of group II PLA_2 activity. The exact mechanism of action and the physiological significance of these accessory proteins on group II PLA_2 activity are still a matter of debate [6, 23].

Long-term regulation of group II PLA_2 activity is accomplished by adjusting the level of gene transcription. In several cell types group II PLA_2 expression is induced by inflammatory cytokines like IL-1, IL-6, and TNFα, or by cAMP-elevating substances like forskolin [24–26]. These observations were substantiated by the demonstration of the presence of cAMP and cytokine responsive elements in the 5′-flanking region of the group II PLA_2 gene [27]. In contrast, the expression of this enzyme is downregulated by a variety of substances like glucocorticoids, TGFβ, aspirin, and tetranactin [24, 28–30]. Recently it was shown that activation of the nuclear transcription factor NF-κB is an essential component of the cytokine signalling pathway responsible for group II PLA_2 gene regulation [28]. That this mechanism of regulation is also operative in cardiac cells is supported by the observation that group II PLA_2 mRNA levels increase in cultured rat neonatal cardiomyocytes upon stimulation with TNFα [15].

The recent discovery of a high-molecular mass phospholipase A_2 (85–110 kD) or cytosolic PLA_2 ($cPLA_2$) led to a different view of the activation of *sn-2* acylhydrolysis through receptor mediated signal transduction [31]. This enzyme selectively cleaves arachidonoyl residues at the *sn-2* position of membrane phospholipids. $cPLA_2$ is equally active against ethanolamine- and choline containing phospholipids, and hydrolyzes both 1-acyl-2-arachidonoyl phospholipids, and 1-alkenyl-2-arachidonoyl phospholipids. $cPLA_2$ has a predominantly cytosolic localization and requires Ca^{2+} in the micromolar range for translocation to its site of action, the phospholipid bilayer. Because of these features, the high molecular mass cytosolic phospholipase A_2 is a likely candidate for the acute liberation of arachidonic acid from membrane phospholipids in cardiac cells under physiological conditions, e.g. by alterations in the cellular Ca^{2+} concentration as a result of ligand-receptor interactions. This notion is substantiated by the observation that $cPLA_2$ itself is a substrate for mitogen-activated protein kineses (MAPK), which serve as important mediators for a variety of receptor-mediated signal transduction pathways [32].

The third type of phospholipase A_2 present in the myocardium was first reported in 1985 by Gross and coworkers [33]. It has a molecular mass of ~40 kD and was shown to be Ca^{2+}-independent for its activity. Although it has a preference for *sn-2*-arachidonoyl plasmalogens, it is also capable of hydrolyzing diacyl-phospholipids or plasmalogens with other fatty acids at the *sn-2* position, albeit at lower rate [34]. Ca^{2+}-independent, plasmalogen specific PLA_2 is localized in the cytosol and can translocate to the cell membrane by interacting with the glycolytic enzyme phosphofructokinase (PFK), forming a large 400 kD protein complex, indirectly regulating the activity of PLA_2 via allosteric modulation of PFK by cytosolic ATP levels [35]. Recently a novel regulatory mechanism was identified whereby the Ca^{2+}-independent phospholipase A_2 is modulated indirectly by calcium ions, i.e. through alterations in the interaction of the phospholipase complex with calmodulin [36].

To summarize, at least three different types of phospholipase A_2 activities have been identified in the heart: a 14 kD Ca^{2+}-dependent group II PLA_2, a plasmalogen-specific, Ca^{2+}-independent PLA_2 and a high molecular mass, cytosolic PLA_2 (cPLA$_2$). Recently, Chen and colleagues cloned another low molecular mass Ca^{2+}-dependent phospholipase A_2 from rat heart that showed only limited homology to group II phospholipase A_2 [37]. Dennis and coworkers demonstrated that group II PLA_2, cytosolic PLA_2 and Ca^{2+}-independent PLA_2 were involved in the release of arachidonic acid following stimulation of P388D$_1$ macrophages [38]. Interestingly, group II PLA_2 was accountable for 60–70 % of the AA release in this particular model system. As far as cardiomyocytes are concerned the contribution of the different PLA_2 types in the ligand-receptor-induced hydrolysis of phospholipids remains to be established.

Effect of ischemia and reperfusion on cardiac phospholipids

Ischemic heart diseases are predominantly caused by impaired coronary perfusion. If the ischemic period has a limited duration, structural and functional recovery of the myocardium is possible by timely restoration of blood flow. If the ischemic period is prolonged, however, injury becomes irreversible, eventually leading to cell death and cardiac dysfunction. During myocardial ischemia phospholipid homeostasis is disturbed. The net degradation of phospholipids results in an increase of their degradation products, mainly fatty acids and lysophospholipids [5, 39–41]. The sarcolemma appears to be the primary target for accelerated phospholipid degradation in oxygen and energy-depleted cardiomyocytes [1]. Although timely restoration of blood flow is required to save the myocardium from irreversible damage, an even more dramatic rise in the tissue levels of lysophospholipids and fatty acids has been observed during the reperfusion phase. Whether this reperfusion-induced injury is a manifestation of cell damage which was already present during ischemia or is a caused by the re-introduction of blood flow *per se* to the previously ischemic myocardium, is still a matter of debate [41, 42].

Currently there are two hypotheses concerning the mechanism involved in the increased phospholipid degradation of the sarcolemma during ischemia and reperfusion [41–43]. The first hypothesis implies that the integrity of the sarcolemma is lost due to physical forces, before the phospholipids are hydrolysed. Mechanical stress will be imposed on the sarcolemma of energy-deprived cells by adjacent non-ischemic, contracting myocytes or by hypercontracture of the ischemic cells themselves. In addition, low-molecular weight substances such as lactate, protons and inorganic phosphate, will accumulate inside ischemic myocytes. This will lead to a shift of water from the extracellular to the intracellular space, imposing an increased osmotic force on the cell membrane. Together with weakening of the anchoring of the cytoskeleton to the sarcolemma, this might result in enhanced membrane fragility. When the sarcolemma is unable to withstand the physical forces anymore the sarcolemma will rupture, after which intracellular structures will become accessible to the extracellular fluid. This will lead to the activation of, amongst others, Ca^{2+}-dependent proteases and phospholipases, which will start to digest cellular components. According to the 'physical forces' hypothesis, degradation of phospholipids is merely an epiphenomenon, reflecting post-mortem autolysis of cardiac cells [41, 43].

In the second hypothesis, the loss of integrity is explained, among others, by a imbalance between hydrolysis and resynthesis of the membrane phospholipids. As indicated before, this may result from increased activity of hydrolyzing enzymes, like phospholipases, or impaired resynthesis due to loss of catalytic activity of the enzymes involved. Acyl-CoA synthetase, an enzyme involved in phospholipid resynthesis, requires ATP as cofactor and is inhibited by AMP and adenosine. Therefore, the ischemia-induced decrease in cellular ATP and elevated levels of AMP and adenosine might lead to impairment of resynthesis [5]. In this light it is also of interest to note that the activity of lysophosphatidyl acyltransferase was found to be compromized in the ischemic pig heart [44] while in several studies PLA_2 activity was found to be increased in the ischemic and reperfused heart [1]. The latter hypothesis implies that the increased degradation of membrane phospholipids plays a pivotal role in the transition to irreversible injury of cardiomyocytes during myocardial ischemia [1, 45].

Role of PLA_2 in ischemia/reperfusion induced phospholipid hydrolysis

Several studies indicate that the activation of especially PLA_2 plays an important role in the transition from reversible to irreversible ischemic myocardial injury. First, in ischemic myocardium various phospholipid degradation products accumulate, including arachidonic acid and lysophospholipids. As arachidonic acid is predominantly (99%) located in the esterified form at the *sn-2* position in phospholipids, its liberation is conceivably due to PLA_2 activity [1]. Second, chemical inhibitors of PLA_2 have been reported to protect the cardiomyocytes against ischemia, anoxia, or energy-deprivation [46, 47].

Earlier attempts to identify the PLA_2 type involved in the enhanced phospholipid hydrolysis during myocardial ischemia and reperfusion have focused on the plasmalogen-specific, Ca^{2+}-independent PLA_2. Hazen and coworkers reported that

in the rabbit heart the activity of the plasmalogen-specific PLA_2 is greatly enhanced already 2 min after the onset of ischemia [9]. Moreover, halo-enol-lactone suicide substrate (HELSS), a specific inhibitor of plasmalogen-specific, Ca^{2+}-independent PLA_2 was found to reduce cell death in cultured neonatal cardiomyocytes exposed to chemical anoxia [47]. However, several other observations raise questions as to whether cardiac plasmalogen-specific, Ca^{2+}-independent PLA_2 plays an important role in the ischemia/reperfusion-induced phospholipid degradation. First, in cardiac homogenates the diacyl-form of phosphatidylethanolamine is hydrolyzed rather than the plasmalogen-form of phosphatidylethanolamine [48]. In line with the latter finding Davies and coworkers observed that the levels of lysoplasmenylcholine and lysoplasmenylethanolamine did not significantly increase in isolated rat hearts subjected to ischemia [49]. Second, the time course of arachidonic acid accumulation during reperfusion does not coincide with that of plasmalogen-specific, Ca^{2+}-independent PLA_2 activation. The enzyme activity quickly goes down to normal levels after ischemia, whereas arachidonic acid continues to accumulate [1, 9]. Thirdly, Vesterqvist and colleagues reported a marked decrease in the activity of plasmalogen-specific, Ca^{2+}-independent PLA_2 in isolated rabbit hearts subjected to a prolonged period of global ischemia [50]. In their study they used intact isolated sarcolemmal membranes as substrate to measure enzyme activity, while Hazen and colleagues used exogenously added phospholipid substrates [9].

In our opinion group II PLA_2 is a likely candidate to be involved in ischemia/reperfusion-induced degradation of membrane phospholipids, because not only arachidonic acid, but also other (un)saturated fatty acids accumulate during prolonged myocardial ischemia (Fig. 3). This argues in favor of the involvement of an aspecific PLA_2 rather than an arachidonoyl-, plasmalogen-specific PLA_2 [43, 45]. Secondly, the observation that cardiac fatty acid levels, including arachidonic acid, continue to rise in the reperfusion phase points toward a role for group II PLA_2 in the process. Reperfusion is known to be accompanied by a substantial increase in intracellular calcium concentration, so that the activation of calcium-dependent phospholipases can be anticipated. Furthermore, during reperfusion peroxidation of membrane phospholipids, as a result of enhanced oxygen free radical production, has been demonstrated [1]. It is of interest to note that peroxidation-damaged phospholipids are more vulnerable for group II PLA_2 attack [51]. Furthermore, in a study by Prasad and colleagues it was shown that pretreatment of isolated hearts with antibodies raised against a snake venom group II PLA_2 effectively blocked the degradation of membrane phospholipids and mitigated the release of cytoplasmic proteins in the acute reperfusion phase [52]. Anti-group II PLA_2 antibodies were also found to decrease the phospholipid degradation in homogenates of rat hearts that had been previously subjected to a period of hypoxia and reoxygenation [48]. Finally, increased expression levels of group II PLA_2 have been found in rat brain after severe forebrain ischemia [53] and in rat small intestinal mucosa after ischemia and revascularization [54]. On the basis of the above, it is tempting to speculate that cardiac group II PLA_2 expression levels will also increase during myocardial ischemia and/or reperfusion.

Alternatively, the observation that group II PLA_2 activity can be greatly enhanced by members of the inflammatory cytokine family, might be of relevance to the process of ischemic injury. First of all it has been shown that serum levels of inflammatory cytokines, like IL-1, IL-6 and TNFα, are increased in various pathophysiological conditions, including myocardial ischemia [55, 56]. Moreover, for other cell types it has been shown that cytokine stimulation caused a very rapid (within 5 min) increase of group II PLA_2 activity [57]. Finally, it has been demonstrated that cardiomyocytes can produce TNFα under hypoxic stress [58], and that stimulation of cultured rat neonatal cardiomyocytes with TNFα resulted in increased group II PLA_2 mRNA levels [15]. Therefore, it can be hypothesized that cytokines secreted by cardiomyocytes could indirectly influence sarcolemmal stability, by increasing the cellular level of group II PLA_2 in an autocrine manner.

Future directions: The use of molecular and transgenic techniques

As indicated above, no definitive proof has been provided that enhanced phospholipid hydrolysis and the induction of irreversible cell damage are causally related. It is also uncertain which type of PLA_2 would be accountable for the ischemia and reperfusion-induced hydrolysis of membrane phospholipids. Studies performed with phospholipase inhibitors lack specificity, which hampers an unequivocal interpretation of the results obtained. For example, in an isolated rat heart preparation subjected to a period of global ischemia followed by reperfusion, the phospholipase inhibitor mepacrine was able to reduce the phospholipid degradation, but also was shown to exert a negative inotropic effect [5] and to interfere with transsarcolemmal calcium fluxes [59]. Accordingly, current conventional pharmacological and/or physiological approaches do not provide conclusive answers as to the role of PLA_2 in ischemia/reperfusion injury.

The advent of molecular biological techniques and transgenic technology allows the generation of mice with either an overexpression ('gain of function') or the absence ('loss of function') of specific genes. By using transgene technology it has been possible to study the role of a specific

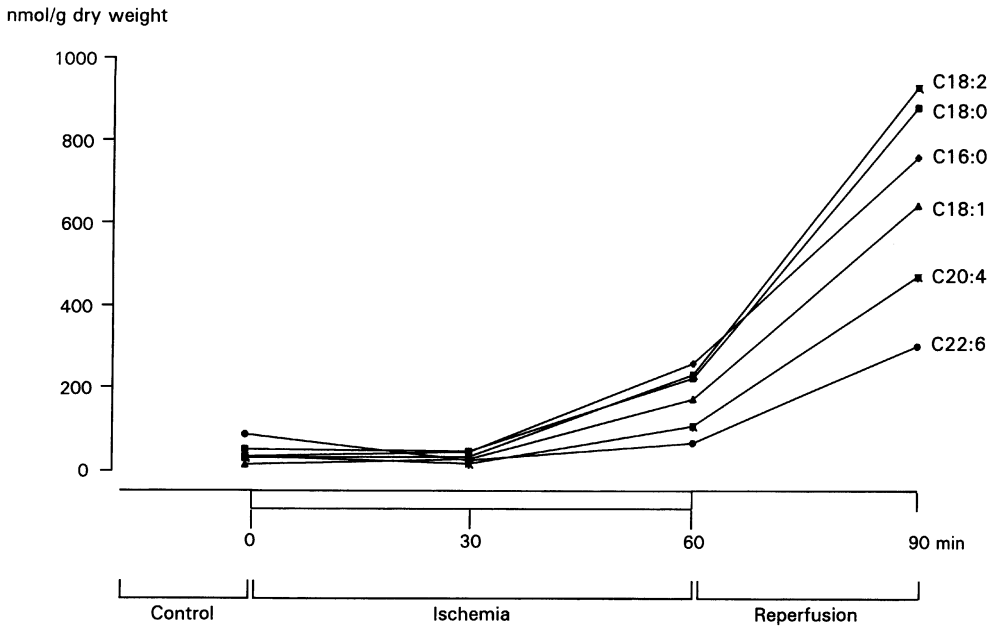

Fig. 2. Effects of ischemia and reperfusion of isolated rat hearts on the tissue content of individual fatty acid species. Values are given in nmol.g^{-1} dry weight and represent the means of 6–10 experiments. C16:0 refers to palmitic acid, C18:0 to stearic acid, C18:1 to oleic acid, C18:2 to linoleic acid, C20:4 to arachidonic acid, and C22:6 to docosahexaenoic acid. (Adapted from: Van Bilsen *et al.* [67]).

gene of interest and to dissect complex cardiovascular phenotypes involving several gene products. This approach has been demonstrated to be effective in creating murine models resembling human myocardial diseases, such as cardiac hypertrophy/failure, congenital heart diseases, and hypertension, just to mention a few (see for some reviews [60, 61]). The transgene technology has also been successfully applied in studies related to ischemia and reperfusion damage. For example, the contribution of oxygen free radicals to ischemia/reperfusion damage was confirmed by the subjecting the hearts of transgenic mice overexpressing superoxide dismutase to transient ischemia [62]. In addition, transgenic mice overexpressing HSP70 were found to be less vulnerable against ischemic injury, clearly indicating the protective effect of HSP70 in this setting [63, 64].

In our laboratories we are currently applying transgene technology in order to modulate group II PLA_2 activity in the heart. With this approach mechanisms underlying disturbances in phospholipid homeostasis as a result of cardiac ischemia and reperfusion will be studied. To achieve this, transgenic mice have been generated that harbor the recently cloned cDNA of rat heart group II PLA_2 downstream to the proximal fragment (250 bp) of the promoter of the myosin light chain-2 (MLC-2) gene. Recent studies indicate that this part of the promoter is sufficient to mediate cardiac-specific expression [65]. In this way, several lines of transgenic mice, differing in copy number and, hence, in the level of cardiac group II PLA_2 activity, will become available for analysis.

The hearts of transgenic and control mice will be subjected to ischemia and reperfusion *ex vivo* to assess the causal relationship, if any, between the level of group II PLA2 and the extent of phospolipid hydrolysis on the one hand and cellular damage and functional recovery on the other. We are currently scaling down the technique of isolated rat heart perfusion, according to the so called 'assisted mode perfusion', to the level of the mouse [66]. This model will allow us to study functional recovery by parameters like the recovery of the left ventricular developed pressure and cardiac output. The development of this isolated left ventricular ejecting mouse heart model will not only be of interest for this particular problem, but will be of great value for future studies to evaluate cardiac function of transgenic mice, whatever the type of transgene they are harboring.

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The role of glucose metabolism in a pig heart model of short-term hibernation

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Abstract

Previously, we reported, alterations in glucose metabolism in a 4 day model of chronic coronary stenosis similar to those described in patients with hibernating hearts. The purpose of this study was 2 fold: (1) to identify whether an acute model of mild, sustained ischemia could effect similar changes, and (2) to determine the effects of pharmacological inhibition of glycolysis. In the first group, extracorporeally perfused, intact pig hearts were subjected to 85 min of a 40% reduction in left anterior descending (LAD) coronary arterial blood flow. A second group was subjected to the same protocol, except after 40 min of LAD regional ischemia, iodoacetate (IAA) was administered to block glycolysis. Ischemia reduced MVO_2 by 10% in both groups with a further 20% reduction noted following IAA treatment. Regional systolic shortening was reduced nearly 50% by ischemia and decreased an additional 40% following treatment with IAA. Glycolysis was increased by over 700% with ischemia in the first group. IAA caused a 3 fold reduction in glycolysis as compared to the preceding ischemic period and inhibited lactate production. Fatty acid metabolism was significantly reduced by ischemia in the first group, but was not reduced in the IAA group. Activity of creatine kinase associated with myofibrils was reduced and may have contributed to the contractile dysfunction. In conclusion, this acute model of short-term hibernation demonstrates several metabolic changes previously reported in chronic hibernation and may prove useful in determining mechanisms of substrate utilization in simulated conditions of chronic coronary stenosis and hibernation. (Mol Cell Biochem **180**: 75–83, 1998)

Key words: creatine kinase, glucose metabolism, mild sustained ischemia

Introduction

Animal modeling has become a central issue in helping define the experimental conditions sufficient to identify the mechanisms operative in chronic ischemia and to explain the clinical entity of hibernating myocardium. The factors determining hibernation are still a source of controversy. It has yet to be determined whether sustained hypoperfusion or repetitive, intermittent ischemia best clarifies and predicts the mechanical down-regulation in this condition [1–4] and to what level of ischemia (mild or severe) is necessary to affect these changes. Several chronic animal preparations of partial coronary stenosis have been developed in an attempt to resolve these differences [3, 5–7]. Moreover, it has now been shown that certain acute models of partial, sustained coronary hypoperfusion [8–13] or intermittent ischemia [14, 15] also are adaptive in down-regulating energy needs to sustain tissue viability. Schulz *et al.* [13], who coined the term 'short-term myocardial hibernation', found that 85 min of a moderate restriction in coronary perfusion caused a decrease in regional contractile function, regeneration of phosphocreatine, depressed levels of ATP, and a leveling off of lactate production. Other investigators have noted similar changes [8–12]. Although these acute models have appeal in terms of their ease of preparation and more controlled experimental conditions, in order to fulfill complete criteria as models of 'short-term hibernation' [16], other alterations in intermediary metabolism, in addition to changes in energy metabolism, must be documented. These alterations have not yet been described.

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Early clinical studies using positron computed tomography suggested that certain subsets of patients with advanced coronary artery disease were compensated metabolically by the accelerated, exogenous utilization of glucose, e.g. 'the flow-metabolic mismatch' [17–20]. Recently, Vanoverschelde and workers [4] showed in patients with hibernating, collateral-dependent myocardium that glycolytic flux was increased despite nearly normal resting coronary blood flows. We showed in a chronic animal model of restricted coronary reserve that during hibernation and reperfusion following hibernation, aerobic metabolism was also mainly preserved based on only modest reductions in oxygen consumption and normal resting coronary blood flows [7, 21]. In these models, glycolysis was also enhanced. Although the amount of energy produced by glycolysis is considered small, it has previously been deemed vitally important in several other models of ischemic heart disease [22, 23]. The purpose of the present studies was thus 2 fold: (1) to characterize the metabolism of acutely altered myocardium and, in particular, to detail substrate utilization in an acute preparation of sustained hypoperfusion previously proposed as a model of short-term hibernation [13], and (2) to determine the importance of glucose utilization in this model by inhibiting glycolysis using treatment with iodoacetic acid (IAA). We hypothesized that this model simulates metabolic trends previously reported in chronically hypoperfused hearts and that accelerated glycolysis is imperative for maintenance of residual contractile function and viability. In addition to describing metabolic rates of oxygen consumption, glucose and fatty acid utilization, observations on the activity of the creatine kinase shuttle system are reported.

Materials and methods

Experimental model

Fourteen adolescent swine weighing between 37–48 kg were studied under general anesthesia induced with pentobarbital (35 mg/kg). A tracheotomy was performed and the animals were placed on controlled positive pressure ventilation using oxygen-supplemented room air to maintain blood gases in the normal range (PO_2 > 100 mm Hg, PCO_2 35–45 mm Hg, and pH 7.35–7.45). Additional pentobarbital was given as needed. Lidocaine HCl (2–3 ml i.v.) was also given if needed to prevent arrhythmias. Hearts were exposed with a sternotomy and bilateral rib resections to achieve the open chest, extracorporeal perfusion preparation previously described [24]. Briefly, the animals were heparinized with an i.v. bolus of 20,000 U heparin followed by additional boluses of 10,000 U/h. The coronary arteries were cannulated and perfused separately via an arterioarterial shunt connected extracorporeally. Blood was withdrawn from the right femoral artery and divided into three independently controlled perfusion pumps, passed through blood filters (40 μm), and infused into the right (RCA), main left, (basically perfusing the circumflex (CFX) artery) and left anterior descending (LAD) coronary arteries. Blood was passed through a 50 ml mixing chamber in the LAD circuit that served as a mixing reservoir for radioactive substrates and indocyanine green. A venous sampling catheter was inserted in the left anterior interventricular vein, which drains the perfusion territory of the LAD coronary artery. The anterior interventricular vein effluent was allowed to drain into the chest cavity. Blood from the chest cavity was continuously pumped into a reservoir, and reinfused into the right femoral vein. This completed the extracorporeal perfusion circuit. A high-fidelity, dual manometer-tipped pressure device (Millar) was placed in the left ventricular chamber to measure left ventricular pressure and aortic pressure. The coronary perfusion pressures were measured using fluid-filled transducers attached to each perfusion line. The pump speeds were adjusted so that the coronary venous oxygen saturation averaged 46 ± 1.5% in both groups and the mean coronary artery pressures matched mean aortic pressure. To maintain a condition of near constant myocardial oxygen demand in all hearts, systemic blood volumes were replenished with 6% dextran in saline so that the systemic pressures were held at 94.5 ± 3 mm Hg. Serum glucose levels were monitored and maintained at 7.75 ± 0.65 μmol/ml. Additional dextrose was given as needed. Plasma concentrations of fatty acids were also monitored throughout the studies and the combined average for both groups was 0.61 ± 0.08 μmol/ml. Ultrasonic crystals were placed at mid-myocardial depth in the LAD perfusion bed to monitor changes in segment shortening, which was used to estimate regional contractile function.

Protocol

Group 1

The first group (n = 8) weighing 41.3 ± 1.1 kg (mean ± S.E.M.) underwent a sustained 40% reduction in LAD blood flow for 85 min based on the hibernation model published by Schulz *et al.* [13]. At time zero, i.e. 30 min prior to ischemia, radioactive [U-^{14}C] palmitate was infused into the LAD mixing chamber at a rate of 1.02×10^6 dpm/min during the aerobic period and then reduced by 40% to 0.61×10^6 dpm/min for the ischemic interval. Simultaneously, [5-^{3}H] glucose was also infused into the mixing chamber in the LAD perfusion line at a rate of 2.35×10^6 dpm/min during the aerobic interval and reduced to 1.41×10^6 dpm/min during the ischemic period. The 30 min aerobic period was used for equilibrium labeling of palmitate and glucose and for baseline measurements. The total experimental time was 115 min.

IAA treatment

A second group, which was treated with iodoacetate (IAA), was also studied in separate non-randomized perfusion trials to evaluate the importance of glucose metabolism in hibernation. This IAA treated group (n = 6), weighing 44.9 ± 0.8 kg, also underwent a 40% reduction in LAD blood flow for 85 min. At time zero, each animal received a continuous infusion of radioactive [U-^{14}C] palmitate at 1.01×10^6 dpm/min and [5-^{3}H] glucose at 2.32×10^6 dpm/min. Ischemia again was induced at time 30 min by reducing the flow to the LAD perfusion bed by 40%. The infusion of radioactive [U-^{14}C] palmitate and [5-^{3}H] glucose was reduced to 0.61×10^6 dpm/min and 1.39×10^6 dpm/min, respectively, to compensate for the reduced blood flow. After 70 min of total elapsed time which corresponded to 40 min of ischemia, IAA at a dose of approximately 1.0 μmol/g of LAD bed weight was injected as a bolus into the LAD perfusion system to inhibit glycolysis.

For both groups, coronary blood flow to the right and circumflex arteries was held at aerobic levels throughout the study. Mechanical and metabolic data were collected every 10 min throughout the study. Blood samples were withdrawn from the LAD coronary arterial perfusion line and the anterior interventricular vein for metabolic and blood gas measurements. Indocyanine green dye was administered in accordance with earlier studies to estimate venous cross contamination and dilution in the LAD circulation [25]. Samples of arterial and venous blood from the LAD perfusion circuit were collected during infusion of indocyanine green dye after the dye had been infused for 5 min. These samples were obtained during the aerobic and ischemic periods in both groups to measure for any unlabeled coronary blood entering the venous system of the LAD perfusion circuit. The mean dilution factors (K) obtained in this study to estimate for this contamination were 0.87 ± 0.03 and 0.77 ± 0.08 for the aerobic and ischemic periods, respectively, for the first group and 0.89 ± 0.03 and 0.81 ± 0.04 for the aerobic and ischemic periods, respectively, for the IAA treated group. The K-factor, was then used in the calculations of exogenous glucose utilization [26] and CO_2 production from labeled fatty acids as previously described [27].

At the end of the study, a section of myocardium was rapidly removed from the center of each of the LAD and CFX perfusion areas. These tissue samples were immediately clamped between tongs cooled in liquid nitrogen and stored at –70°C for later analysis. A second set of tissue samples was removed from Group 1 hearts and used at once for isolation of mitochondria and myofibrils. In both groups, the LAD perfusion region was then dyed with India Ink (injected via the LAD cannula) to demarcate the LAD from the CFX and RCA perfusion beds, which were then dissected free and weighed. The weight of the LAD perfusion bed was used to normalize the blood flow and metabolic data.

Analytical methods

Estimates of mechanical performance as well as arterial and venous samples for metabolic measurements were collected every 10 min during the experiments. A blood gas analyzer (Radiometer ABL2) was used to determine pH, PCO_2 and PO_2. The hemoglobin concentration and oxygen saturation were measured on a hemoximeter (Radiometer OM1). Regional myocardial oxygen consumption (MVO_2) was calculated according to the formula previously reported and expressed in mmol × h^{-1} × g dry wt^{-1} [28]. Serum glucose levels and fatty acid concentrations were also obtained every 10 min. Serum glucose was measured using a Glucometer II and glucose stix, while free fatty levels were determined using a kit from Wako. Blood samples from the LAD artery and vein were immediately deproteinized in ice cold 7% perchloric acid (1:2 vol:vol), weighed, centrifuged and analyzed for lactate using previously described enzymatic methods [29]. From these measurements, lactate extraction/production from or into the coronary perfusate was calculated as described previously [26]. Plasma samples from artery and vein were analyzed for tritiated water released from labeled glucose according to the procedures of Rovetto *et al.* [30] using a Dowex-1 borate column. The tritiated water was eluted from the column with water and counted for radioactivity. The radioactive counts of the tritiated water were used to calculate glucose utilization as described previously [26]. Labeled CO_2 from fatty acid oxidation was determined by adding 1 ml of blood in triplicate from the LAD artery and vein to sealed Erlenmeyer flasks containing sulfuric acid and a disposable centerwell with a CO_2 absorber, which was removed and counted for radioactivity. The CO_2 produced from labeled palmitate was then used to estimate fatty acid oxidation as described previously [27].

Frozen tissue from both beds in all animals was extracted with 6% perchloric acid and neutralized. Lactate was determined enzymatically from the neutralized extract [29]. The adenine nucleotides and phosphocreatine were measured from the neutralized extract by HPLC with a μBondapak C_{18} column (Waters). The compounds were eluted by varying a linear gradient consisting of mobile phase A (50 mM KH_2PO_4 and 2 mM Tetrabutylammoniumphosphate) and mobile phase B (5 mM KH_2PO_4 and acetonitrile, 2:1) from 5–75% of mobile phase B [31]. The adenine nucleotides were detected at 260 nm while phosphocreatine was detected at 210 nm on a multichannel UV detector.

Glycogen was enzymatically digested to obtain free glucose from tissue in both LAD and circumflex perfusion beds in all animals according to Bradley and Kaslow [32]. The glucose concentration was measured using a glucose kit from Sigma. Total lipids were extracted from powdered, frozen tissue by a mixture of water:chloroform:methanol in

a 0.8:1:2 ratio by the methods of Bligh and Dyer [33]. The triglyceride content was measured spectrophotometrically using a commercially available kit (Sigma).

To examine the mechanisms behind the contractile dysfunction and preservation of high energy phosphates found in the short-term hibernation model [13], creatine kinase in isolated myofibrils and mitochondria was measured. Purified myofibrils were prepared as described by Solaro *et al.* [34]. Freshly harvested tissue from both perfusion beds was washed with 0.9% NaCl and minced. The minced tissue was homogenized in 0.3 M sucrose and 10 mM imidazole buffer (pH 7.0) and centrifuged. The myofibrils were further washed in a buffer containing 60 mM KCl, 30 mM imidazole and 2 mM $MgCl_2$. The washed myofibrils were treated with Triton X-100 to remove all additional membranes. Mitochondria were also isolated from fresh tissue as previously described [31] using homogenization media containing 20 mM Tris buffer (pH 7.4) and 0.25 M sucrose. Protein for both mitochondria and myofibrils was determined by the method of Bradford [35]. Creatine kinase activity in the isolated mitochondria and myofibrils was measured using spectrophotometric methods with a commercially available kit (Sigma).

Statistics

All data are reported as mean ± S.E.M.. Statistical comparisons were performed between the average of the data points obtained during the aerobic period and the average of the data points obtained during the second half of the ischemic period (time 80, 90, 100, 110, 115 min) with a paired Student's *t*-test. A paired Student's *t*-test was also employed to compare differences between the aerobic CFX bed and the treated LAD bed in the same group. Statistical significance was defined for probability values of $p < 0.05$.

Results

Group 1

Heart rate averaged 128 ± 5 beats/min and was stable over the course of the experiments. Reducing the blood flow by 40% in these studies caused no significant changes in peak left ventricular systolic pressure during the entire perfusion period (98 ± 3 mmHg). Perfusate glucose (8.0 ± 0.69 μmol/ml) sampled every 10 min throughout the studies was near constant. The concentration of fatty acids in the blood was also sampled every 10 min during the protocol and found to be stable at 0.58 ± 0.09 μmol/ml throughout the perfusion trials.

Mechanical functions

The first 40 min of the 85 min ischemic period was used for stabilization and adaption commensurate with the previous protocol used for short-term hibernation [13]. Contractile function was expressed as percent systolic shortening (% SS) and normalized to the value at the start of the experiment (Fig. 1, panel A). Regional contractile function in Group 1 fell from 95 ± 4% during aerobic perfusion (t = 0–20 min) to 50 ± 17% during the last 45 min of ischemia (t = 70–115 min), a 47.4% reduction in performance ($p < 0.04$ compared to the aerobic value).

Metabolic functions

Myocardial oxygen consumption (MVO_2) dropped from its initial aerobic value of 1.01 ± 0.06 mmol × h^{-1} × g dry wt^{-1} to 0.77 ± 0.04 mmol × h^{-1} × g dry wt^{-1} during the last half of ischemia ($p < 0.0004$) (Fig. 1, panel B).

Glucose utilization increased on average over 7 fold during the last 45 min of ischemia in Group 1 hearts ($p < 0.002$) (Fig. 1, panel C), which is similar to that seen in chronic hibernation. Tissue glycogen levels were significantly lower in the ischemic LAD bed as compared with the aerobic CFX bed (103 ± 18 vs. 182 ± 20 in μmol/g dry; $p < 0.001$ in Group 1 hearts).

There was net lactate consumption during the initial aerobic period, which reversed to lactate production at the onset of ischemia. (Fig. 1, panel D). Lactate production did not increase further despite the continuing ischemia during the later times of the perfusion trials, but leveled off similar to what was noted in the Schulz model of hibernation [13]. The lactate production caused a relative accumulation of lactate in the tissue of the ischemic LAD perfusion bed (25.6 ± 2.2 μmol/g dry) at end-ischemia compared to the aerobic CFX bed (14.1 ± 1.6 μmol/g dry) ($p < 0.001$).

Ischemia caused an anticipated decrease in fatty acid oxidation as estimated by CO_2 production from infusion of exogenously labeled palmitate. After equilibration, fatty acid oxidation fell significantly from an aerobic value of 26.69 ± 6.54 μmol × h^{-1} × g dry^{-1} to 17.81 ± 3.34 μmol × h^{-1} × g dry^{-1} ($p < 0.05$) in the last 45 min of ischemia. Tissue content of triglycerides were not different between perfusion beds despite the lower fatty acid oxidation due to ischemia (Table 1).

Table 1. Tissue content of triglycerides in μmol/g dry taken from left anterior descending coronary artery (LAD) perfusion bed and tissue from the aerobic circumflex artery (CFX) perfusion bed at the end of the studies

Triglycerides	LAD	CFX	p-value
Group 1	3.66 ± 0.58	3.18 ± 0.73	ns
IAA	4.83 ± 1.03	4.46 ± 1.07	ns

Group 1, n = 8; IAA group, n = 6. All values are expressed as means ± S.E.M. ns = not significantly different.

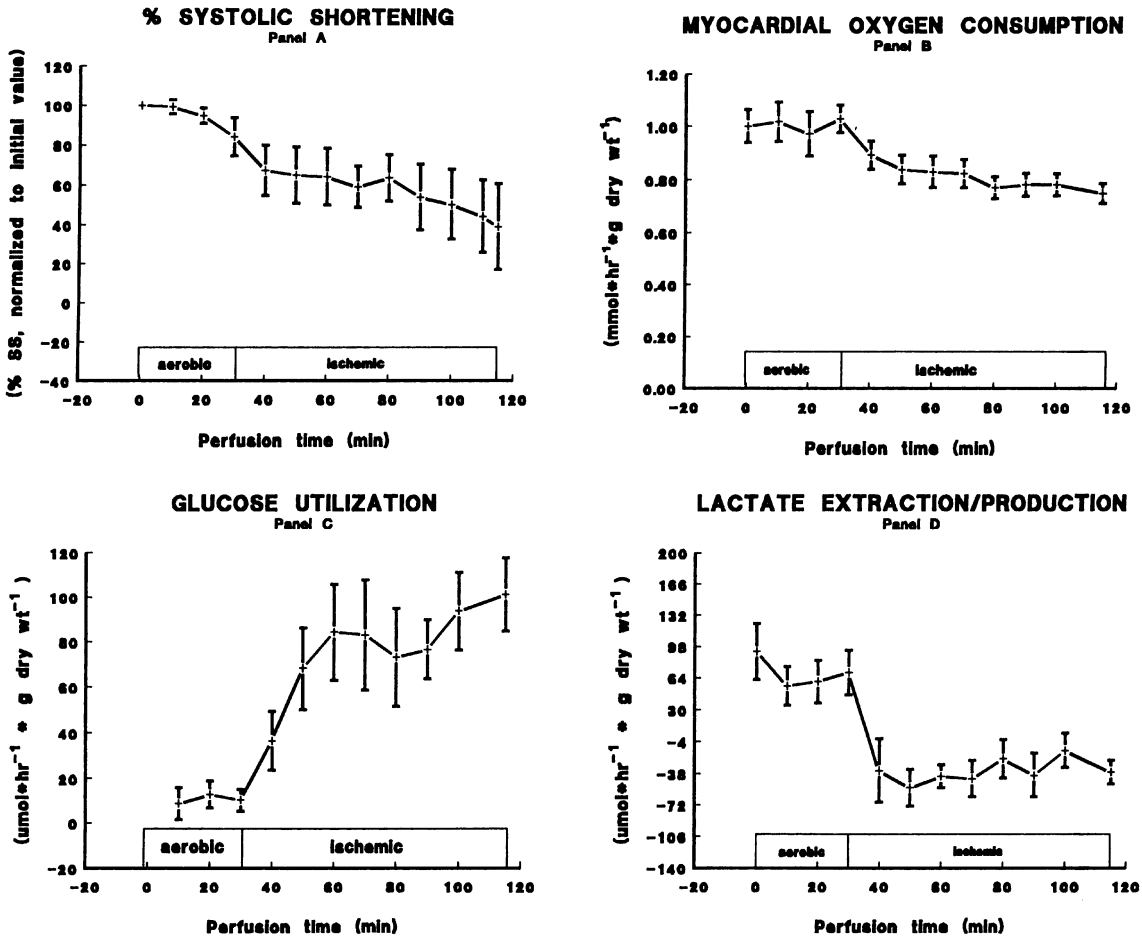

Fig. 1. Group 1 – Panel A: Changes in regional systolic shortening (% SS) over the course of the perfusion trials, which were normalized to the initial aerobic value; Panel B: Plot of myocardial oxygen consumption (MVO_2) in mmol × h^{-1} × g dry wt^{-1} vs. perfusion time; Panel C: Rates of glycolysis from exogenous glucose are also shown vs. perfusion time in µmol × h^{-1} × g dry wt^{-1}; Panel D: Rates of net lactate production or extraction are expressed in µmol × h^{-1} × g dry wt^{-1} and plotted vs. time. All data is expressed as mean ± S.E.M., n = 8.

Another marker of myocardial adaption previously used in confirming hibernation was preserved high energy phosphates. In Group 1 hearts, there was no change in phosphocreatine content between the two perfusion beds. A fall in the tissue content of ATP in the LAD perfusion bed as compared to the aerobic CFX perfusion bed was observed. These are the same changes previously reported in the model by Schulz *et al.* [13] (Table 2).

Creatine kinase activity

A reduction in creatine kinase activity may in part explain the contractile dysfunction in hibernating myocardium. The activity of mitochondrial creatine kinase from the treated LAD bed was not significantly different from the aerobic CFX bed (Table 3). However, the maximal activity of the creatine kinase associated with the m-line of the myofibrils in the LAD bed was significantly reduced as compared to the untreated CFX bed (Table 3). This incomplete utilization of PCr at the myofibrils may partially explain the mechanism of contractile dysfunction.

IAA treated group

As explained above, this group was prepared to specifically address the significance of increased glucose utilization in

Table 2. Tissue from the ischemic left anterior descending coronary artery (LAD) perfusion bed and tissue from the aerobic circumflex artery (CFX) perfusion bed was taken at the end of the experiments to determine the content of phosphocreatine (PCr), adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP)

	LAD	CFX	p-value
ATP			
Group 1	18.50 ± 0.96	27.00 ± 1.22	0.001
IAA	10.09 ± 0.90	20.68 ± 3.52	0.024
ADP			
Group 1	4.64 ± 0.20	5.65 ± 0.26	0.01
IAA	3.31 ± 0.22	4.06 ± 0.43	ns
AMP			
Group 1	0.65 ± 0.06	0.59 ± 0.05	ns
IAA	0.22 ± 0.033	0.068 ± 0.018	0.011
PCr			
Group 1	43.22 ± 4.01	44.30 ± 4.27	ns
IAA	56.76 ± 4.80	59.72 ± 9.07	ns

All values are expressed as means ± S.E.M. in μmol/g dry (Group 1, n = 8; IAA group, n=6). ns = not significantly different.

Table 3. Creatine kinase activities of Group 1 from the ischemic left anterior descending coronary artery perfusion bed (LAD) and the aerobic circumflex bed (CFX)

	LAD BED	CFX BED	p-value
Mito-CK (U/mg protein)	4.44 ± 0.75	4.60 ± 0.67	ns
MM-CK (U/mg protein)	0.78 ± 0.15	1.20 ± 0.25	0.04

One unit (U) of activity is the amount of enzyme which produces one μmole of NAD or NADH per min under the assay conditions. Mean ± S.E.M, n = 8. Mito-CK = mitochondrial creatine kinase; MM-CK = myofibrillar creatine kinase; ns = not significantly different.

hibernating hearts. In the IAA group, heart rate (131 ± 4 beats/min), peak left ventricular systolic pressure (91 ± 3 mmHg), perfusate glucose (7.5 ± 0.61 μmol/ml) and perfusate fatty acids (0.63 ± 0.07 μmol/ml) were all stable throughout the perfusion trials.

Mechanical functions

Once again the first 40 min of the 85 min ischemic period was used for stabilization and adaptation [13]. After 40 min of ischemia, IAA was injected into the LAD coronary circulation to inhibit regional glycolysis. Data were collected for the next 45 min, averaged and compared to the initial aerobic conditions.

Contractile function was expressed as percent systolic shortening (% SS) and normalized to the value at the start of the experiment (Fig. 2, panel A). Regional contractile function fell from 101 ± 7% during aerobic perfusion to 8 ± 9% following the IAA injection, a 92% reduction ($p < 0.0004$ compared to the aerobic value).

Metabolic functions

In the IAA treated group, aerobic MVO_2 (0.70 ± 0.03 mmol × h^{-1} × g dry wt^{-1}) was significantly reduced by the combined effects of ischemia and IAA to 0.48 ± 0.04 mmol × h^{-1} × g dry wt^{-1} ($p < 0.0025$ compared to the aerobic value) (Fig. 2, panel B).

In the IAA group, treatment strikingly decreased glucose utilization from the first half of ischemia, although, the modest level of glycolysis at end reperfusion was still twice that of the preischemic values ($p < 0.003$) (Fig. 2, panel C). Tissue glycogen levels were significantly lower in the ischemic LAD bed as compared with the aerobic CFX bed (113 ± 11 vs. 199 ± 38 in μmol/g dry; $p < 0.03$ in IAA treated hearts).

Induction of ischemia caused lactate consumption to reverse to lactate production. As anticipated, IAA treatment effectively inhibited glycolysis, which prevented lactate production and thus accounted for the lactate extraction seen after IAA treatment (Fig. 2, panel D). Since lactate extraction was re-established late in ischemia, it followed that there would be no accumulation in tissue lactate between the two beds at end ischemia and such was the case (40.8 ± 4.4 LAD vs. 36.7 ± 16.7 CFX; in μmol/g dry).

Fatty acid oxidation slightly fell throughout the perfusion trials in the IAA treated group, but never reached significance verses the initial conditions (12.58 ± 2.53 in the aerobic period vs. 9.09 ± 1.03 during the IAA treated period in μmol × h^{-1} × g dry^{-1}). There was also no change in the tissue levels of triglycerides between perfusion beds (Table 1).

The IAA treatment did little to affect the levels of high energy phosphates despite the presumed decrease in energy supply by the inhibition of glycolysis (Table 2). Phosphocreatine was unchanged and ATP was depressed.

Discussion

The results of this study demonstrate that myocardium subjected to an acute bout of mild ischemia, e.g. short-term hibernation, simulates trends previously reported in chronically hypoperfused hearts. In particular, the mild ischemia caused a decrease in regional contractile function, an increase in glucose utilization, a leveling off of lactate production, and a partial preservation of high energy phosphates. In addition, for the first time in this model of short-term hibernation, it was demonstrated that myofibrillar creatine kinase activity is reduced and that glycolysis as illustrated in the IAA group is necessary to maintain contractile function.

Limitations of experimental approach

A bolus of IAA was use to inhibit glycolysis as described by Jennings [36]. IAA is a sulfhydryl-oxidizing agent and will

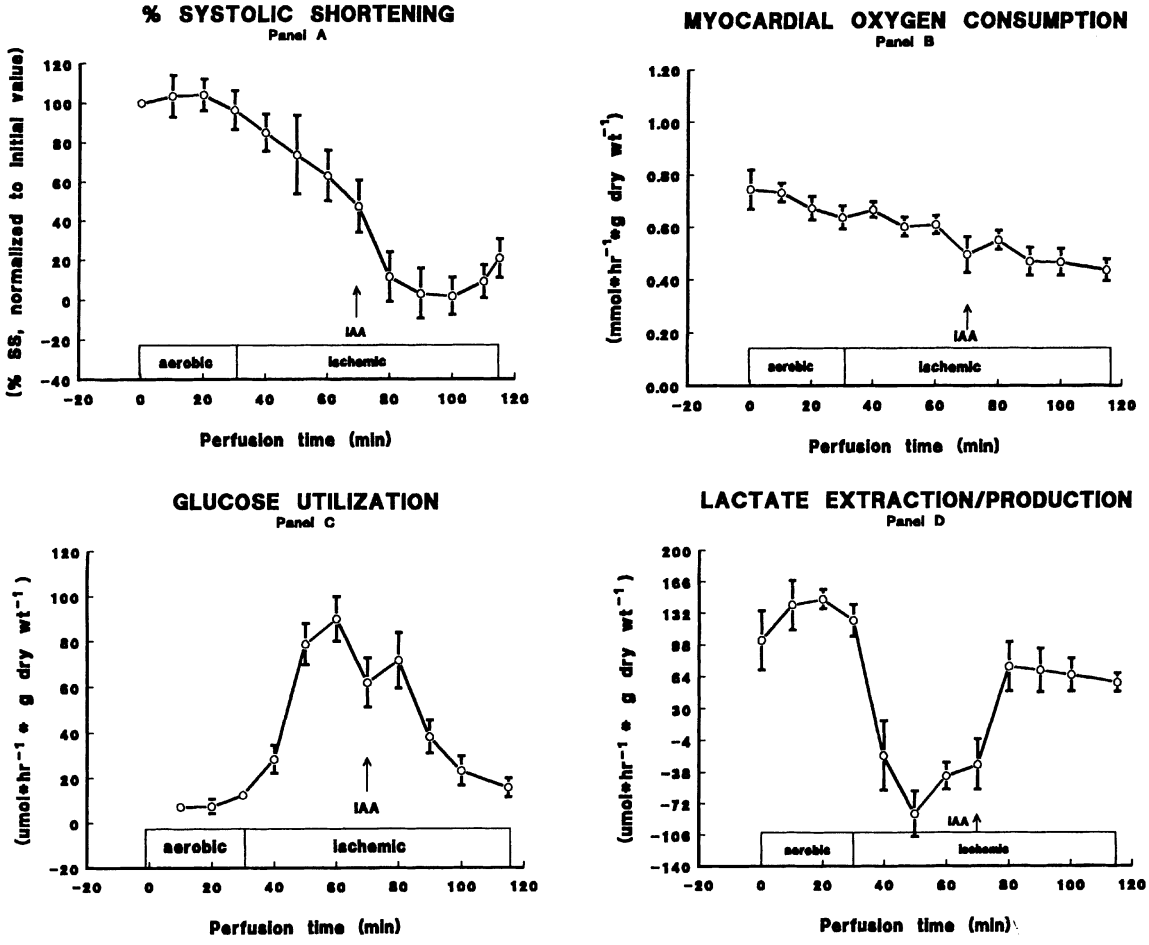

Fig. 2. IAA Group – Panel A: Changes in regional systolic shortening (% SS) over the course of the perfusion trials, which were normalized to the initial aerobic value; Panel B: Plot of myocardial oxygen consumption (MVO_2) in mmol × h^{-1} × g dry wt^{-1} vs. perfusion time; Panel C: Rates of glycolysis from exogenous glucose are also shown vs. perfusion time in µmol × h^{-1} × g dry wt^{-1}; Panel D: Rates of net lactate production or extraction are expressed in µmol × h^{-1} × g dry wt^{-1} and plotted vs. time. All data is expressed as mean ± S.E.M., n = 6.

inhibit enzymes containing sulfhydryl groups such as glyceraldehyde 3-phosphate dehydrogenase. Several studies have shown that the primary effect of IAA is on this enzyme and does not influence oxidative myocardial function [37]. It has also been shown that IAA does not directly affect sarcolemma or sarcoplasmic reticulum function or influence excitation-contraction coupling [38]. However, IAA was effective in reducing lactic acid production and tissue accumulation of lactate, which may have ameliorated the acidosis that normally occurs with ischemia. Although tissue pH was not measured, it is possible that it was higher in the IAA group than in Group 1 hearts. This diminished acidosis would probably improve function and lessen acidotic tissue injury rather than cause more deleterious effects. This would support the argument that accelerated glycolysis despite the effect on pH is more important in maintaining contractile function than the negative inotropic effect of lactic acidosis due to ischemia. The increased glycolysis in the setting of otherwise near normal aerobic behavior in this model of short-term hibernation is similar to the data reported in a recent clinical study which also showed accelerated glycolysis in hibernating myocardium of patients [4]. Examination of tissue specimens from these patients showed that cells from the hibernating hearts have an accumulation of glycogen such that the contractile filaments are diminished in volume [4]. However, it would appear that these hibernating cells are still viable and

will resume contracting after some time upon normalization of blood flow. Although the production of ATP from glycolysis probably only accounts for 3–8% of the energy needs during low-flow ischemia [37], it is qualitatively important. ATP from glycolysis is more beneficial in delaying or preventing ischemic contracture than the same amount of ATP produced by oxidative phosphorylation [23]. In addition, protocols that preserved glycolysis during ischemia with high glucose and insulin had higher ATP levels and contractile function during ischemia and improved recovery after ischemia [39, 40]. The present study confirms that glycolysis from exogenous glucose is necessary to maintain contractile function. Although ATP content falls during the course of the study, it is thought that compartmentation of the glycolytically produced ATP maintains either membrane and/or contractile function. This compartmentation becomes more important when one considers that the breakdown of the phosphocreatine energy shuttle, as seen in this study, may exaggerate the cytosolic ATP compartmentation further, resulting in an increased dependence on ATP produced near the contractile apparatus.

The myofibrills are dependent on the production of ATP from creatine phosphate via creatine kinase for contraction. This creatine kinase is localized and tightly bound at the myofibrillar M-band, where the creatine kinase is functionally coupled to the myofibrillar actin-activated Mg ATPase. The reduction in activity found in this study of this M-band bound creatine kinase (MM-CK) would lead to a reduction in ATP production for utilization by the myofibrils and perhaps contractile dysfunction. This reduction in MM-CK activity is in concordance with the findings of Greenfield and Swain [41] even though their dog model had a more severe ischemic insult (total occlusion for 15 min). In addition, Greenfield and Swain calculated the free ADP content to be below the Km of ADP for the MM-CK. Therefore they suggested two mechanisms for contractile dysfunction; lack of substrate and loss of enzyme activity [41]. In isolated rat hearts, specific inhibition of CK decreased the contractile reserve in hearts stressed with norepinephrine infusion, or under hypercalcemic or hypoxic conditions [42]. They speculated that an alteration in the functional coupling of CK and the myofibrillar ATPase within the micro compartment of the myofibrillar bundle could alter contractile performance.

In summary, these experiments indicate that the pig model of short-term hibernation described in this report may be suitable for simulating trends seen in clinical myocardial hibernation that exists for weeks to months. In addition, the data support the argument that glycolysis is necessary for maintenance of contractile function and that the reduction observed in contractile function may be linked to the decrease in activity of myofibril bound creatine kinase.

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Glucose metabolism, H^+ production and Na^+/H^+-exchanger mRNA levels in ischemic hearts from diabetic rats

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Abstract

Glycolysis uncoupled from glucose oxidation is a major reason for the intracellular acidosis that occurs during severe myocardial ischemia. The imbalance between glycolysis and glucose oxidation, and the resultant H^+ produced from glycolytically derived ATP hydrolysis in the diabetic rat heart is the focus of this study. Isolated working hearts from 6 week streptozotocin diabetic rat hearts were perfused with 11 mM glucose and 1.2 mM palmitate and subjected to a 25 min period of global ischemia. A second series of experiments were also performed in which hearts from control, diabetic, and islet-transplanted diabetic rats were subjected to a 30 min aerobic perfusion, followed by a 60 min period of low-flow ischemia (coronary flow = 0.5 ml/min) and 30 min of aerobic reperfusion. H^+ production from glucose metabolism was measured throughout the two protocols by simultaneous measurement of glycolysis and glucose oxidation using perfusate labelled with [5-^{3}H/U-^{14}C]-glucose. Rates of H^+ production were calculated by measuring the difference between glycolysis and glucose oxidation. The H^+ production throughout the perfusion was generally lower in diabetic rat hearts compared to control hearts, while islet-transplantation of diabetic rats increased H^+ production to rates similar to those seen in control hearts. This occurred primarily due to a dramatic increase in the rates of glycolysis. Despite the difference in H^+ production between control, diabetic and islet-transplanted diabetic rat hearts, no difference in mRNA levels of the cardiac Na^+/H^+-exchanger (NHE-1) was seen. This suggests that alterations in the source of protons (i.e. glucose metabolism) are as important as alterations in the fate of protons, when considering diabetes-induced changes in cellular pH. Furthermore, our data suggests that alterations in Na^+/H^+-exchange activity in the diabetic rat heart occur at a post-translational level, possibly due to direct alterations in the sarcolemmal membranes. (Mol Cell Biochem **180:** 85–93, 1998)

Key words: Na^+/H^+-exchanger, NHE-1, glycolysis, glucose oxidation, ischemia

Introduction

In the myocardium, accelerated Na^+/H^+-exchange activity during and following myocardial ischemia can lead to accumulation of intracellular Na^+, and has been implicated in the pathogenesis of ischemic injury [1, 2]. Na^+ accumulation via the Na^+/H^+-exchanger during ischemia can exchange with Ca^{2+} via the Na^+/Ca^{2+}-exchanger leading to Ca^{2+} overload during reperfusion. In the diabetic rat heart, studies in both isolated sarcolemmal (SL) membranes [3] and in the intact heart [4] have shown that a decrease in the Na^+/H^+-exchanger activity occurs. This defect has been suggested to convey some protection to the severely ischemic diabetic rat heart secondary to a decrease in Na^+ accumulation both during ischemia and during reperfusion following ischemia. Consistent with a defect in the Na^+/H^+-exchanger, recovery of heart function following ischemia is significantly better in the diabetic rat hearts following severe no flow or very low flow ischemia [4–6]. Furthermore, recovery of pH following ischemia is slower in diabetic rat hearts compared to control hearts [4], and follows a time course similar to hearts perfused in the presence of amiloride, an inhibitor of the myocardial amiloride-sensitive Na^+/H^+ exchanger (NHE-1). To date, the reason why Na^+/H^+-exchange activity is

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decreased in the diabetic rat heart has not been determined.

A major source of acidosis during ischemia originates from glucose metabolism [7]. In aerobic hearts, if glycolysis is coupled to glucose oxidation the H^+ production from glucose metabolism is zero [7]. During ischemia, however, acceleration of glycolysis and a decrease in glucose oxidation creates an imbalance between these two pathways. This results in an overall increase in H^+ derived from hydrolysis of ATP originating from anaerobic glycolysis. However, regardless of the acidosis that occurs during ischemia myocardial levels of NHE-1 mRNA do not change dramatically during short episodes of ischemia [8]. In diabetes, myocardial glycolytic rates are depressed even in the absence of ischemia, secondary to a decrease in insulin-dependent glucose uptake and an increase in circulating fatty acid levels [9–11]. As a result, the potential for H^+ production from glycolytically derived ATP hydrolysis is chronically decreased in the diabetic rat heart. This raises the possibility that depressed Na^+/H^+-exchange activity in diabetic rat hearts is due to a decreased expression of NHE- 1 mRNA secondary to decreased glycolytic ATP production.

Recently, mRNA levels of NHE-1 have been shown to increase in response to various extracellular and intracellular stimuli [12–15]. Rao *et al.* [16] have demonstrated increases in NHE-1 mRNA levels as high as 25 fold in vascular smooth muscle cells treated with serum growth factors, platelet-derived growth factors and fibroblast growth factors. Moe *et al.* [17] have also shown that acid preincubation of mouse renal cortical tubule cells causes Na^+/H^+-exchanger mRNA levels to increase.

In the present study we determined H^+ production from glucose metabolism by directly measuring glycolysis and glucose oxidation in isolated working hearts from control and diabetic rats. H^+ production was determined during an aerobic perfusion, as well as during an extended period of severe ischemia and during aerobic reperfusion following ischemia. We also determined, using a rabbit myocardial cDNA clone [18], if the decrease in Na^+/H^+-exchanger activity observed in the diabetic rat heart can be explained by a decrease in the expression of the mRNA for NHE-1.

Material and methods

Materials

D-[U-^{14}C] glucose and D-[5-^{3}H(N)] glucose were purchased from New England Nuclear, Boston, Massachusetts, USA. Bovine serum albumin (fraction V) was obtained from Boehringer Mannheim, West Germany. Hyamine hydroxide (methylbenzethonium; 1 M in methanol solution) was obtained from New England Nuclear Research Products (Boston, MA, USA). Dowex 1-X4 anion exchange resin (200–400 mesh chloride form) was obtained from Bio-Rad Laboratories (Richmond, CA). ACS Aqueous counting scintillant was purchased from Amersham Canada (Oakville, Ontario, Canada). All other chemicals were reagent grade.

Experimental animals

Male Sprague-Dawley (protocol 1) or Wistar-Furth rats (protocol 2) ranging in weight from 250–275 g body weight were injected with a single i.v. injection of streptozotocin (55 mg/kg) dissolved in a citrate buffer solution (pH 4.5). In the first series of experiments (protocol 1) animals were used 6 weeks following the onset of diabetes. In the second series of experiments (protocol 2) the diabetic animals were divided into two groups. The first group were untreated diabetic controls. The second, experimental groups were diabetic rats that were transplanted with 1200 syngeneic islets of Langerhans. Islet transplantation was carried out by the laboratory of Dr. Ray Rajotte (Surgical Medical Research Institute, University of Alberta, Canada), as described in detail previously [19]. Control and both diabetic groups were then kept for an additional 8 week period prior to experimentation.

Heart perfusions

Hearts from sodium pentobarbital anesthetized rats were excised, the aorta cannulated, and a retrograde perfusion with Kreb's-Henseleit buffer (pH 7.4, gassed with 95% O_2-5% CO_2) initiated, as previously described [20]. During this initial perfusion the hearts were trimmed of excess tissue, the pulmonary artery cut, and the opening to the left atrium cannulated. Hearts were then switched to the working mode and perfused at an 11.5 mm Hg left atrial filling pressure and 80 mm Hg hydrostatic aortic afterload with buffer containing 11 mM glucose, 1.2 mM palmitate, and 100 μU/ml insulin. Palmitate was prebound to 3% bovine serum albumin. Heart rate, peak systolic pressure development (PSP), and developed pressure (ΔP) were monitored throughout the perfusion using a Spectramed P 23XL pressure transducer (Oxnard, CA, USA) in the aortic afterload line. Signals were recorded with a Gould RS-3600 physiograph (Cleveland, Ohio, USA).

Protocol 1

Hearts from control and 6 week streptozotocin diabetic rats were perfused for a 15 min aerobic perfusion, after which time a global no-flow ischemia was produced by clamping off

both left atrial inflow and aortic outflow lines. Following a 25 min period of ischemia, flow was re-initiated and hearts perfused under aerobic conditions for a further 60 min period. This type of perfusion protocol has been previously shown by us to produce only slight changes in the 5 kb NHE-1 message in hearts [8].

At the end of the reperfusion period, hearts were quickly frozen with Wollenberger tongs cooled to the temperature of liquid N_2.

Protocol 2

In this series of perfusions, glycolysis and glucose oxidation were measured simultaneously in hearts from control, diabetic, and islet-transplanted diabetic rats. Hearts were perfused with Krebs'-Henseleit buffer containing 11 mM [5-^{3}H/U-^{14}C] glucose and 1.2 mM palmitate and 100 μU/ml insulin. Hearts were initially perfused for a 40 min period under aerobic conditions, during which time glycolysis and glucose oxidation were measured. In this protocol hearts were paced throughout the procedure at 280 beats/min Following this, hearts were switched to the Langendorff mode and perfused with the same recirculating buffer at a coronary flow of 0.5 ml/min for a further 60 min period. At the end of this low-flow ischemia, hearts were switched back to the working mode and perfused under aerobic conditions for a further 30 min period. Hearts were then quickly frozen with Wollenberger tongs cooled to the temperature of liquid N_2.

Measurement of glycolysis and glucose oxidation

Glucose oxidation and glycolysis were measured in hearts perfused in protocol 2 by simultaneous measurement of $^{14}CO_2$ (glucose oxidation) and ^3H_2O (glycolysis) production. $^{14}CO_2$ and ^3H_2O production were measured at 10 min intervals prior to ischemia, at 20 min intervals during the 60 min period of low flow ischemia, and at 10 min intervals during aerobic reperfusion. Steady state oxidative rates of glucose were determined as described previously [20, 21]. After the retrograde drip-out perfusion, hearts were perfused in a closed system that allowed quantitative collection of both gaseous and perfusate $^{14}CO_2$. Glucose oxidation rates were expressed as nanomoles glucose oxidized per min per g dry weight. Steady state glycolytic rates were determined as previously described [20]. Glycolytic rates were expressed as nanomoles of glucose metabolized per minute per g dry weight.

Tissue workup

Frozen ventricular tissue from perfused hearts was weighed and powdered in a mortar and pestle cooled to the temperature of liquid N_2. A portion of the powdered tissue was used to determine the dry-to-wet weight ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 h at 100°C, and weighed. With the dried atrial tissue, total frozen ventricular weight, and the ventricular dry-to-wet weight ratio, the total dry weight of the heart was determined.

RNA isolation and analysis

Poly (A^+) RNA was prepared from isolated heart tissue using a modified procedure similar to that described earlier [8]. Ten μg of Poly (A^+) RNA was separated on 1.5% Formaldehyde gels and the mRNA was transferred to Immobilon-N transfer membranes and probed with [^{32}P] labelled random primed cDNA . The fragment of DNA used as the probe originated from the first 688 base pairs (coding region) of the rabbit cardiac cDNA clone [8]. To confirm that the mRNA samples were of the same quantity and quality, all Northern blots were stripped and reprobed with a cDNA probe encoding for actin and/or ribosomal 28S subunit. Hybridization and washes were as described earlier, with blots being routinely washed with 1 × SSC at 58°C for a total of 1 h.

Statistical analysis

The unpaired *t*-test was used for the determination of statistical difference of two group means. For groups of three, analysis of variance followed by the Neuman-Keuls test was used. A value of $p < 0.05$ was considered significant. All data are presented as mean ± S.E.M.

Results

Protocol I

Characteristics of control and diabetic rats

In the first experimental protocol rats were injected with a 60 mg/kg i.v. dose of streptozotocin. Within 24 h this resulted in the characteristic symptoms of diabetes (glucosuria, polyphagia, polydypsia, polyuria, and weight loss). At sacrifice, blood glucose levels were 23.5 ± 5.6 mM, compared to 6.3 ± 1.4 mM in control hearts. Animals were used 6 weeks following the onset of diabetes, since this is a time period in

Table 1. Recovery of mechanical function in diabetic rat hearts following 25 min of global no flow ischemia

	Heart rate (beats/min)	Peak systolic pressure (mm Hg)	HR × PSP ($\times 10^{-3}$)
Pre-ischemia			
–control	173 ± 10	115.2 ± 2.7	19.91 ± 1.23
–diabetic	150 ± 11	110.5 ± 3.3	16.73 ± 1.50
Post-ischemia			
–control	177 ± 9	94.9 ± 6.1*	16.70 ± 1.35*
–diabetic	166 ± 10	102.5 ± 5.6	18.24 ± 1.39

–Values are the mean ± S.E.M. of 22 control and 10 diabetic rat hearts. Ischemia was induced by clamping off both left atrial inflow and aortic outflow. Mechanical function in diabetic rat hearts was measured 30 min after reperfusion was restored. HR – heart rate; PSP – peak systolic pressure. *Significantly different than pre-ischemic function.

which the changes in cardiac Na^+/H^+-exchange occur. Table 1 shows the mechanical function observed in isolated working hearts perfused at a 11.5 mm Hg left atrial preload and a 80 mm Hg aortic afterload. A slight decrease in mechanical function was observed in the heart rate-pressure product of the diabetic rat hearts compared to control.

NHE-1 mRNA levels in control and diabetic rat hearts

We recently demonstrated that mRNA levels for NHE-1 from isolated hearts were not effected by short periods of ischemia and reperfusion. In this study, isolated working rats hearts were subjected to a 30 min period of global no-flow ischemia followed by a 60 min period of aerobic reperfusion. As shown in Table 1 and Fig. 1, a 109% recovery of function following ischemia was seen in the diabetic rat hearts, compared to a 83% recovery of function in the control hearts. This better relative recovery of function of diabetic rat hearts from a short period of no-flow ischemia parallels previous studies, and has been attributed to a slower recovery of pH during reperfusion due to a defect in Na^+/H^+-exchange activity.

At the end of the 60 min reperfusion period, hearts were quick frozen and the mRNA isolated. Figure 2 shows a Northern blot of control and diabetic rat heart mRNA probed with a fragment of rabbit NHE-1 cDNA. Although a decrease in myocardial Na^+/H^+-exchange activity in diabetic rat hearts has previously been suggested, no significant difference in mRNA levels of NHE-1 was observed. Attempts to measure actual levels of NHE-1 in the heart tissue were not made in this study due to the low amounts of NHE- 1 protein in the cardiac sarcolemmal membrane, and the inability of our existing antibodies to detect NHE-1 in whole heart tissue.

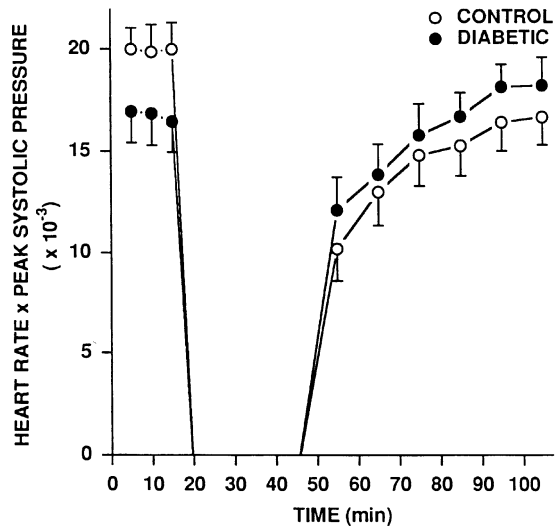

Fig. 1. Effects of a 25 min period of global no-flow ischemia on reperfusion recovery of heart function in control and diabetic rat hearts. Values are the mean ± S.E. of 22 control and 10 diabetic rat hearts. Hearts were perfused as described in Materials and methods.

Protocol 2

H^+ production in the cell is an important stimulus for both Na^+/H^+-exchange activity and for Na^+/H^+-exchange expression. Glycolysis uncoupled from glucose oxidation is a major source of acidosis during ischemia in the heart, which occurs secondary to glycolytically produced ATP hydrolysis. However, using a no-flow ischemia (such as used in protocol 1) does not allow for the direct measurement of glycolysis and glucose oxidation during ischemia. We therefore modified the protocol to a low-flow ischemia that allows

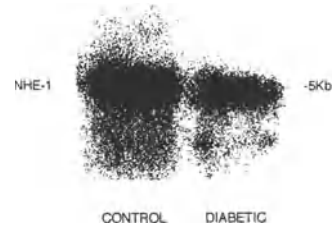

Fig. 2. NHE-1 mRNA levels in control and diabetic rat hearts. Shown is a representative Northern blot analysis, performed as in the Materials and methods section, of mRNA prepared from hearts obtained from control and diabetic rat heart subjected to 30 min global ischemia and 60 min of aerobic reperfusion. The figure is an autoradiograph performed after probing with ^{32}P-NHE-1.

for the measurement of glycolysis and glucose oxidation during the actual ischemic period. Even in the absence of ischemia, an imbalance between glycolysis and glucose oxidation can occur in fatty acid perfused hearts, resulting in a significant H^+ production derived from glucose metabolism. Due to the marked alterations in glucose metabolism seen in the diabetic rat hearts, we directly measured glucose metabolism in control and diabetic rat hearts prior to, – during, – and following an episode of severe ischemia. In addition, a group of diabetic rats was studied in which diabetes symptoms were normalized by transplantation of 1200 islets of Langerhans in the subrenal capsule. This was done to determine if normalizing the symptoms of diabetes could overcome the abnormalities in glucose metabolism and/or NHE-1 mRNA expression differences between the control and the diabetic rats.

As with protocol 1, administration of streptozotocin (55 mg/kg i.v.) resulted in the characteristic symptoms of diabetes. Plasma glucose levels rose to 29.8 ± 1.9 mM in the diabetic rats. Islet transplantation 3 weeks after the onset of diabetes resulted in a drop in both plasma glucose with the animals returning to baseline (7.5 ± 0.2 mM) by 3 weeks post transplantation and remaining at this level throughout the 8 weeks following this period.

Heart function in control, diabetic and islet-transplanted diabetic rats

Table 2 shows the mechanical function of hearts obtained from control, diabetic, and islet-transplanted diabetic rats. In this study, hearts were paced at 280 beats/min. As with protocol 1, a significant decrease in heart rate-pressure product was observed in the diabetic rat heart compared to control hearts. A significant decrease in the ΔP was also observed in the diabetic rat hearts. Islet transplantation prevented the decrease in PSP, heart rate-pressure product, and in ΔP.

At the end of a 30 min period of aerobic perfusion, hearts were subjected to a 60 min period of ischemia. A low flow ischemia was chosen so that glycolytic rates could be measured during the actual ischemic period. During the 60 min period, heart function could not be measured. Figure 3A and Table 2 show the recovery of heart function following ischemia. In control hearts, the severity of the ischemic insult resulted in a marked depression of functional recovery during reperfusion (Fig. 3A). This was reflected in both a decrease in heart rate, peak systolic pressure, and DP compare to preischemic rates (Table 2). It should be noted that even though hearts were paced during the reperfusion period, a rate of 280 beats/min was not achieved in any of the experimental groups during reperfusion. The high variability in heart rate seen in hearts during reperfusion can be explained by the fact that a number of hearts failed to recover, resulting in a heart rate value of zero being assigned to these hearts. Although functional recovery was also depressed in the diabetic rat hearts, recovery of function was significantly improved compared to control hearts. No significant difference in functional recovery was seen between the diabetic and islet-transplanted diabetic rat hearts.

Effects of H^+ production from glucose metabolism in diabetic rat hearts

Figure 3 shows the rates of glycolysis (Fig. 3B), glucose oxidation (Fig. 3C), and H^+ production from glucose metabolism (Fig. 3D) obtained at 10–20 min intervals throughout the 120 min perfusion period. As expected, rates of glycolysis were significantly depressed in the diabetic rat hearts compared to control hearts. In addition, a marked depression in glucose oxidation rates was also observed in diabetic rat hearts compared to control hearts. In the islet-transplanted diabetic rat hearts, this decrease in glycolysis and glucose oxidation did not occur.

Confirming our previous studies, in fatty acid perfused control hearts most of the glucose that passed through glycolysis to pyruvate was not oxidized. Calculated H^+ produced from glucose metabolism is shown in Fig. 3D. The amount of H^+ production derived from glycolytically derived ATP hydrolysis in aerobically perfused control hearts was similar to previously reported values [23]. Due to the lower rates of glycolysis seen in aerobically perfused diabetic rat hearts, H^+ production rates from glucose metabolism was significantly lower than rates seen in control hearts. This data demonstrates that the H^+ load derived from glucose metabolism in non-ischemic hearts is dramatically lower in diabetic rat hearts. In islet transplanted hearts, glycolysis, glucose oxidation, and H^+ production from glucose metabolism were all increased compared to untreated diabetic rat hearts, and were now similar to control.

During ischemia, glycolytic rates increased in all experimental groups, while glucose oxidation rates were abolished. This resulted in an increase in H^+ production rates from glucose metabolism during ischemia in all experimental groups, with the largest increase occurring in the islet-transplanted diabetic rat hearts. During reperfusion, glycolytic rates decreased and glucose oxidation rates increased in all groups. This decreased the imbalance between glycolysis and glucose oxidation, resulting in a dramatic drop in H^+ production rates from glucose metabolism. During this reperfusion period, very little difference in H^+ production rates from glucose metabolism were seen between the experimental groups.

Table 3 shows the steady state rates of H^+ production rates from glucose metabolism obtained during the aerobic,

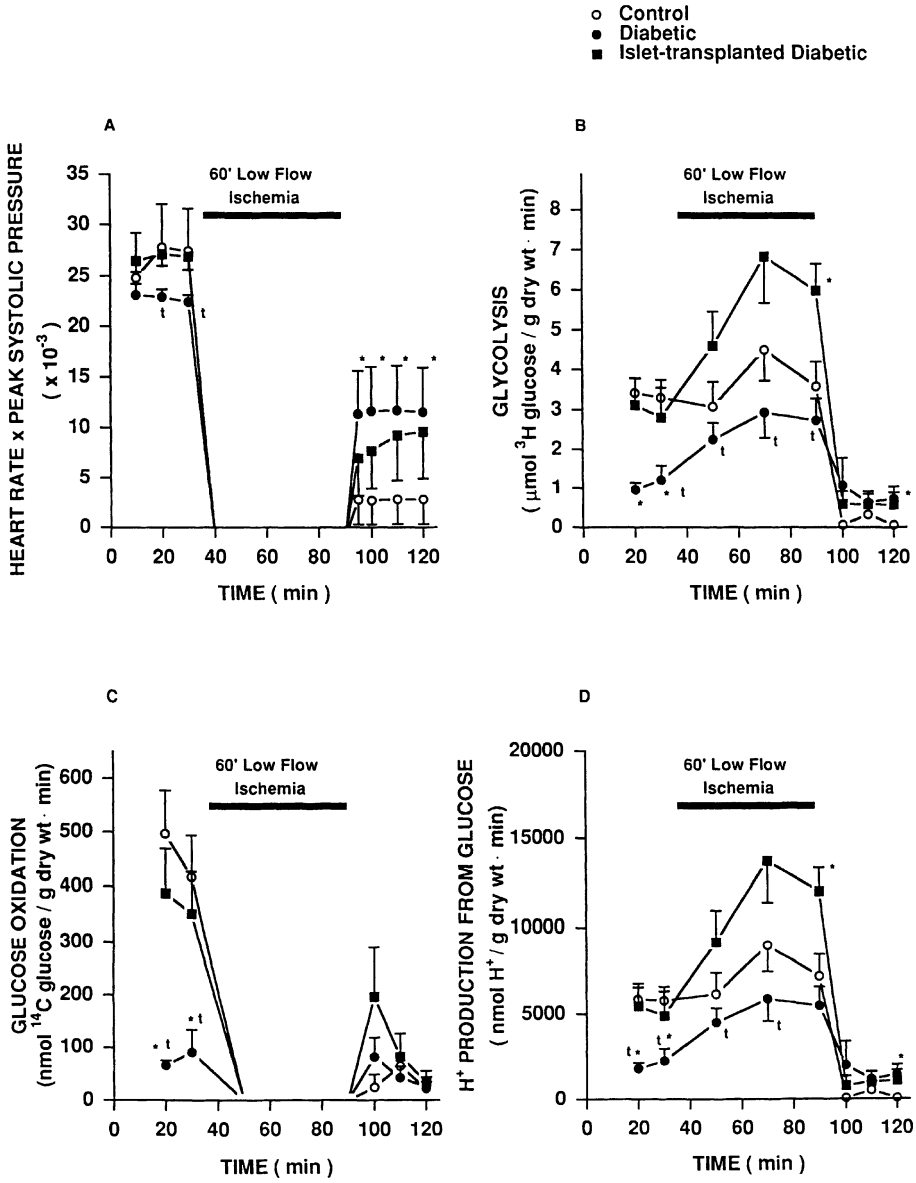

Fig. 3. Effect of a 60 min period of low flow ischemia on heart function, glycolysis, glucose oxidation, and H^+ production from glucose metabolism in control, diabetic, and islet-transplanted diabetic rat hearts. Values are the mean ± S.E. of 8 control, 7 diabetic, and 8 islet-transplanted diabetic rat hearts. Mechanical function (3A), glycolysis (3B), glucose oxidation (3C), and H^+ production (3D) were measured as described in Materials and methods.

ischemic, and reperfusion periods. H^+ production tended to be lower in the diabetic rat hearts, compared to either control or islet-transplanted diabetic rat hearts, especially during the initial aerobic period. Of importance was that H^+ production from glucose metabolism was never higher in the diabetic group than in the control of islet-transplanted group during the 120 min perfusion period.

Table 2. Recovery of mechanical function of hearts from control, diabetic, and islet transplanted rats subjected to 60 min of low flow ischemia

Perfusion condition	Heart rate (beats/min)	Peak systolic pressure (mm Hg)	Developed pressure (ΔP) (mm Hg)	HR×PSP ($\times 10^{-3}$)	HR×ΔP ($\times 10^{-3}$)
Pre-ischemia					
–control (n = 8)	280 (paced)	99.3 ± 3.4	45.3 ±4.6	28.1 ± 4.4	12.8 ± 2.3
–diabetic (n = 7)	280 (paced)	81.8 ± 3.3*	28.5 ±4.9*	22.9 ± 0.9*	7.9 ± 1.4*
–islet-transplanted diabetic (n = 8)	280 (paced)	98.5 ± 2.7[a]	38.0 ±4.5	27.6 ± 0.8[a]	10.6 ± 1.2
Post-ischemia					
–control (n = 8)	35 ± 35†	10 ± 10†	3.25 ± 3.3†	2.8 ± 2.5†	0.9 ± 0.8†
–diabetic (n = 7)	140 ± 53*†	41.3 ± 15.7†	10.5 ±4.3†	11.5 ± 4.4*†	2.9 ± 1.2†
–islet-transplanted diabetic (n = 8)	105 ± 51†	34.2 ± 16.7†	9.5 ±4.7†	9.6 ± 4.7†	2.7 ± 1.3†

–Values are the mean ± S.E.M. of the number of hearts shown in brackets. Hearts were perfused for 30 min under aerobic conditions before being subjected to a 60 min period of low flow ischemia (coronary flow = 0.5 ml/min) and a 30 min period of aerobic reperfusion. Heart function was measured prior to low flow ischemia or following 30 min of aerobic reperfusion following low flow ischemia. Spontaneous heart rates prior to pacing were 223 ± 17, 155 ± 18, and 214 ± 22 beats/min in control, diabetic, and islet-transplanted diabetic rat hearts, respectively. *Significantly different than control hearts; †significantly different than comparable hearts prior to ischemia; [a]significantly different than untreated diabetic rat hearts.

NHE-1 mRNA levels in control, diabetic, and islet-transplanted diabetic rat hearts

At the end of the 40 min reperfusion period, hearts were quick frozen with liquid N_2 and used for mRNA isolation. Figure 4 shows the Northern blots from the 3 experimental groups when probed with the cDNA for NHE-1. No significant differences in mRNA levels were observed. The data confirms our earlier results indicating that despite a consistently lower H^+ production from glucose metabolism during the extended perfusion protocol, the levels of NHE-1 mRNA were not depressed in the diabetic rat hearts compared to either control or islet-transplanted diabetic rat hearts.

Table 3. Steady state H^+ production from glucose in control, diabetic, and islet-transplanted diabetic rat hearts

	H^+ production from glucose (mmol H^+/g dry wt.min)		
	Aerobic	Ischemia	Reperfusion following ischemia
–control (n = 8)	6131 ± 1252	7432 ± 1325	1617 ± 1617
–diabetic (n = 7)	1993 ± 397*	5273 ± 753	2638 ± 939
–islet-transplanted diabetic (n = 8)	5172 ± 1339†	11613 ± 1726†	2518 ± 1168

–Values are the mean ± S.E.M. of the number of hearts shown in brackets. Note: only 1 control heart recovered glucose oxidation to the point where the H^+ production could be determined during reperfusion. *Significantly different than control hearts; †significantly different than untreated diabetic rat hearts.

Discussion

It has long been recognized that glycolytically derived ATP hydrolysis is a major reason for the intracellular acidosis that occurs during severe ischemia in the heart (see [10] for review). However, it has only recently been recognized that glycolytic rates can markedly exceed glucose oxidation rates even in the absence of ischemia [20, 22, 23). In heart perfused aerobically with high levels of fatty acids, glucose oxidation is inhibited to a much greater extent then glycolysis [20]. This is due to a marked inhibition of the mitochondrial pyruvate dehydrogenase complex by fatty acids [24]. In the presence of physiological levels of fatty acids, glucose uptake and glycolysis can be 5 times higher than glucose oxidation rates. At high levels of fatty acids (such as can be seen in diabetes or during and following an acute myocardial infarction in man) this difference can exceed 10 fold [20, 22]. As shown in Fig. 3 and Table 2, this can result in a significant H^+ production from ATP hydrolysis derived from glycolysis. In contrast, H^+ production derived from glycolysis coupled to glucose oxidation is zero. This is

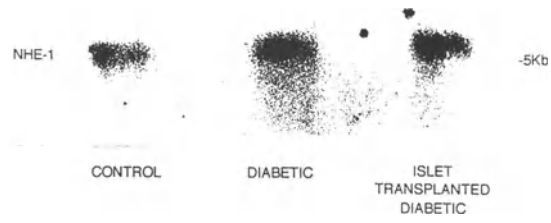

Fig. 4. NHE-1 mRNA levels in control, diabetic, and islet-transplanted diabetic rat hearts. Shown is a representative Northern Blot analysis, performed as in the Materials and methods section, of mRNA prepared from hearts obtained from control and diabetic rat heart subjected to a 60 min period of low flow ischemia followed by 30 min of aerobic reperfusion. The figure is an autoradiograph performed after probing with ^{32}P-NHE-1.

because as many H^+ are incorporated into ATP during synthesis as are released during hydrolysis [7]. The imbalance between glycolysis and glucose oxidation, and the resultant H^+ produced from this imbalance, may be one of the important reasons for Na^+/H^+-exchange activity in the heart.

Although glucose oxidation rates were low in the diabetic rat hearts compared to control hearts (Fig. 3), glycolytic rates were also significantly lower. Since glycolytic rates always far exceeded glucose oxidation rates in all hearts, the result was a lower H^+ production from glucose metabolism in the diabetic rat heart. It is possible that this decrease suggests that changes in SL membrane protein function in diabetes may be occurring secondary to changes in the membrane itself. As a result, decreased Na^+/H^+-exchange activity in the diabetic rat heart may not result from alterations in protein expression, but rather from secondary alterations in diabetes induced changes in the SL membrane. It cannot be concluded from our studies, however, that a post-translational modification of the exchanger may be occurring in the diabetic rat hearts. The exact nature of these alterations are not yet known. Further studies are needed to clarify this in diabetes.

In conclusion, we demonstrate that when considering diabetes-induced changes in cellular pH, alterations in the protons derived from glucose metabolism are as important as alterations in the fate of protons.

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Regulation of carbohydrate and fatty acid utilization by L-carnitine during cardiac development and hypoxia

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Abstract

This study is designed to investigate whether substrate preference in the myocardium during the neonatal period and hypoxia-induced stress is controlled intracellularly or by extracellular substrate availability. To determine this, the effect of exogenous L-carnitine on the regulation of carbohydrate and fatty acid metabolism was determined during cardiac stress (hypoxia) and during the postnatal period. The effect of L-carnitine on long chain (palmitate) and medium chain (octonoate) fatty acid oxidation was studied in cardiac myocytes isolated from less than 24 h old (new born; NB), 2 week old (2 week) and hypoxic 4 week old (HY) piglets. Palmitate oxidation was severely decreased in NB cells compared to those from 2 week animals (0.456 ± 0.04 vs. 1.207 ± 0.52 nmol/mg protein/30 min); surprisingly, cells from even older hypoxic animals appeared shifted toward the new born state (0.695 ± 0.038 nmol/mg protein/30 min). Addition of L-carnitine to the incubation medium, which stimulates carnitine palmitoyl-transferase I (CPTI) accelerated palmitate oxidation 3 fold in NB and approximately 2 fold in HY and 2 week cells. In contrast, octanoate oxidation which was greater in new born myocytes than in 2 week cells, was decreased by L-carnitine suggesting a compensatory response. Furthermore, oxidation of carbohydrates (glucose, pyruvate, and lactate) was greatly increased in new born myocytes compared to 2 week and HY cells and was accompanied by a parallel increase in pyruvate dehydrogenase (PDH) activity. The concentration of malonyl-CoA, a potent inhibitor of CPTI was significantly higher in new born heart than at 2 weeks. These metabolic data taken together suggest that intracellular metabolic signals interact to shift from carbohydrate to fatty acid utilization during development of the myocardium. The decreased oxidation of palmitate in NB hearts probably reflects decreased intracellular L-carnitine and increased malonyl-CoA concentrations. Interestingly, these data further suggest that the cells remain compliant so that under stressful conditions, such as hypoxia, they can revert toward the neonatal state of increased glucose utilization. (Mol Cell Biochem **180**: 95–103, 1998)

Key words: L-carnitine, fatty acid oxidation, neonatal metabolism, glucose oxidation, hypoxia

Introduction

The utilization of metabolic substrates changes considerably during the development of the heart. The myocardium of neonatal animals has a great capacity to utilize glucose and lactate, while that of adult animals utilizes fatty acids as its major energy source. Non-esterified fatty acids provide 60–70% of ATP production in the normal adult heart under aerobic conditions [1–3]. Metabolism of glucose produces most of the remainder, with lactate and ketone body utilization contributing very little. In contrast, in 1 day old rabbit, glycolysis provides 48% of ATP production in the myocardium whereas, palmitate oxidation provides as little as 13%, and lactate utilization provides 25% of the ATP production [4]. This marked difference between neonatal and adult myocardial substrate utilization could be explained, at

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least in part, by the huge difference in the levels of circulating substrates during development of the heart. In fetal circulation lactate concentration is extremely high reaching 10 mM, while free fatty acid levels are less than or equal to 0.025 mM [5, 6]. This could cause a reciprocal inhibition of fatty acid uptake secondary to increased levels of lactate oxidation. Alternatively, as indicated in several studies the mitochondrial capacity for oxidizing substrates is also altered in the neonate which could affect substrate usage [7, 8]. In addition, Tomec and Hoppel [9] have found that the activity of carnitine palmitoyltransferase I (CPT I), the enzyme responsible for the mitochondrial transport of long chain fatty acids, is reduced in neonatal hearts compared to adult hearts. Furthermore, studies by Prip-Buss and coworkers [10] have suggested that the sensitivity of CPT I to malonyl-CoA is greater in new born hearts than adult hearts, which could lead to inhibition of CPT I and decreased long chain fatty acid oxidation. Furthermore, in neonatal cardiac tissue, expression of the liver CPT I isoform (which has a K_m for carnitine of only 30 μM compared to a value of 500 μM for the muscle isoform) is increased; however, gradually during the several weeks after birth, the muscle isoform increases [11]. Thus, whether the transition in substrate utilization from neonate to adult, depends on changed substrate availability or intracellular changes remains to be determined.

Several studies have shown that new born hearts are better able to withstand hypoxia than mature hearts [12–15]. Greater tolerance to hypoxia in new born hearts is probably due to the marked ability of the myocardium to utilize glucose anaerobically through glycolysis. Recent studies have demonstrated the importance of ATP derived from glycolysis in protecting cardiac function during ischemia [16, 17]. Thus, the cardiac response to stress may be aided by altering substrate usage.

The present study is undertaken to investigate if the transition in substrate utilization during the maturation of the myocardium is controlled by intracellular metabolic signals independent of levels of circulating substrates and whether during periods of cardiac stress exogenous L-carnitine which alters the balance between fatty acid oxidation and glycolysis in the adult heart alters this relationship as it does during development.

Materials and methods

Animals

Newborn piglets were obtained from Charles River Laboratory (Raleigh, NC, USA). Induction of anesthesia was accomplished with Ketoset (22 mg/kg) and maintained with IV Pentothal (2.5%). Once adequate anesthesia has been attained, a median sternotomy was accomplished. The animal was exsanguinated and the heart was removed. The animal protocol was approved by Duke University's Institutional Care and Animal Use Committee.

Hypoxic animals

Briefly, four newborn piglets were placed into custom-built holding tanks in which the oxygen content was controlled at 12%. Animals were exposed to this environment for 1 month. A control series of six piglets exposed to the same routine care at room air were used. At the end of this period, these animals were sacrificed and cardiac myocytes were isolated and used in these studies.

Materials

[U-^{14}C]-D-glucose, [1-^{14}C]pyruvate, [2-^{14}C]pyruvate, [1-^{14}C]palmitate, [1-^{14}C]octanoate, [6-^{14}C]glucose and [1-^{14}C]lactate were purchased from New England Nuclear (Boston, MA, USA). Sigma was the source of bovine serum albumin (BSA, essentially fatty acid free). Joklik essential medium was purchased from Gibco Laboratories (NJ, USA). Collagenase type II was purchased from Worthington (NJ, USA).

Isolation of myocytes

Myocytes were isolated from newly born, 2 week old, and hypoxic pigs using the published method of Frangakis *et al.* [18]. Myocytes were isolated with Joklik essential medium containing 5.55 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The viability of myocytes isolated by this procedure was 90–95% as determined by trypan blue exclusion.

Metabolic studies using myocytes

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM HEPES (pH 7.4), were placed in a 25 ml Erlenmeyer flask. Cells were preincubated with or without L-carnitine (5 mM) for 10 min at 37°C. To this cell suspension was added 0.1 ml of a single labelled metabolic substrate yielding a final concentration of 2 mM [1-^{14}C]pyruvate (2×10^5 dpm), 2 mM [2-^{14}C]pyruvate (2×10^5 dpm), 5.55 mM [U-^{14}C]-D-glucose (2×10^5 dpm), 0.2 mM [1-^{14}C]-palmitic acid (2.2×10^5 dpm), 0.2 mM [1-^{14}C]octanoate (2.5×10^5 dpm), 2 mM [1-^{14}C]lactate (2.2×10^5 dpm), or 5.55 mM [6-^{14}C]-D-glucose (2×10^5 dpm). The Erlenmeyer flask was then closed with a rubber septum

containing a plastic center well. The incubation was continued during shaking at 37°C for 30 min. An injection of 0.4 ml of 1 M hyamine hydroxide was administered through the septum into the center wells to absorb the released CO_2, and the reaction was terminated by injecting 0.4 ml of 7% perchloric acid through the septum into the incubation medium. The flasks were then shaken continuously for 2 h at 37°C, at which time the plastic center well was removed, placed into a scintillation vial containing 10 ml of Scinti Verse BD, and counted in a liquid scintillation counter. Control experiments with $NaH^{14}CO_3$ added to the cell suspension showed that the release of $^{14}CO_2$ was complete 1 h after the addition of perchloric acid.

Stock solutions of substrates were prepared by dissolving glucose, pyruvate, lactate, or octanoate in a myocyte suspension buffer and by dissolving palmitic acid in a solution of defatted serum albumin in the cell suspension buffer. The molar ratio of palmitate to albumin was 2:1.

Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, VA).

Determination of malonyl-CoA levels

Biopsies from the left ventricular free wall were quickly removed, placed in liquid nitrogen and stored at –70°C. Samples were extracted with 6% perchloric acid and supernatants were neutralized to a pH 3.0. Malonyl-CoA levels were determined in these extracts using the HPLC method of Todd *et al.* [19].

Analysis of acetylcarnitine by tandem mass spectrometry

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, and 0.5 mM $CaCl_2$ were preincubated with L-carnitine (5 mM), or L-carnitine plus enoximone (0.5 mM) for 10 min at 37°C. Myocytes were then incubated for an additional 30 min at 37°C after the addition of 2 mM pyruvate and 0.2 mM palmitate. At the end of this period, cell suspensions were centrifuged for 2 min. To 0.1 ml aliquots of the supernatant, 20 pmol of [$^2H_3$]acetylcarnitine was added as internal standard. The mixture was extracted with 0.8 ml methanol, centrifuged and clear supernatant was dried under nitrogen and immediately prepared for analysis of acetylcarnitine. The dried aliquots containing acetylcarnitine were incubated with 100 µl of 3 M HCl in n-methanol at 50°C for 15 min in a capped 1 ml glass vial. The esterifying agent was removed by evaporation under nitrogen and the derivatized sample was dissolved in 50 µl of methanol:glycerol (1:1; v/v) containing 1% octyl sodium sulfate (matrix). A QUATTRO tandem quadrupole mass spectrometer (Fisons-VG Instruments, Danvers, MA) equipped with a liquid secondary ionization source and a cesium ion gun was used for the analysis of acetylcarnitine. This method of analysis is based on the detection of a common fragment ion of acetylcarnitine methyl esters produced by collision-induced dissociation as previously described by Millington *et al.* [20]. Approximately 2 µl of sample matrix was analyzed and the data recorded and processed as previously described. The final spectra displayed the relative intensities of ions corresponding to the molecular weights of the individual acylcarnitine methyl esters. The concentrations of acetylcarnitine corresponding to m/z 218 were determined based on their intensities relative to the internal standard, labeled acetylcarnitine, which corresponds to m/z 221.

Statistical analysis

Data are presented as the mean ± S.D. of 4 separate experiments. Statistical significance was determined by using paired and unpaired *t*-test and analysis of variance. Neuman-Keuls analysis was used to determine statistical difference between groups ($p < 0.05$ was considered significant).

Results

Figure 1A shows the oxidation of palmitate in the presence and absence of L-carnitine in myocytes isolated from new born, 2 week old, and hypoxic (4 weeks) pigs. Palmitate oxidation was severely depressed in new born myocytes when compared to 2 week old or hypoxic myocytes (0.456 ± 0.04 vs. 1.207 ± 0.52 and 0.695 ± 0.038 nmol/mg protein/30 min, respectively).

The addition of L-carnitine to the incubation medium accelerated palmitate oxidation 3 fold in new born and approximately 2 fold in hypoxic cells and 2 week old cells (Fig. 1B).

Table 1 shows the level of malonyl-CoA, a potent inhibitor of CPT I, in cardiac tissue isolated from new born and 2 week old piglets. Malonyl-CoA levels were significantly higher in the new born group.

The oxidation of octanoate, a medium-chain fatty acid which is independent of CPT I activity, was determined in these myocytes to investigate if only CPT I-dependent fatty

Table 1. Malonyl-CoA concentration in cardiac tissue from new born and 2 week old piglets (nmol/mg wet tissue)

2 week old	New born
16.11 ± 3.46	23.10** ± 3.38

Values are presented as the mean ± S.D. of five separate experiments. **Indicate a $p < 0.01$ of 2 week old vs. new born.

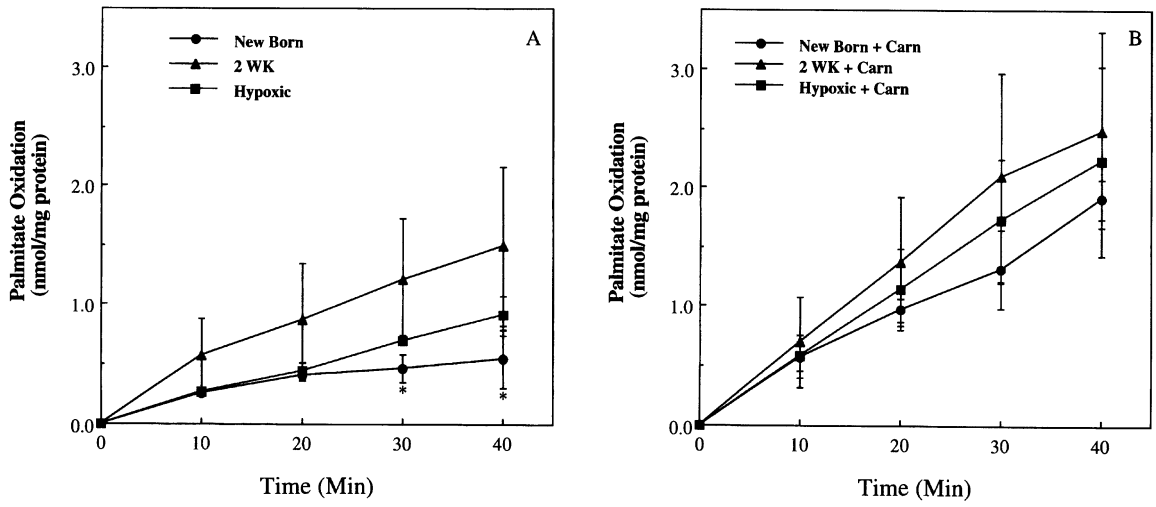

Fig. 1. The oxidation of palmitate in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. *indicates a $p < 0.05$ of new born vs. 2 week old. The p value of new born + carn vs. new born at 20 and 40 min was < 0.05. 2 week + carn vs. 2 week at 20, 30 & 40 min ($p < 0.01$, 0.05 and 0.01). Hypox + carn vs. hypox at 10, 20, 30 & 40 min ($p < 0.05$).

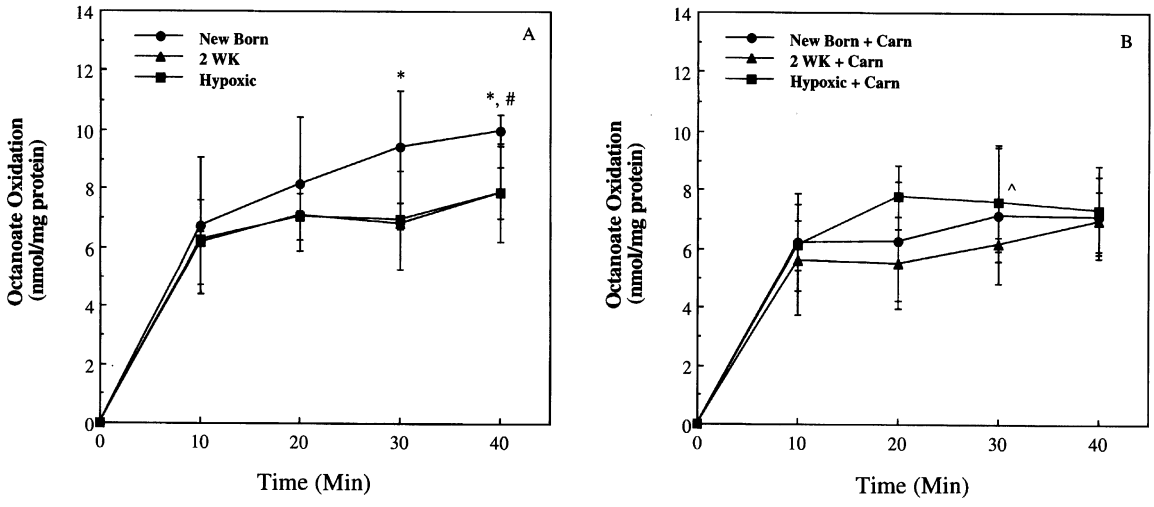

Fig. 2. The oxidation of octanoate in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. *indicates a $p < 0.05$ of new born vs. 2 week old; $p < 0.05$ of new born vs. hypox. The p value of new born + carn vs. new born at 20, 30 and 40 min was < 0.05. 2 week + carn vs. 2 week at 30 min ($p < 0.05$). Hypox + carn vs. 2 week + carn at 30 min ($p < 0.05$).

acid oxidation was decreased in new born myocytes. As shown in Fig. 2A octanoate oxidation was not decreased in new born myocytes. Instead, octanoate oxidation was significantly higher in new born myocytes at 30 and 40 min. Octanoate oxidation was decreased in these groups by addition of L-carnitine, particularly in the new born group where this decrease was statistically significant at 20, 30 and 40 min (Fig. 2B).

Figure 3 shows the oxidation of [U-^{14}C] glucose in the presence and absence of L-carnitine in myocytes isolated

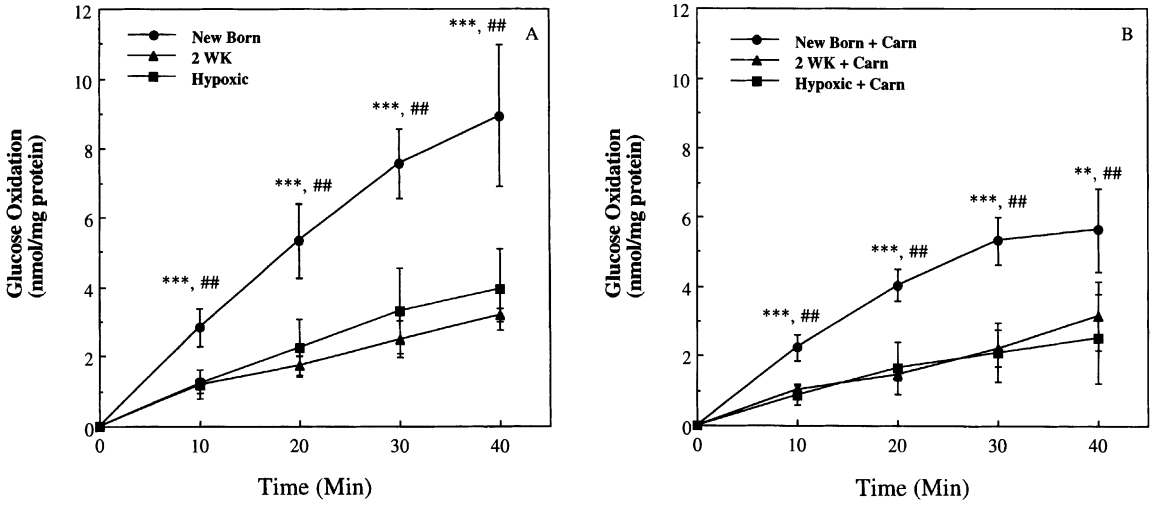

Fig. 3. The oxidation of [U-^{14}C]glucose in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. ***indicate a $p < 0.001$ of new born vs. 2 week old; $^{\#\#\#}p < 0.001$ of new born vs. hypox (Fig. 3A). **indicate a $p < 0.001$ of new born + carn vs. 2 week; $^{\#\#}p < 0.01$ of new born + carn vs. hypox (Fig. 3B).

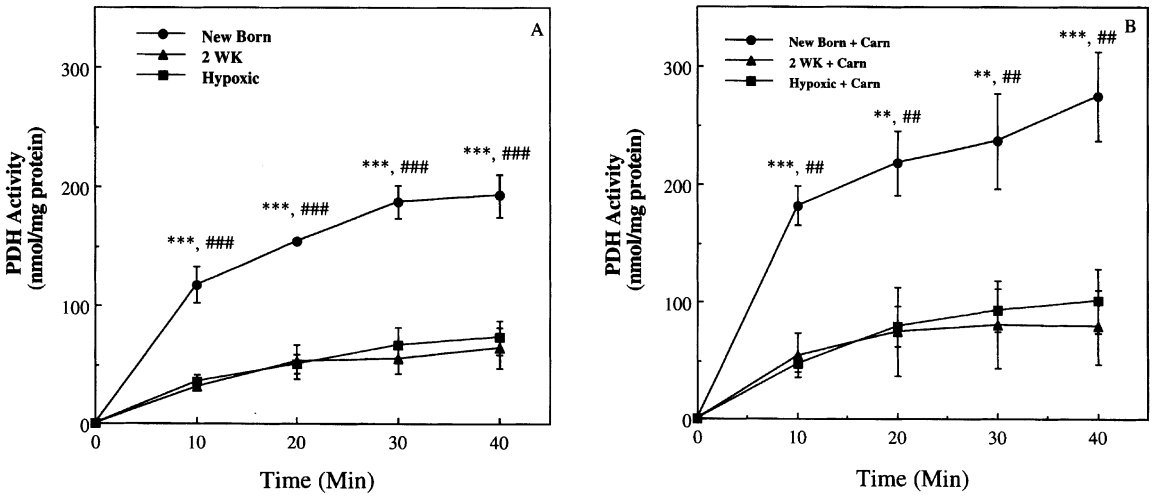

Fig. 4. The pyruvate dehydrogenase (PDH) activity in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. ***indicate a $p < 0.001$ of new born vs. 2 week old; $^{\#\#\#}p < 0.001$ of new born vs. hypox (Fig. 4A). **,***indicate a $p < 0.01$ and 0.001 of new born + carn vs. 2 week, $^{\#\#}p < 0.01$ of new born + carn vs. hypox (Fig. 4B).

from new born, 2 week old, and hypoxic (4 weeks) pigs. Glucose oxidation was greatly increased in new born myocytes when compared to 2 week old or hypoxic myocytes (7.56 ± 1.01 vs. 2.5 ± 0.52 and 3.30 ± 1.22 nmol/mg protein/30 min, respectively).

Glucose oxidation in hypoxic cells was intermediate between new born and 2 week old (Fig. 3A). The addition of L-carnitine to the incubation medium resulted in decreased glucose oxidation in both the new born and hypoxic groups (Fig. 3B). However in the new born group glucose oxidation

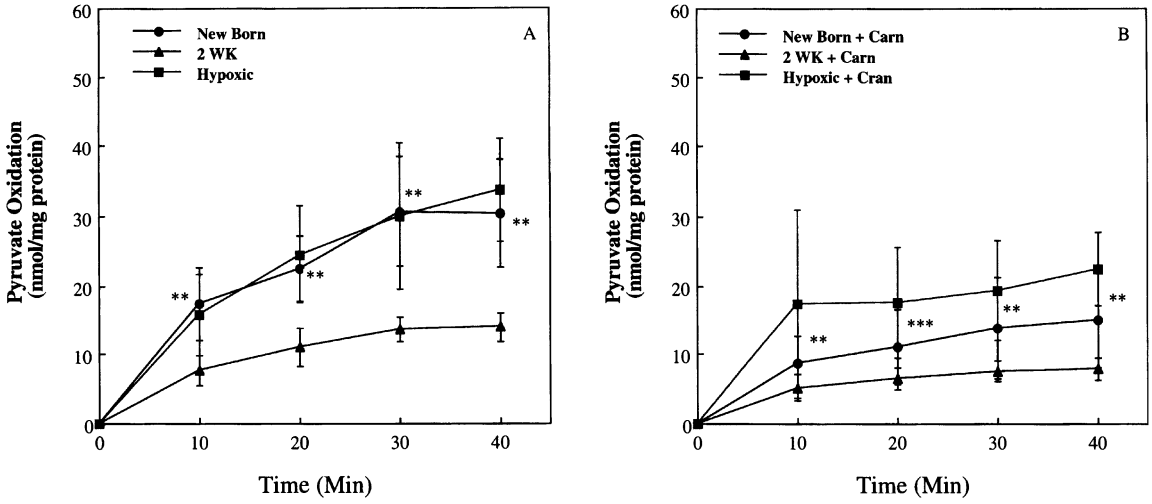

Fig. 5. The oxidation of pyruvate in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. **indicate a $p < 0.01$ of new born vs. 2 week; hypox vs. 2 week at 10, 20, 30 and 40 had $p < 0.05$, 0.01, 0.05 and 0.01 (Fig. 5A). New born + carn vs. new born had $p < 0.01$, 0.001, 0.01 and 0.01 (Fig. 5B).

was still significantly higher than in the other groups.

The pyruvate dehydrogenase complex (PDH) activity was determined in these myocytes to investigate if this enzyme is responsible for increased rates of glucose oxidation in new born myocytes. The PDH activity was determined by quantitating the amount of CO_2 released from 11-^{14}C]pyruvate. The PDH activity was remarkably higher in the new born group when compared with the 2 week or hypoxic groups. The PDH activity of the 2 week and hypoxic cells were similar (Fig. 4A). The increase in PDH activity by L-carnitine was only significant in the hypoxic cells (Fig. 4B).

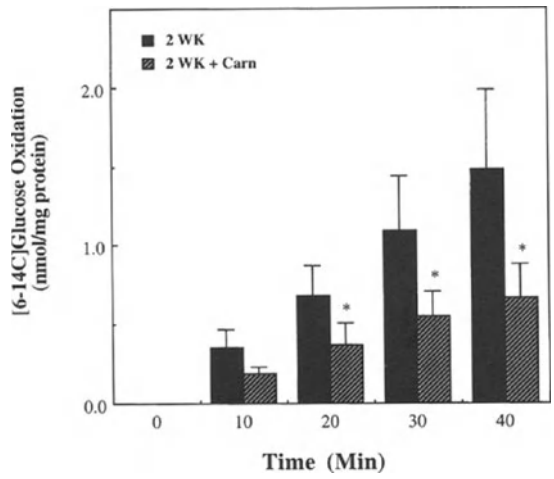

Fig. 6. The oxidation of [6-^{14}C]glucose in myocytes from 2 week old pigs in presence and absence of L-carnitine. Values are presented as the mean ± S.D. of at least 4 separate experiments. *indicates a $p < 0.05$ of 2 week vs. 2 week + carn.

The oxidation of [2-^{14}C]pyruvate, an index of the acetyl-CoA flux through the Krebs cycle in these myocytes is shown in Fig. 5. Pyruvate oxidation was much higher in new born and hypoxic groups than the 2 week group. The addition of L-carnitine to incubation medium significantly decreased the oxidation of pyruvate in these groups.

The effects of L-carnitine on the oxidation of [6-^{14}C]glucose was determined in only one group of myocytes (2 week). As shown in Fig. 6, L-carnitine significantly decreased the oxidation of [6-^{14}C]glucose in these myocytes.

Figure 7 shows the oxidation of lactate in the presence and absence of L-carnitine in myocytes isolated from new born, 2 week old, and hypoxic (4 weeks) pigs. Lactate oxidation was greatly increased in new born myocytes relative to 2 week old myocytes, whereas hypoxic myocytes were intermediate (54.58 ± 25.65 vs. 7.99 ± 1.23 and 21.75 ± 5.66 and nmol/mg protein/30 min, respectively). The addition of L-carnitine to the incubation medium resulted in the decrease of lactate oxidation in the 2 week old and hypoxic groups.

Discussion

Results from the present study clearly showed the preference of new born myocytes for utilizing glucose and lactate which

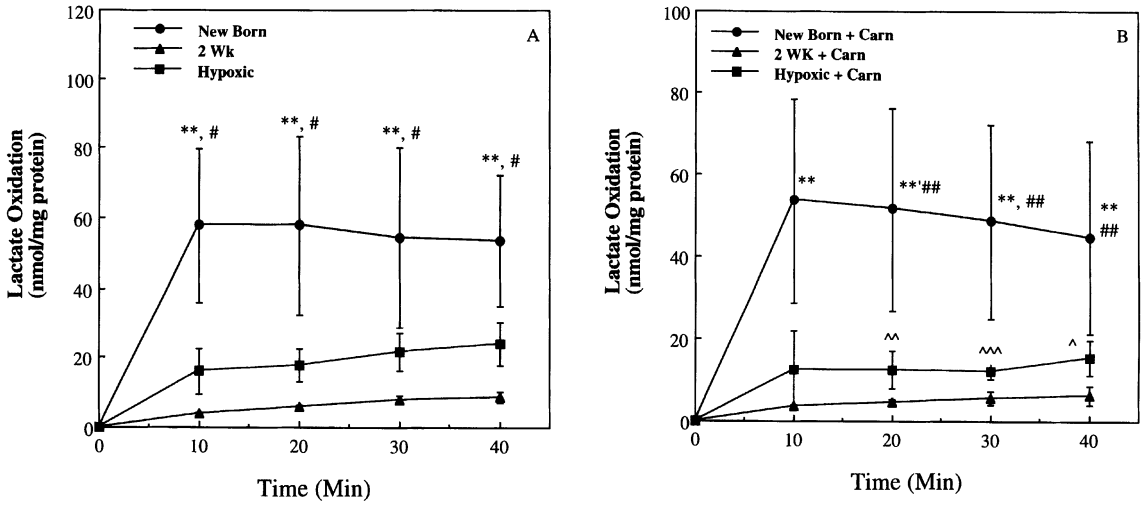

Fig. 7. The oxidation of lactate in absence (A) and presence of L-carnitine (B) in new born, 2 week old and hypoxic myocytes. For experimental details see section under Materials and methods. Values are presented as the mean ± S.D. of at least 4 separate experiments. **indicate a $p < 0.01$ of new born vs. 2 week; $^{\#}p < 0.05$ of new born vs. hypox (Fig. 6A). **indicate a $p < 0.01$ of new born + carn vs. 2 week + carn; $^{\#\#}p < 0.01$ of new born + carn vs. hypox + carn; ^'^^'^^^p < 0.05, 0.01, 0.001 of hypox + carn vs. 2 week + carn (Fig. 5B).

shifts to palmitate oxidation by 2 weeks after birth. This finding is consistent with several studies which documented the change in substrate utilization during the postnatal development of the heart [4–8]. In these studies, myocytes from new born animals showed a greater capacity for utilizing glucose and lactate despite the fact that they were incubated with the same concentration of substrates as cells from 2 week old pigs.

The regulation of intermediary metabolism in the heart depends on several factors: (1) levels of circulating substrates, (2) hormonal regulation of key enzymes that control substrate utilization, (3) work performed by the heart, (4) coronary flow and oxygen delivery to the heart, and (5) intracellular metabolic signals that coordinate the shift in substrate utilization. Since isolated cardiac myocytes in the present study were incubated with fixed concentration of substrates, the marked difference in oxidation of these substrates during the development of the myocardium is due to, at least in part, alterations of intracellular metabolic signals that coordinate carbohydrate and fatty acid utilization in the myocardium.

The marked decrease of palmitate oxidation in newly born myocytes could occur: (1) secondary to a decrease of L-carnitine and/or CPT I activity. L-carnitine is an essential cofactor for CPT I, a key enzyme for long chain fatty acid oxidation [21]. (2) subsequent to an increase of malonyl-CoA levels. Malonyl-CoA is a potent inhibitor of CPT I and long chain fatty acid oxidation [22], or (3) secondary to increased carbohydrate derived acetyl-CoA in the mitochondria. This acetyl-CoA could depress oxidation of fatty acid by inactivating 3-ketoacyl-CoA thiolase, the final enzyme of the β-oxidation pathway. Acetyl-CoA has been shown to be a potent inhibitor of 3-ketoacyl-CoA thiolase purified from pig heart tissue [23]. Findings from the present study are consistent with all these possibilities because the increase of palmitate oxidation by L-carnitine, and the lack of inhibition of octanoate oxidation (whose oxidation is independent of carnitine or CPT I), support the first hypothesis. This finding is also consistent with the lack of inhibition of palmitoylcarnitine in mitochondria from neonatal hearts [24]. The oxidation of palmitoylcarnitine, like that of octanoate, is independent of carnitine or CPT I. While increased malonyl-CoA levels, and increased carbohydrate-derived acetyl-CoA in new born myocytes support the other two hypotheses.

L-carnitine could increase palmitate oxidation by two independent mechanisms. First, by increasing the mitochondrial uptake of palmitate which is controlled by CPT I. Low levels of L-carnitine were found in neonatal myocardium [9], therefore addition of L-carnitine could increase palmitate oxidation by increasing its transport into the mitochondria. A second hypothesis is that, stimulation of palmitate oxidation by L-carnitine could occur secondary to decreasing the acetyl-CoA derived from carbohydrate metabolism. Presumably, L-carnitine reduces acetyl-CoA levels by interacting with acetyl-CoA to produce acetylcarnitine which is catalyzed by carnitine:acetylcarnitine transferase. Findings from the present study are consistent with both hypotheses because the increase of palmitate oxidation in new born and 2 week old myocytes by L-carnitine was paralleled by the decrease of oxidation of

acetyl-CoA derived from [2-^{14}C]pyruvate and [6-^{14}C]glucose.

In contrast to the depression of palmitate oxidation in new born myocytes, octanoate oxidation was increased in these cells. Since octanoate enters the mitochondria independent of the CPT system, this would suggest an alteration of long chain fatty acid oxidation in new born cells at extra site(s). Octanoate oxidation was likely increased in new born cells because of decreased utilization of endogenous long chain fatty acid in these cells compared to 2 week old cells. The increase of octanoate oxidation in the present study is consistent with the earlier findings of Itoi [24], which demonstrated the increase of palmitoylcarnitine in mitochondria from neonatal hearts.

The increased glucose utilization in new born cells compared to that of 2 week old and hypoxic cells is consistently correlated with the PDH levels in these cells. The PDH complex is a key enzyme in the pathway of oxidative glucose metabolism and increased levels of this enzyme could increase glucose oxidation [25]. Surprisingly, L-carnitine decreased glucose oxidation in the new born and hypoxic groups and had no effect on that of 2 week old. This could be explained by examining the mechanism by which L-carnitine decreases acetyl-CoA oxidation. L-carnitine decreases the acetyl-CoA levels by interacting with this compound and generating acetylcarnitine, thus preventing acetyl-CoA from being oxidized through the Krebs cycle. To further examine the effect of L-carnitine on decreasing glucose oxidation, we determined the effect of L-carnitine on the oxidation of [6-^{14}C]glucose. The radiolabelled CO2 in this case is released only through the Krebs cycle, thus avoiding interference with radiolabeled CO_2 produced from the PDH reaction as in case of [U-^{14}C]glucose. Although, the decrease of glucose oxidation by L-carnitine was insignificant with [U-^{14}C]glucose, its oxidation was significantly decreased when [6-^{14}C]glucose was used in the 2 week old cells. Previous studies in this laboratory have demonstrated the decrease of glucose oxidation by L-carnitine in adult rat myocytes [26]. These findings disagree with the study by Broderick *et al.* [27] who reported an increase of glucose oxidation by L-carnitine. Differences between the two studies could be due to the use of different experimental conditions. As mentioned previously, the study by Broderick *et al.* [27] have examined the effect of L-carnitine on glucose oxidation using [U-^{14}C]glucose to investigate this process. Furthermore, their study was performed using the isolated working heart where the affinity of carbohydrate-derived acetyl-CoA for Krebs cycle could be greater than that of isolated myocytes.

Lactate oxidation was markedly increased in the new born myocytes and was also increased, but to a lesser extent, in the hypoxic cells. High levels of PDH complex in new born cells may shift the equilibrium of lactate dehydrogenase towards pyruvate production. However, this mechanism could not account for the increase of lactate utilization in hypoxic cells since the PDH levels were not different from those in 2 week old cells. These findings argue against a previous study by Rolph and Jones [28] which indicated that the mitochondria of new born hearts have a limited oxidative capacity.

Since complete oxidation of metabolic substrates occurs finally inside the mitochondria, increased utilization of lactate, glucose, and octanoate in new born cells in the present study strongly support the hypothesis that the oxidative capacity of the mitochondria in these cells is not reduced as previously suggested by Goodwin and coworkers [7] and Glatz and Veerkamp [8].

In conclusion, these results demonstrated that the depression of long chain fatty acid oxidation in new born hearts may be due to over-reliance of the myocardium on glucose utilization, decreased levels of intracellular L-carnitine, and increased malonyl-CoA levels. These studies have also demonstrated that the oxidative capacity of the mitochondria is not reduced in new born hearts. Data from this study indicated that hypoxia appears to revert substrate utilization toward the neonatal state.

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Identification of nucleoside transport binding sites in the human myocardium

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Abstract

The role of nucleoside transport in ischemia-reperfusion injury and arrhythmias has been well documented in various animal models using selective blockers. However, clinical application of nucleoside transport inhibitors remains to be demonstrated in humans. It is not known whether human heart has nucleoside transport similar to that of animals. The aim of this study is to pharmacologically identify the presence of nucleoside transport binding sites in the human myocardium compared to animals.

Myocardial tissue was obtained from guinea pig left and right ventricle, canine left ventricle, human intraoperative right atrium and human cadaveric right atrium and right and left ventricles. Myocardial preparations were obtained from tissue samples after homogenized and a differential centrifugation.

Equilibrium binding assays were performed using [^{3}H]-p-nitrobenzylthioinosine (NBMPR) at room temperature in the presence or absence of non-radioactive NBMPR or other nucleoside transport blockers such as p-nitrobenzylthioguanosine dipyridamole, lidoflazine, papaverin, adenosine and doxorubcine. From saturation curves and inhibition kinetics, we determined the relative maximal binding (B_{max}) and dissociation constant (K_d) of [^{3}H]-NBMPR binding of human myocardial preparations.

Results demonstrated that the fresh human myocardial preparations have a specific binding site for NBMPR with a B_{max} of 283 ± 32 fmol/mg protein and K_d of 0.56 ± 0.12 nM. These values are lower than those obtained from guinea pigs (B_{max} = 1440 ± 187 fmol/mg protein and K_d = 0.21 ± 0.03 nM) and canine atrium (B_{max} 594 ± 73 fmol/mg protein, and K_d = 1.12 ± 0.22 nM).

Displacement kinetics studies revealed the relative potencies (of certain unrelated drugs as follow: p-nitrobenzylthioguanosine > dipyridamole > lidoflazine > pavaverine > Diltazam > adenosine > doxyrubicin. It is concluded that human myocardium contains an active nucleoside transport site which may play a crucial role in post-ischemic reperfusion-mediated injury in a wide spectrum of ischemic syndromes. (Mol Cell Biochem**180**: 105–110, 1998)

Key words: adenosine, atrium, cadaveric, coronary artery bypass grafting, cardiac surgery, diltiazam, dipyridamole, dogs, doxyrubicin, guinea pig, lidoflazine, paverine, ventricle

Introduction

The biological significance of endogenous and exogenous nucleosides has long been of medical interest and research continues to attempt to elucidate the role of these agents. Many analogues of the physiological nucleobases and nucleosides have been synthesized as potential therapeutic agents. A major impetus in this area was provided by the search for analogues with antineoplastic activity; more recently the cardiovascular activity and anti-viral activity of various synthetic nucleosides have become a focus of many recent investigations [1–7].

The nucleoside adenosine plays a major role in the autoregulation of coronary, cerebral, and renal blood flow. The action of adenosine and its nucleoside analogues at extracellular receptor sites is terminated by either rapid uptake into surrounding cells by a membrane located nucleoside transporter or deamination by adenosine deaminase to inosine. The nucleoside transporter is a membrane-bound, nonconcentrative, bidirectional nucleoside transport mechanism of broad specificity [8, 9]. Inhibition of nucleoside transporter, which might be expected to potentiate the effects of adenosine and other nucleosides, has been proposed as the mechanism responsible for the vasodilator effects of dipyridamole and

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of the amnesic agent diazepam. Nucleoside transport inhibition has also been shown to decrease uptake of numerous nucleoside-like chemotherapeutic agents into host cells [10]. We have demonstrated that inhibition of nucleoside transport effectively traps adenosine and inosine within cardiac tissue during ischemia and prevents free radical-mediated injury caused by post-ischemic reperfusion [1–5].

Previously, nucleoside transporter was extensively characterized in human and animal erythrocytes and lymphoma cell lines using either uptake of a permeant or a site specific binding of the potent and selective nucleoside transport inhibitor *p*-nitrobenzylthioinosine (NBMPR) [11, 12]. Work done with cardiac membranes in a variety of mammals revealed a rather wide species variability in the maximal binding capacity of bovine, guinea pig, canine, rabbit, mouse and rat tissue [13]. Species-related differences in nucleoside transport rates and in the binding of NBMPR to erythrocytes has also been demonstrated [14]. We also demonstrated age-related differences in the binding of [^{3}H]-NBMPR to myocardial membrane preparations [15]. Work from our laboratory has focused on characterization of the physiologic and pathophysiologic role of myocardial nucleoside transporter *in vivo* using small and large animal models and isolated cardiomyocytes. Nucleoside trapping during ischemia or only during early reperfusion have been shown to have anti-arrhythmic and anti-stunning cardioprotective actions [16–18]. However, nucleoside transport binding sites have not been identified in the human heart. Therefore, the aim of this study was to characterize the selective binding of NBMPR to human cardiac membranes and compare binding kinetics to tissue preparations from animals.

Materials and methods

Tissue sources and membrane preparations

Myocardial tissue was obtained from the following sources: (1) guinea pig left and right ventricle, (2) canine left ventricle, (3) human intraoperative right atrium, and (4) cadaveric human right atrium, right ventricle, and left ventricle. Hearts were excised from anesthetized guinea pigs and the left and right ventricles and inter-ventricular septum were retained for study. Mongrel dogs were anesthetized with intravenous injection of sodium pentobarbital (35 mg/Kg), a left thoracotomy was performed and the heart was excised. A transmural biopsy (approximately 3 g) of the left ventricle was retained for each study. Intraoperative human right atrial tissue was obtained from patients undergoing cardiac surgery for either coronary artery bypass grafting or cardiac valve replacement. After median sternotomy, an approximately 0.5–2 g biopsy of the right atrial appendage was taken during venous cannulation for extracorboreal circulation (cardiopulmonary bypass). Human cadaveric tissue was obtained during post-mortem autopsies with acquisition of transmural biopsies of the right atrium, left ventricle, and right ventricle in each patient.

Biopsies (1–5 g) were immediately placed in ice-cold 0.34 M sucrose, deprived of associated fat and coronary vessels, and weighed. Tissues were washed and homogenized on ice in 10–20 vols (w/v) of ice cold 0.32 M sucrose. Polytron homogenization (setting 6 for 10 sec) was followed by hand homogenization until complete. The suspension was then centrifuged at 1000 g for 10 min at 4°C. The supernatant was aspirated exclusive of the pellet and recentrifuged at 22,000 g for 25 min at 4°C to obtain a crude membrane pellet. The pellet was resuspended using vortex mixing in 20–50 volume of 50 mM Tris-HCl buffer, pH 7.4, to a final protein concentration of 0.5–1.0 mg/ml. Protein concentrations were spectrophotometrically determined using bovine serum albumin as standard as described by Lowry [19].

[^{3}H]-NBMPR binding assay

Equilibrium binding assays were performed at room temperature (23°C) by adding protein suspension (0.2 mg) to an incubation medium (l ml) containing graded concentrations of [^{3}H]-NBMPR (0.01–4.0 nM) in a buffered medium containing 50 mM Tris-HCl, pH 7.4. All samples were incubated for 30 min at 23°C. The incubation was terminated by filtration under vacuum through Whatman GF/B filters. Each filter was washed twice with 5 ml of an ice cold buffer. [^{3}H]-NBMPR bound to membranes was counted by a liquid scintillation counter. Non-specific NBMPR binding was determined by preincubating tissue preparations first with cold NBMPR (10 uM) before the addition of [^{3}H]-NBMPR.

The specific [^{3}H]-NBMPR binding was calculated by subtracting the non-specific binding from the total [^{3}H]-NBMPR binding. Dissociation constants (K_D) and maximal binding capacities (B_{max}) for [^{3}H]-NBMPR were determined from Scatchard analysis of the displacable binding component. The inhibitory effects of several known selective and non-selective nucleoside transport inhibitors and other pharmacologic agents on the [^{3}H]-NBMPR binding to human right atrial cardiac membranes similarly were studied. Inhibition constants (K_i) were obtained through double reciprocal and log-probit competition analysis of the site-specific binding component for various concentrations of *p*-nitrobenzylthioguanosine (HNBTG), dipyridamole, lidoflazine, papaverine, diltiazem, adenosine, and doxorubicin.

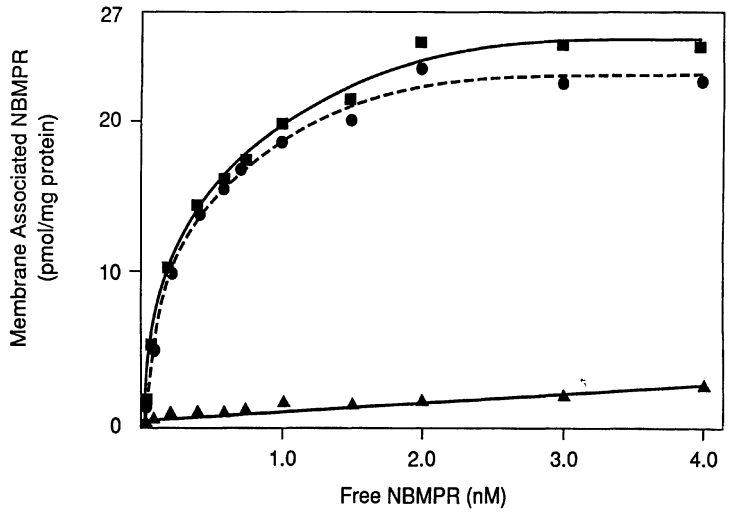

Fig. 1. Nucleoside transport binding: The figure illustrates the saturation curves of the total and non-specific binding of [^{3}H]-NBMPR binding in fresh human myocardial preparations. Tissue preparations were incubated with varying concentrations of [^{3}H]-NBMPR The specific binding was calculated from the difference between the [^{3}H]-NBMPR binding in the absence and presence of non-radioactive NBMPR (25 μM), respectively. Data presented as means of 3–5 replicates from at least 3 different preparations.

Results

The site-specific binding of [^{3}H]-NBMPR to cardiac membrane preparations in all tissues studied was proportional to the protein concentration in the assay mixtures. Binding occurred rapidly and equilibrium was attained by 12 min. The total amount of [^{3}H]-NBMPR associated with cardiac membrane preparations of all tissues studied consisted of two components (Fig. 1): one of which was site-specific and saturable whereas the other (nonspecific binding) was proportional to free [^{3}H]-NBMPR concentration. Non-specific binding was similar when determined in the presence of either cold NBMPR (10 μM) or HNBTG (30 μM). Specific binding was calculated by subtraction of non-specific bound radioligand from the total [^{3}H]-NBMPR bound at each concentration of radioligand. Figure 1 depicts a representative of saturation curves obtained from fresh right atrial preparations. Analysis of the inhibitable binding component by the method of Scatchard yielded straight line plots which indicated the binding sites were of a single type (Fig. 2). The maximal binding capacity (B_{max}) and the apparent dissociation constant (K_D) of [^{3}H]-NBMPR at these sites is shown in Table 1. B_{max} was greater in the guinea pig followed by canine, intraoperative right atrium, and finally cadaveric chambers.

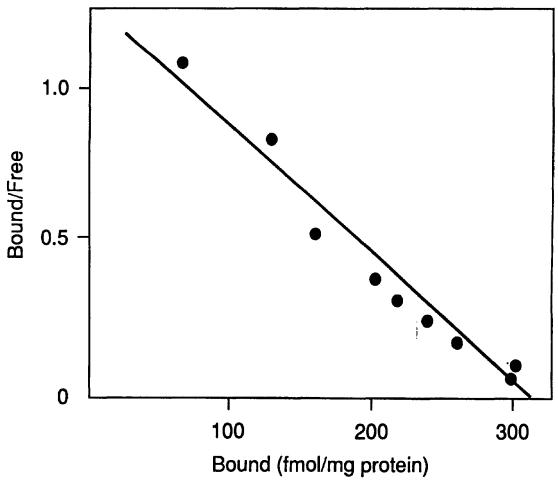

Fig. 2. Scatchard plot demonstrating inhibition kinetics of [^{3}H]-NBMPR binding to human myocardial membranes. Data are presented as mean of 3–5 replicates of at least 3 different preparations.

Table 1. Nucleoside transport binding in the human myocardium

	[^{3}H]-NBMPR binding in animal and human myocardium	
	Maximum binding* B_{MAX} (fmol/mg protein)	Dissociation constant* K_D(nM)
Guinea pig ventricle	1440 ± 187	0.21 ± 0.03
Canine ventricle	594 ± 73	1.12 ± 0.22
Human ventricle (cadaveric)	283 ± 32	0.56 ± 0.12

* Results are presented as mean ± S.E.M., n = 3–5 preparations and at least 3 replicates in each preparation.

K_D was seen to be highest in the canine species and lowest in the guinea pig with human intermediate in value.

All tissue samples in the intraoperative and cadaveric groups were obtained from patients who were receiving either oral or intra-venous medication; however, due to the small number of patients in each group, no trends or significance in the variables determined could be attributed to the type or quantity of medication. All experiments were performed (otherwise stated) using freshly prepared membrane suspensions and each study was performed in 3–5 replicate per each preparation with averaging of at least 3 different preparations prior to Scatchard analysis. Each degree of freedom represents a single biopsy specimen, as no tissue pooling was required. Binding parameters were shown to be unaffected by repeated washing of the tissue or membrane suspension during preparation. Both boiling (100°C for 10 min) and trypsin digestion (0.5 mg/ml) of the membrane preparations resulted in complete elimination of the specific binding component of [^{3}H]-NBMPR.

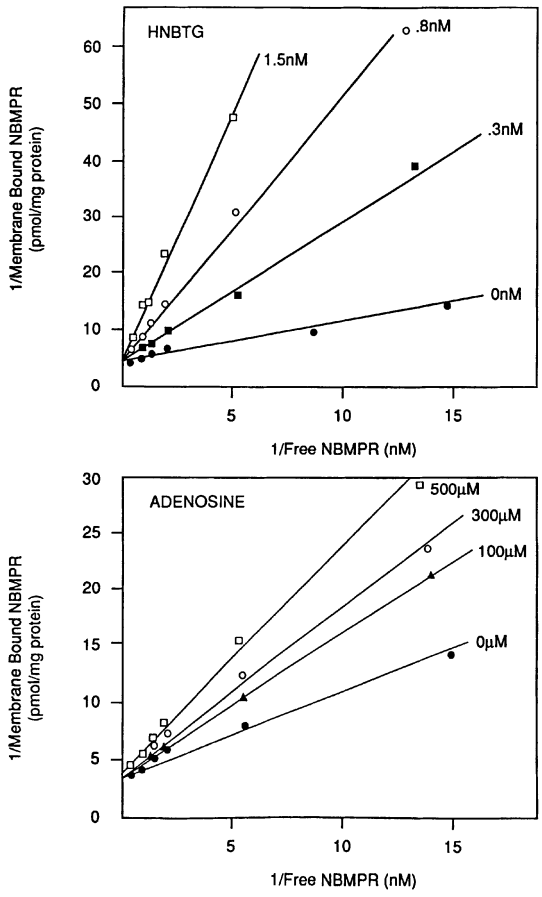

Fig. 3. Scatchard plot demonstrating inhibition kinetics of [^{3}H]-NBMPR binding to human myocardial membranes by different concentrations of the nucleoside transport blocker HNBTG or adenosine. Results are presented as mean of 3–5 replicates from at least 3 different preparations.

Table 2. Inhibition constant of certain inhibitors of [^{3}H]-NBMPR binding to human myocardial preparations

Inhibitor	Ligand inhibition K_i*
HMBG	0.14 ± 0.024 nM
Dipyridamole	19.3 ± 9.3 nM
Lidoflazine	81.3 ± 21.7 nM
Papaverine	28.7 ± 5.9 μM
Diltazam	215.0 ± 63.9 μM
Adenosine	272.7 ± 86.7 μM
Doxyrubicin	5–400 μM

* Inhibition constant; results are presented as mean ± S.E.M., n = 3–5 preparations and at least 3 replicates in each preparation.

Mass law analysis using the double reciprocal plot method of the inhibition of the site-specific binding of [^{3}H]-NBMPR to cardiac membranes of human intraoperative right atrial tissue revealed that inhibition was apparently competitive (plots of the reciprocals of bound NBMPR and the corresponding equilibrium concentration of free NBMPR intersected on the ordinate) for all agents studied. Representative examples for HNTGB and adenosine are shown in Fig. 3.

The recognized nucleoside transport inhibitors HNBTG and dipyridamole were potent inhibitors of the selective binding of [^{3}H]NBMPR (Table 2). Similarly, lidoflazine, papaverine, diltiazam and adenosine inhibited the binding of [^{3}H]NBMPR in concentration dependent manner. Doxorubicin was incapable of displacing [^{3}H]NBMPR even at concentrations up to 400 μM.

Discussion

This study demonstrates that saturable high affinity binding sites for the recognized nucleoside transport inhibitor NBMPR exist on human myocardial membranes. Selective binding of [^{3}H]NBMPR occurred to a single class of sites, was entirely eliminated by both denaturation and proteolytic digestion, and could be inhibited in a competitive fashion by other known nucleoside transport inhibitors and pharmacologic agents. The data presented for canine and guinea pig species are in concordance with that previously observed in the literature with respect to maximal binding capacity and binding affinity [20]. The rank order of magnitude of inhibition by pharmacologic agents demonstrated here in human myocardium is also similar to that seen in other mammalian species [21]. Therefore, we suggest that the human myocardial nucleoside transport system, displayed characteristics similar to that described in other human and mammalian tissues. In

fresh human right atrial tissue the maximal binding capacity (B_{MAX}) was seen to 395 fmol/mg protein as the constant of dissociation (K_d) was 0.31 nM. This indicates a somewhat lower concentration of binding sites but a slightly higher affinity of binding in human right atrium when compared to the canine species.

The ability to obtain fresh human right atrial tissue in the present study is thought to be important. Freezing and thawing of the myocardial membrane fraction or prolonged storage of human erythrocytes was seen to diminish the binding characteristics of high affinity ligands for the nucleoside transport sites in previous studies. Our results reveal a reduction in both maximal binding capacity and affinity when comparing fresh right atrial to cadaveric right atrial tissue as mean time to membrane preparation after death was 8.5 h (range 5–16 h) in the latter. This prolonged time to processing may explain the differences between the fresh and cadaveric right atrial data. Binding capacity and affinity were seen to differ slightly between the various cardiac chambers but no statistical analysis was attempted to show differences.

It was not possible in this study to control patient medications, an obvious shortcoming. It might be predicted that certain medications such as dipyridamole may and should well alter the binding characteristics of NBMPR. The numbers in this study were not sufficient to make inferences concerning this; however, results in two patients taking dipyridamole and one patient taking diltiazem revealed binding capacities and affinities within one standard deviation of the mean. Additional more highly controlled patient studies would be required to determine the effectiveness of commonly used dosages to effect a change in binding characteristics. No attempts were made in this study to define the specific site at which the transporter exists. Prior studies in human erythrocytes reveal that it is a membrane associated proteinaceous species. Our data are consistent with this finding; however, we cannot conclude whether it lies on endothelial, myocyte, sarcolemmal, or mitochondrial membranes or on any other component of the microsomal fraction. Further membrane purification techniques may be helpful to elucidate this important point.

Our results demonstrate that a nucleoside transport system exists in the human myocardium. Therefore, specific efforts should be directed to design and develop selective nucleoside blockers that can modulate nucleoside uptake and release of nucleosides of therapeutic potentials. Nucleoside trapping before or after ischemia attenuated free radical production, reperfusion-mediated arrhythmias and ventricular dysfunction (stunning). Site specific trapping of endogenous adenosine may also promote endogenous mechanisms of protection via adenosine signaling and activation of A_1 and A_3-receptors and K_{ATP} channels. This property may be useful in trapping nucleoside intracellularly to diminish depletion intracellular nucleoside pool during post-ischemic reperfusion.

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Mutagenesis and characterization of specific residues in fatty acid ethyl ester synthase: A gene for alcohol-induced cardiomyopathy

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Abstract

Fatty acid ethyl ester synthase-III metabolizes both ethanol and carcinogens. Structure-function studies of the enzyme have not been performed in relation to site specific mutagenesis. In this study, three residues (Gly 32, Cys 39 and His 72) have been mutated to observe their role in enzyme activity. Gly to Gln, Cys to Trp and His to Ser mutations did not affect fatty acid ethyl ester synthase activity, but His to Ser mutant had less than 9% of control glutathione S-transferase activity. The apparent loss of transferase activity reflected a 28 fold weaker binding constant for glutathione. Thus, this study indicates that Gly and Cys may not be important for synthase or transferase activities however, histidine may play a role in glutathione binding, but it is not an essential catalytic residue of glutathione S-transferase or for fatty acid ethyl ester synthase activity. (Mol Cell Biochem **180**: 111–115, 1998)

Key words: ethanol, catalysis, enzymes, carcinogens, mutations, cardiomyopathy

Abbreviations: FAEE – Fatty acid ethyl ester; FAEES-III – Fatty acid ethyl ester synthase-III; GST – Glutathione S-transferase; SFFV-neo – Friend spleen focus-forming virus-neo; SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Alcohol-induced heart disease is common in the United States and is similar to idiopathic cardiomyopathies, with the defining difference being the chronic consumption of ethanol. The disease is characterized by arrhythmias, cardiomegaly, and congestive heart failure. The biochemical link between ethanol ingestion and most of its acute and chronic effects on the heart had not been clearly established until we reported a new pathway for alcohol metabolism [1–3].

Fatty acid ethyl esters (FAEE), metabolic products of ethanol, have been detected in concentrations as high as 115 uM in human hearts obtained at autopsy of individuals who were either acutely intoxicated or were chronic ethanol abusers [1–7]. Recently, we showed that fatty acid ethyl ester synthase/glutathione S-transferase can also catalyze synthesis of fatty acid ethyl esters from hydrophobic carboxylic acids and ethanol [1–7]. This observation not only expanded the role of this enzyme in processing toxic xenobiotics, but it also provided another catalytic reaction for monitoring structure-function studies. Because of this new kinetic finding, we have begun a series of studies to determine if this synthetic reaction (formation of ethyl esters) is catalyzed by the same amino acid residues as those used in the transferase reaction or if there are two separate reaction pathways.

The first insight into this question was provided by a kinetic and structural comparison between FAEES-III and GSTπ-1 [7]. These two proteins contain 210 amino acids per subunit and differ by only four residues in the total primary structure. Despite this high degree of homology, GSTπ-1 does not catalyze the synthase reaction while FAEES-III can catalyze both fatty acid ethyl ester synthesis and the

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glutathione transferase reaction. This striking difference is most easily explained by assuming that each reaction has a different set of residues which are essential for catalysis.

The sequence of FAEES-III cDNA, recently cloned in our laboratory, contains Gly 32, Cys 39 and His 72 and is different from GSTπ-1. In this work, we have used site-directed mutagenesis of FAEES-III to probe the effect of a single amino acid substitution on both fatty acid ethyl ester synthase and glutathione transferase.

Materials and methods

Materials

Mutagenesis reagents, the corresponding bacterial cells, nitrocellulose paper, and electrophoresis reagents were obtained from Bio-Rad. Restriction enzymes and T4 DNA ligase were obtained from Promega. DEAE-dextran was purchased from Pharmacia LKB Biotechnology, Inc. The primers used in the study were provided by Midland Certified Reagent Company (Midland, TX).

Site specific mutagenesis

Oligonucleotide-directed, site-specific mutagenesis was performed according to Kunkel *et al.* [8]. The full length human FAEES-III cDNA (7), containing the EcoR1 restriction sites at both ends of the cDNA, was subcloned into the EcoR1 site of bacteriophage M13 mp18 [9]. The recombinant DNA was used to transfect the dut- and ung- *E. coli* CJ236 cells, allowing the incorporation of uracil into DNA in place of thymidine. Single stranded DNA was then isolated from these cells and used as a template for the mutagenesis experiments.

Oligonucleotide primers were designed to substitute the (codon CAA), a Gln in place of Gly (codon GGC), Trp (codon TGG) in place of Cys (codon TGC) and a Ser residue (codon AGC) in place of histidine (codon CAC) at residue 32, 39 and 72 respectively. The mutagenic oligonucleotide primers were then used to hybridize with the single stranded M13 DNA template. In each experiment, heteroduplex plasmid was formed by adding the Klenow fragment of DNA polymerase 1 and T4 DNA ligase and incubating for 2 h at 37 degrees C. Aliquots of the reaction were then used to transform competent *E. coli* DH5*a*F' cells. The presence of uracil in the parent strand resulted in its specific hydrolysis and the selection of the non-uracil containing mutagenized daughter strand. The resultant clones were further screened by digestion with EcoR1 of the M13 replicative form of DNA. The selected clones were propagated in *E. coli* cells, and the DNA was analyzed further by complete nucleotide sequencing [10]. The sequences revealed no additional mutations within the FAEES-III cDNA.

Transfection and expression of FAEES-III cDNA

The native or mutant forms of FAEES-III cDNA were isolated as a 0.7 kb fragment by digestion with EcoR1. The cDNA fragment containing the entire coding region was then subcloned into a similarly digested SFFV-neo plasmid for propagation. Recombinant plasmid containing the FAEES-III cDNA was identified by sequencing and the orientation of the cDNA in the plasmid was verified by restriction mapping analysis. The plasmids containing the FAEES-III cDNA in proper orientation were used to transfect COS-7 cells by the DEAE-Dextran method [11]. The transfected cells were then lysed and the supernatant was analyzed for FAEES-III activity [2] and GST activity [1].

Gel electrophoresis and immunoblotting procedures

The cell supernatant containing 50 ug of protein was precipitated by addition of 1% sodium deoxycholate (30 ul) and 100% trichloracetic acid (80 ul). The samples were centrifuged at 5000 × 9 for 30 min at 4°C, resuspended in sodium hydroxide and separated by SDS-PAGE [12]. After transferring the proteins to nitrocellulose paper, the paper was washed 4 times with 300 ml of phosphate-buffered saline containing 0.3% (v/v) Tween 20 and then incubated for 3 h with rabbit anti-FAEES-III antibody and 1% ovalbumin. The paper was washed 3 times with phosphate-buffered saline and incubated with [^{125}I]-protein A (150 ml, 105 cpm/pmol) containing 1% ovalbumin. The nitrocellulose paper was then washed again with phosphate-saline, dried and exposed to x-ray film for 8–12 h.

Results and discussion

Although there have been numerous reports on the specificity of the various fatty acid ethyl ester synthase isoenzymes towards substrates (ethanol and fatty acids), only recently has there been progress in elucidating the catalytic mechanism and involvement of specific amino acids in substrate recognition and catalysis [13]. The ability of the enzyme to lower the pKa of the thiol may be one aspect of the synthase catalytic mechanism. This process could be achieved by positioning the thiol in the active site of the enzyme near a positively charged electrostatic field, such as that provided by histidine, lysine or arginine. (The differences between FAEES-III and GSTπ-1 cDNAs are shown in Tables 1 and 2.)

Table 1. FAEES-III and GSTπ-1. The star on the top of differences in nucleotide sequences of FAEES nucleotides shows the difference in the nucleotides

	91	* *		
FAES-III	GAG	GGA	GTG	GTG
GSTπ-1	GAG	GAG	GTG	GTG
	112	*		
FAEES-III	ACG	TGC	CAG	GAG
GSTπ-1	ACG	TGG	CAG	GAG
	151	*		
FAEES-III	GGG	GAG	CTC	CCC
GSTπ-1	GGG	CAG	CTC	CCC
	574		*	*
FAEES-III	GCC	TTC	GTG	GCG
GSTπ-1	GCC	TTC	CTG	GCC

Table 2. Comparison of amino acid sequences between FAEES-III and GSTπ-1. The star on top of the amino acid marks the difference in amino acids

	30		*		34
FAEES-III	Lys	Glu	Gly	Val	Val
GSTπ-1	Lys	Glu	Glu	Val	Val
	36			*	40
FAEES-III	Val	Gb	Thr	Cys	Gln
GSTπ-1	Val	Glu	Thr	Trp	Gln
	50		*		54
FAEES-III	Tyr	Gly	Glu	Leu	Pro
GSTπ-1	Tyr	Gly	Gln	Leu	Pro
	191			*	195
FAEES-III	Lys	Ala	Phe	Val	Ala
GSTπ-1	Lys	Ala	Phe	Leu	Ala

Table 3. Synthase activity and GST activity of native FAEES-III and its mutants

Samples	FAEES-III activity* (nmol/mg/h)	GST activity* (nmol/mg/h)
Native FAEES-III	35.0	43.0
Mutant FAEES-III		
Gly 32 to Gln 32	35.5	44.0
Cys 39 to Trp 39	36.1	43.2
His 72 to Ser 72	34.0	4.0
Control SFFV-neo plasmid vector	3.0	4.05

*Ref 2

In the present study, we have shown that Gly to Gln 32, Cys to Trp 39 mutations have no effect on synthase and transferase activities (Table 3) by mutating Gly 32, and Cys 39 residues of FAEES-III cDNA. These data indicate that these residues may not be important for catalysis of the synthase reaction. A mutation at the histidine 72 codon was also performed and all these mutations were verified by sequencing (Table 4). The mutagenized cDNAs transfected into COS-7 cells yielded a single immunoreactive protein, (M_r of 26,000), while control plasmid (SFFV-neo without cDNA) did not yield this protein (Fig. 1).

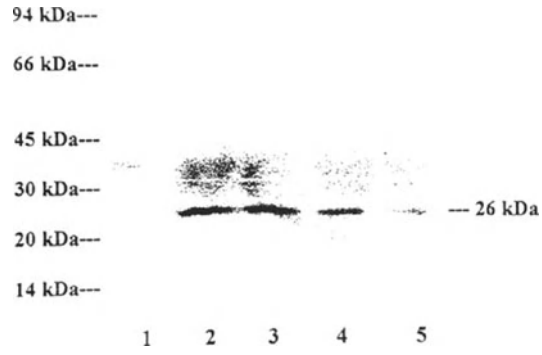

Fig. 1. Immunoblot analysis of transfected COS-7 cells: Lane 1. Protein, transfected with the vector SFFV-neo without cDNA insert; Lane 2. Protein, transfected with FAEES-III mutant cDNA with codon for Gln at residue 32; Lane 3. with mutant cDNA with codon for Trp at residue 39; Lane 4. with mutant cDNA with codon for Ser at residue 72 and Lane 5. Protein, transfected with native FAEES-]II cDNA.

The supernatant from these transfected COS-7 cells was assayed for GST and synthase activities. Gly to Gln 32, Cys to Trp 39, and His to Ser 72 demonstrated normal synthase activity. However, when these same supernatants were assayed for GST activity, His 72 mutant retained little activity when measured under standard GST assay conditions (1 mM glutathione; 1 mM1-chloro-2, 4-dinitro benzene) (Table 3). This apparent loss of activity of His 72 mutation was investigated further by assaying the mutant supernatant in the presence of increasing concentrations of glutathione (2–20 mM). As the concentration of glutathione was increased in the assay mixture, activity became readily measurable. Since synthase/GST catalysis has been described as an ordered bi-reactant reaction, the data were plotted as a Hanes-Woolf plot to determine the new binding constant for glutathione (Fig. 2). For comparison, a similar plot is also shown for the native, transfected enzyme (Fig. 2, inset). From the x intercept, the glutathione binding constant for the mutated enzyme is 1.4 mM while that for the native enzyme is .05 mM. Thus, the apparent loss of activity of the mutated enzyme is due to a 28 fold decrease in the binding constant for glutathione.

For FAEES-III, the present results suggest that Glycine, Cysteine, and Histidine are not essential components for the actual catalytic step of promoting the attack of a nucleophile, such as glutathione or ethanol, or an electrophilic acceptor. The hypothesis that histidine enhances the nucleophilicity of ethanol/glutathione cannot be supported. Rather, it appears that His 72 interacts with an anionic substrate to facilitate its binding to the enzyme. This is supported further by the observation that ethanol, a simple molecule with no charge, is largely unaffected by this amino acid replacement. This differential effect on synthase and transferase activity emphasizes that while Histidine may play a role in substrate

Table 4.

Residues 25–37		Gln	Ser	Trp	Lys	Glu	Gly	Val	Val	Thr	Val	Glu	
Coding strand	5'	CAG	AGC	TGG	AAG	GAG	GGA	GTG	GTG	ACC	GTG	GAG	3'
Complement	3'	GTC	TCG	ACC	TTC	CTC	CCT	CAC	CAC	TGG	CAC	CTC	5'
Oligoprimers 32	3'	TC	TCG	ACC	TTC	CTC	GTC	CAC	CAC	TGG	CAC	CTC	5'
Coding strand 1	5'	AG	AGC	TGG	AAG	GAG	CAA	GTG	GTG	ACC	GTG	GAG	3'
Amino acid		Gln	Ser	Trp	Lys	Glu	Gln	Val	Val	Thr	Val	Glu	
Residues 35–43		Thr	Val	Glu	Thr	Cys	Gln	Glu	Gly	Ser			
Coding strand	5'	ACC	GTG	GAG	ACG	TGC	CAG	GAG	GGC	TCA	3'		
Complement	3'	TGG	CAC	CTC	TGC	ACG	GTC	CTC	CCG	AGT	5'		
Oligoprimers 39	3'	GG	CAC	CTC	TGC	ACC	GTC	CTC	CCG	AGT	5'		
Coding strand 2	5'	CC	GTG	GAG	ACG	TGG	CAG	GAG	GGC	TCA	3'		
Amino acids		Thr	Val	Glu	Thr	Trp	Gln	Glu	Gly	Ser			
Residues 69–76		Ile	Leu	Arg	His	Leu	Gly	Arg	Thr				
Coding strand	5'	ATC	CTG	CGT	CAC	CTG	GGC	CGC	ACC	3'			
Complement	3'	TAG	GAC	GCA	GTG	GAC	CCG	GCG	TGG	5'			
Oligoprimers 72	3'	AG	GAC	GCA	TCG	GAC	CCG	GCG	TGG	5'			
Coding strand 3	5'	TC	CTG	CGT	AGC	CTG	GGC	CGC	ACC	3'			
Amino acids		Ile	Leu	Arg	Ser	Leu	Gly	Arg	Thr				

Total three oligonucleotide primers were designed for these experiments. The first oligo primer 32 was based on the sequences for residues 25 –37 of FAEES-III. Second oligo primer 39 was based on the sequences for residues 35–43 of FAEES-III and third oligo primer 72 was based on the sequences for residues 69 –76 of FAEES-III [7]. The mutated amino acids and their corresponding nucleotide sequences are underlined.

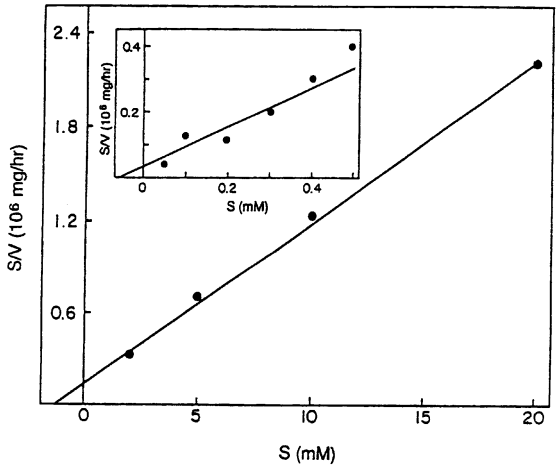

Fig. 2. Hanes-Woolf plot for mutated enzyme (Ser 72). The binding constant for glutathione (1.4 mM) was determined from the x intercept. Insert. Hanes-Woolf plot for native, transfected FAEES-III cDNA. The binding constant for glutathione was .05 mM.

binding, this residue is not an essential component of the catalytic mechanism and since Gly and Cys mutations did not change synthase or GST activities, we also think these residues may not play any role in the catalytic mechanism of the enzyme. Since FAEE were shown to bind to mitochondria *in vivo* and to cause a concentration-dependent reduction in the respiratory control ratio index of oxidative phosphorylation [14, 15], and that these FAEE could be the metabolic link between ethanol abuse and changes in mitochondrial function, the generality of this conclusion for the fatty acid ethyl ester synthase can be known only by direct determination. Nonetheless, a recent report by Ballester *et al.* [16] may support this conclusion that the FAEEs are deemed to be the mechanisms of tissue damage in those organs that lack oxidative metabolism of alcohol.

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Palmitate oxidation by the mitochondria from volume-overloaded rat hearts

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Abstract

In this work, an attempt was made to identify the reasons of impaired long-chain fatty acid utilization that was previously described in volume-overloaded rat hearts. The most significant data are the following: (1) The slowing down of long-chain fatty acid oxidation in severely hypertrophied hearts cannot be related to a feedback inhibition of carnitine palmitoyltransferase I from an excessive stimulation of glucose oxidation since, because of decreased tissue levels of L-carnitine, glucose oxidation also declines in volume-overloaded hearts. (2) While, in control hearts, the estimated intracellular concentrations of free carnitine are in the range of the respective K_m of mitochondrial CPT I, a kinetic limitation of this enzyme could occur in hypertrophied hearts due to a 40% decrease in free carnitine. (3) The impaired palmitate oxidation persists upon the isolation of the mitochondria from these hearts even in presence of saturating concentrations of L-carnitine. In contrast, the rates of the conversion of both palmitoyl-CoA and palmitoylcarnitine into acetyl-CoA are unchanged. (4) The kinetic analyses of palmitoyl-CoA synthase and carnitine palmitoyltransferase I reactions do not reveal any differences between the two mitochondrial populations studied. On the other hand, the conversion of palmitate into palmitoylcarnitine proves to be substrate inhibited already at physiological concentrations of exogenous palmitate. The data presented in this work demonstrate that, during the development of severe cardiac hypertrophy, a fragilization of the mitochondrial outer membrane may occur. The functional integrity of this membrane seems to be further deteriorated by increasing concentrations of free fatty acids which gives rise to an impaired cooperation between palmitoyl-CoA synthase and carnitine palmitoyltransferase I. In intact myocardium, the utilization of the *in situ* generated palmitoyl-CoA can be further slowed down by decreased intracellular concentrations of free carnitine. (Mol Cell Biochem **180**: 117–128, 1998)

Key words: cardiac hypertrophy, isolated working heart, isolated mitochondria, fatty acid oxidation, palmitoyl-CoA synthase, carnitine palmitoyltransferase I

Introduction

Recent studies on mechanically overloaded hearts revealed that the development of cardiac hypertrophy is frequently associated with an impaired fatty acid oxidation [1, 2, 3], the mechanism of which remains still to be elucidated. Parallel to impaired fatty acid utilization, hypertrophied hearts present also elevated glycolytic enzyme activities and high rates of glycolysis [2, 4]. Therefore, some authors suggested that during the development of cardiac hypertrophy a shift from fatty acid to glucose utilization may occur [5]. This possibility was recently analysed by Allard *et al.* [2] who made simultaneous measurements of rates of glycolysis and those of glucose oxidation in hearts of rats with a moderate cardiac hypertrophy. According to these authors, the acceleration of glycolysis that occurs in pressure overloaded hearts is not followed by a stimulation of glucose oxidation. In contrast, because of decreased intracellular concentrations of L-carnitine [3, 6] the imbalance between glycolysis and glucose oxidation [7] may be amplified, thus leading to a further

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dissociation between these two metabolic pathways [2]. As a result, the contribution of carbohydrates to mitochondrial acetyl-CoA production decreases and, therefore, cannot account for the prospective feed-back inhibition of fatty acid oxidation [8]. One of the objectives of the present work was to verify this point in severely hypertrophied hearts from rats with chronic aorto-caval fistula [1]

A more conventional hypothesis consists in the assumption that the aforementioned decrease in tissue carnitine [1, 6, 9] may directly affect the activities of the enzymes involved in the control of long-chain fatty acid oxidation. It has been suggested that, in healthy tissues, carnitine concentrations are not found in excess, but rather at set levels appropriate for optimal substrate metabolism [10]. Therefore, in mechanically overloaded hearts, the estimated intracellular concentrations of L-carnitine may be lower than its respective saturating concentrations for mitochondrial carnitine palmitoyl transferase I [11]. This *per se* could result in impaired kinetics of long-chain acyl transfer from CoA to carnitine and, thus, in decreased oxidative utilization of long-chain fatty acids. In addition, low tissue carnitine concentrations may also compromise the activities of enzymes of β-oxidation, i.e. 3-ketoacyl-CoA thiolases [12], since L-carnitine proved to have stimulatory effects on palmitoylcarnitine oxidation by isolated rat heart mitochondria [13]. Recently, it has been suggested that L-carnitine-induced stimulation of palmitate oxidation by isolated cardiac myocytes [14] may be related to the same mechanism. In fact, it has been reported that L-carnitine decreases mitochondrial levels of acetyl-CoA generated by oxidative metabolism by promoting its conversion to acetylcarnitine [8, 14]. Since acetyl-CoA has been shown to be a potent inhibitor of 3-ketoacyl-CoA thiolase [12, 13], the possibility that L-carnitine restores its activity is not excluded. Finally, it has been suggested that modifications of membrane phospholipides may occur in mechanically overloaded hearts [15]. Such changes may affect the kinetic properties of different membrane-associated enzymes [16] and, particularly, the functional cooperation between palmitoyl-CoA synthase and carnitine palmitoyltransferase I which reside on the opposite sides of mitochondrial outer membrane [17, 18]. Intrinsic alterations of one of these enzymes, although less plausible [19], are also not excluded. Such a change was previously suggested to occur in the mitochondria from ischemic hearts. In this particular preparation, the impaired oxidation of long-chain fatty acids persisted despite the fact that all necessary cofactors were kept at saturating concentrations [20].

In this work, we tried to verify whether the amplitude of carnitine depletion occurring in volume-overloaded hearts from rats with chronical aorto-caval fistula [1, 9] can be compatible with a kinetic limitation of carnitine palmitoyltransferase I *in situ* [10, 11]. The respective kinetic constants of palmitoyl-CoA synthase were also compared with the intracellular concentrations of free CoA and ATP. The ability of mitochondria isolated from volume-overloaded hearts to oxidize different lipid substrates was then tested in presence of saturating concentrations of all cofactors necessary. The saturation kinetics of palmitoyl-CoA synthase and carnitine palmitoyltransferase I were also studied in order to verify whether intrinsic properties of these enzymes have not been modified during the development of cardiac hypertrophy. Finally, we measured the rate of palmitoylcarnitine formation from exogenous palmitate to assess the structural integrity of mitochondrial membranes [20] as well as the state of the prospective functional cooperation between palmitoyl-CoA synthase and overt carnitine palmitoyltransferase [17].

Materials and methods

Animals

A chronical volume-overload was induced in 2 month old rats of Wistar strain (IFFA-CREDO) by a surgical opening of the aorto-caval fistula. Sham-operated animals from the same litters were used as controls. Our technique of the induction of the aorto-caval shunt has been described in detail in our preceding paper [1]. Three months after the surgery, the surviving animals (about 90%) were sacrificed using a light ether anaesthesia and the hearts of rats presenting a significant increase in the heart weight were used for the *in vitro* experiments.

Heart perfusions

The hearts utilized for studies of glucose and palmitate oxidation were perfused at a moderate workload as defined in our earlier papers [1, 21]. After excision, the hearts were rapidly fastened to the aortic cannula and perfused for 10 min with a bicarbonate buffer containing 11 mM glucose and 2.5 mM free calcium. They were then recirculated via left atrium for 20 min at 10 Torr preload and 70 Torr afterload with the same buffer containing 11 mM glucose, insulin and 1.2 mM palmitate bound to 3% BSA (Sigma, fraction V). The rates of palmitate oxidation [22] were estimated from rates of ^3H_2O production from [9, 10-^{3}H] palmitate added into the perfusion system (spec. activity 160 000 dpm/ml). Samples of the recirculated perfusion media were collected in 5 min intervals and treated with a mixture of chloroform and methanol (1/2, vol:vol). The aqueous phase was then re-extracted in a mixture of chloroform, methanol and 2 M KCl. The samples of the resulting aqueous phase were taken for counting. The rates of glycolysis and glucose oxidation were determined in a separate group of hearts recirculated under the same loading

conditions in a system closed to ambient air [2]. The perfusion medium contained [5-^{3}H] glucose (spec. activity 400 000 dpm/ml) and [U-^{14}C]glucose (spec. activity 400 000 dpm/ml). The rates of glycolysis were measured by quantitative determination of the amount of ^3H_2O liberated at the enolase level from [5-^{3}H] glucose [2, 3]. The rates of oxidative utilization of glucose were estimated from the rates of $^{14}CO_2$ production from [U-^{14}C] glucose [2, 3]. Both gaseous $^{14}CO_2$ and $^{14}CO_2$ present as bicarbonate were determined according to the technique described in our earlier paper [1].

Biochemical assays

At the end of the perfusion, the hearts were frozen and lyophilized in order to determine the ventricular dry weight. The samples of ventricular myocardium were deproteinized in 0.6 N perchloric acid (4°C) and the supernatant brought to pH 5.8. Tissue levels of ATP were determined by standard enzymatic procedure [21]. Tissue levels of CoA and acetyl-CoA were determined fluorimetrically using the α-keto-glutarate oxidase and phosphotransacetylase reactions [23]. Total tissue CoA and long-chain acyl-CoA were determined as CoA released from dry tissue samples or perchloric acid precipitates by alkaline hydrolysis [23]. Free L-carnitine was assayed in neutralized perchloric acid extracts by the radio-enzymztic method of McGarry and Foster [24]. Total and long-chain acylcarnitine was determined as free carnitine after alkaline hydrolysis of tissue samples and perchloric acid precipitates respectively [23]. Tissue concentrations of free carnitine were calculated with the assumption that the intracellular water space equals 2.25 ml/g dry weight [21].

Preparation of isolated mitochondria

Crude cardiac mitochondria were isolated according to the technique derived from that of Lindenmayer *et al.* [25]. The hearts were chilled in 10 ml of cold isolation medium (250 mM sucrose, 10 mM Tris HCl, 1.0 mM EDTA, pH 7.4 containing 0.5% BSA). The left and right ventricles were homogenized for 20 sec using a Polytron adjusted to position 3 and the homogenate was spun at 600 g for 10 min. The supernatant was collected and centrifuged at 8000 g for 15 min.

The preparation of purified mitochondria consisted in a resuspension of the pellet resulting from the previous spin in 0.25 M sucrose and 35% Percoll, pH 7.2 [26] followed by a centrifugation at 40000 g for 45 min. (Kontron A8-24 rotor). At the end of this step, two distinct bands could be observed in Percoll containing tubes: a thin upper band enriched in catalase and NADPH-cytochrome *c* reductase activities, which was discarded, and a lower band enriched in mono-amine oxidase and cytochrome *c* oxidase activities. This fraction was collected and washed in order to eliminate the Percoll and used for the assessment of enzyme kinetics. Fresh mitochondria prepared according to this technique presented a RCI close to 10 when they were supplied with 10 μM palmitoylcarnitine or with 10 mM glutamate-malate. Mitochondria stored at –20°C in a medium without albumin were used for the measurements of marker enzyme activities. The protein was determined according to Gornall *et al.* [27].

Marker enzyme activities

The activity of the monoamine oxidase (a marker of the external mitochondrial membrane) was determined spectrophotometrically at 360 nm using 300 μM 3-(2 amino-phenyl)-3-oxopropanamine (Kynuramine) as substrate [28]. The activity of the cytochrome *c* oxidase (marker enzyme of inner mitochondrial membrane) was determined at 550 nm in presence of 25 μM ferrocytochrome *c* [29]. The reduced cytochrome *c* was prepared by an exposure of ferricyto-chrome *c* to sodium ascorbate. The two compounds were separated by a Sephadex G-25 desalting chromatography. The quantification of NADPH-cytochrome *c* reductase (marker enzyme of the microsomal fraction) was done in presence of 1 mM KCN according to the method of Sottocasa using 100 μM NADPH as substrate [29]. The activity of the catalase (a microperoxisomal marker) was evaluated from H_2O_2 disappearance measured spectrophotometrically at 405 nm in presence of titanylsulfate. The units, as defined by Leighton [30], were employed to quantify the enzyme activity.

Conversion of [1-^{14}C]palmitate and [1-^{14}C]palmitoyl esters of CoA and carnitine into acid soluble products

For these experiments we used 0.5 ml of a buffer of the following composition (in mM): 5 potassium phosphate, 120 KCl and 25 Hepes, pH 7.4. The oxidation of [1-^{14}C]palmitate (200 HM) was studied in presence of 1.5 mM ATP, 1.5 mM $MgCl_2$, 50 μM CoA and 1.24 mM L-carnitine [31]. The same incubation medium kept at 30°C was used to study [1-^{14}C]-palmitoyl-CoA (50 μM) oxidation, while L-carnitine was omitted when [1-^{14}C]palmitoyl carnitine (12.5 μM) was used as substrate. The reaction was started by addition of 200 μg of mitochondrial protein and, at the end of 10 min period, the reaction was stopped by 10 μl of 70% perchloric acid. The acidified samples were maintained in ice for 1 h in order to ensure the complete precipitation of remaining long-chain acyls. They were then spun for 5 min at 5000 g and 200 μl aliquots of the clear supernatant were used for counting. In some experiments, [U-^{14}C]palmitate was used to enhance ^{14}C-

short-chain acyl production and, for the same reason, 200 µM malate was added into the incubation medium.

Palmitoyl-CoA synthase activity

The activity of the palmitoyl- CoA synthase was determined in 250 µl of a standard medium containing (in mM): 2.5 ATP, 5 $MgCl_2$, 0.8 CoA, 5 dithiothreitol, 0.3 [1-^{14}C]palmitate (s.a. 0.45 nCi/nmol) bound to albumin (molar ratio 3/1), 150 Tris-HCl; final pH 7.4. The reaction was initiated by the addition of 100–150 µg of mitochondrial fraction and it was stopped 120 sec later by 1 ml of Dole's reagent [18] The remaining palmitate was then separated from the palmitoyl-CoA by use of 4 times repeated extraction in 600 µl of hexane. An aliquot of the resulting water phase was then added into 10 ml of scintillation liquid.

Carnitine palmitoyltransferase activity

The incubation medium used for the measurements of carnitine palmitoyltransferase was composed of (in mM): 75 KCl, 80 mannitol, 1 EGTA, 1 dithiothreitol and 0.1 [1-^{14}C]palmitoyl-CoA bound to albumin (molar ratio 3/1). 200 µg of mitochondrial protein were preincubated for 2 min at 37°C in 0.5 ml of the reaction mixture and the reaction was started by addition of 1.24 mM L-carnitine (s.a. 0.62 nCi/nmol). 4 min later, the reaction was stopped by 1 ml of 1.2 N HCl. The resulting palmitoylcarnitine was extracted in 3 ml of water-saturated butanol. 2 ml of the organic phase were rinsed twice in 3 volumes of butanol-saturated water and, finally, 1 ml aliquot of the organic phase was used for counting [32].

Carnitine palmitoyltransferase activity in presence of the in situ *generated palmitoyl-CoA*

For this work, we used essentially the same technique as the above described method applied for measurements of carnitine palmitoyltransferase activity. The only difference consisted in use of palmitate (10–200 µM) bound to albumin (molar ratio 3/1) instead of palmitoyl-CoA. This was generated by palmitoyl-CoA synthase that resides on the overt side of the outer mitochondrial membrane. It has been suggested that kinetic studies of coupled reaction between palmitoyl-CoA synthase and carnitine palmitoyltransferase allow to assess the functional cooperation between these two enzymes [17]. The concentrations of different cofactors necessary for palmitate activation were as follows (in mM): 1.5 ATP, 1.5 $MgCl_2$; 0.05 CoA. After a brief preincubation of mitochondria (200 µg of mitochondrial protein), the coupled reaction was started by addition of [3H]-L-carnitine (1.24 mM, s.a. of 0.62 nCi/nmol). The reaction was arrested by the extraction of palmitoylcarnitine as described above [32].

Analysis of saturation kinetics

The analysis of saturation kinetics of the palmitoyl-CoA synthase and carnitine palmitoyltransferase were done by fitting of the experimental data to the Michaelis-Menten model by a method of non-linear regression (SIGMA PLOT for Apple-Macintosh) which provides the values of V_{max} and K_m. The kinetics of coupled reaction catalyzed by the palmitoyl-CoA synthase and carnitine palmitoyltransferase were analyzed by use of a substrate inhibition model [33]. This allows to determine the inhibition constant K_{ss} corresponding to the intersections of regression lines obtained from the respective Dixon's plots [$1/v = f([S])$] with the x axis. The equations of these latter were calculated from the experimental data by a linear regression method (SIGMA PLOT for Apple-Macintosh).

Statistical analysis

The experimental data of each experiment were expressed as means ± S.E.M. for $n > 6$. Statistical analysis was done by use of Student's *t*-test for unpaired comparisons and difference were considered significant when $p < 0.05$.

Results

Ponderal data

The heart weights of rats exposed for 3 months to aorto-caval fistula were increased by about 75% when compared with sham-operated controls (1.34 ± 0.14 instead of 0.76 ± 0.09 g wet weight). The cardiac hypertrophy was bilateral and both ventricles were only slightly dilated. The hearts of the animals presenting a macroscopic evidence of congestive heart failure (less than 10% of surviving rats) were discarded from further study.

Glucose and exogenous palmitate utilization by volume-overloaded hearts

As in our previous work [1], the rates of exogenous palmitate oxidation were significantly decreased (from 1287 ± 41 to 709 ± 28 nmol/min g dry wt) in volume overloaded hearts exposed to moderate workload. This inhibition of palmitate

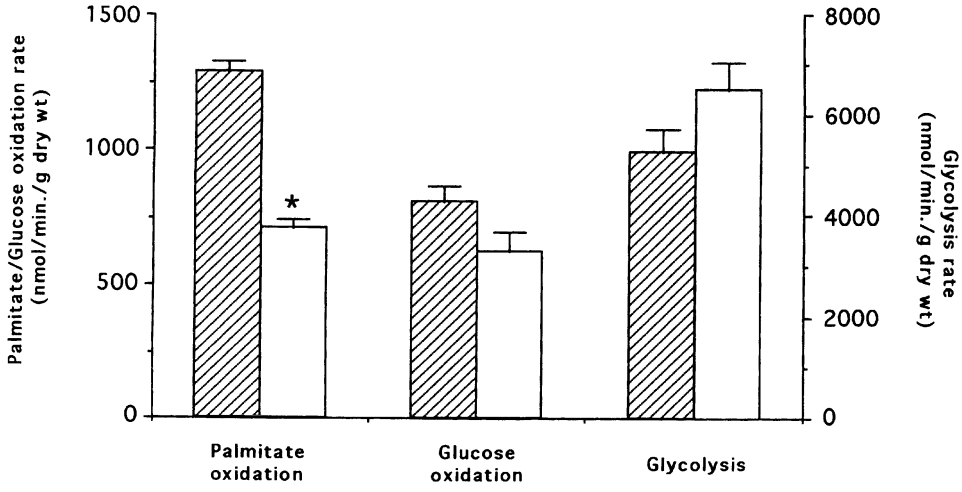

Fig. 1. Steady state palmitate (glucose) oxidation and rates of glycolysis in control (▨) and volume-overloaded (□) hearts determined during the perfusions at moderate work load (20 min lasting perfusion at 10 Torr preload and 70 Torr afterload). The values are means ± S.E.M. for n = 6; *p < 0.05.

oxidation could not be related to a prospective shift of myocardial metabolism from fatty acid to glucose utilization [5] since glucose oxidation also decreased in volume-overloaded hearts (from 802 ± 60 to 621 ± 76 nmol/min g dry wt). This slowing down of oxidative pathways involved in ATP production was not entirely compensated by the concomitant acceleration of glycolysis (Fig. 1). As a result, the overall energy turnover, as suggested by oxygen consumption data [21] and calculated ATP production rates [34], was decreasing. This was accompanied by a depression of left ventricular mechanical performance during the perfusions at both moderate (this work) and high work loads [1, 21].

Tissue contents of ATP, CoA and carnitine that we obtained at the end of the perfusion period are given in Table 1. We could confirm the presence of a significant depletion of total tissue carnitine and a decrease in free carnitine content in volume-overloaded hearts. Tissue contents of both total and free CoA were also slightly depressed while tissue ATP levels were decreased but not significantly different from control values. When intracellular concentrations of CoA and carnitine were compared with kinetic constants of palmitoyl-CoA synthase and carnitine palmitoyltransferase I as determined in this work (Table 3), it occurred that only the changes in intracellular carnitine concentrations (0.63 instead of 1.07 mM) might be of functional significance [11, 35]. Table 1 also shows that tissue contents of different intermediates of lipid metabolism were significantly decreased in volume-overloaded hearts. This finding suggested that, during the perfusions used in the present study, a slowing down of long-chain fatty acid utilization did occur. A 30% depletion of acetyl-CoA was of particular interest since, previously, it had been suggested [8] that intracellular concentrations of acetyl-CoA are proportional to cytosolic concentrations of malonyl-CoA. Therefore, the decrease of fatty acid oxidation in volume-overloaded hearts did not appear to be related to malonyl-CoA induced inhibition of carnitine palmitoyltransferase I [36].

Characterization of isolated mitochondria

To avoid the interference from the prospective extramitochondrial carnitine palmitoyltransferase and long-chain acyl-CoA synthase, we preferred to work with a purified mitochondrial fraction obtained by differential centrifugation of crude

Table 1. Tissue contents of ATP, CoA and carnitine in control and hypertrophied rat hearts perfused at moderate workloads with 11 mM glucose and 1.2 mM palmitate

	ATP µmol/g.dry wt	CoA	Acetyl-CoA nmol/g.dry wt	Acyl-CoA nmol/g.dry wt	Total CoA	Free carnitine	Acylcarnitine nmol/g dry wt	Total carnitine
Controls	20.05 ± 0.87	324.78 ± 11.69	73.69 ± 8.09	158.79 ± 3.38	608 ± 50	2404 ± 113	544 ± 27	5802 ± 150
Fistulae	17.84 ± 1.20	293.55 ± 18.89*	51.34 ± 5.02*	110.70 ± 3.65*	550 ± 38	1405 ± 74*	370 ± 25*	3864 ± 174*

Considering the intracellular water space as 2.25 ml/g dry wt [20], tissue contents of selected metabolises can be converted to intracellular concentrations. Values are means ± S.E.M. for n = 10; *p < 0.05.

Table 2. Activities of mitochondrial and microsomal enzymes in the mitochondria from control and hypertrophied hearts

	Controls	Fistulae
Monoamine oxidase	5.88 ± 0.19	5.62 ± 0.21
Cytochrome *c* oxidase	1.23 ± 0.06	1.16 ± 0.04
Catalase	7.40 ± 0.50	6.98 ± 0.14
NADPH-cytochrome *c* reductase	0.75 ± 0.08	0.62 ± 0.07
Microsomal contamination	2.6%	2.1%

Units are mIU/mg protein and values are mean ± S.E.M. for n = 6. Estimated by using NADPH-cytochrome *c* reductase as microsomal marker.

mitochondria on Percoll gradient [26]. This allowed to eliminate about 75% of microperoxisomes (catalase activity) and 83% of microsomes (NADPH-cytochrome *c* reductase activity) present in crude mitochondria (Table 2). At the same time, the activities of mitochondrial marker enzymes, i.e. the monoamine oxidase and cytochrome *c* oxidase increased by 50 and 26% respectively. Comparing the two purified populations, we did not note any significant difference in the above enzyme activities between the mitochondria from control and volume-overloaded hearts (Table 2). In addition, both mitochondrial populations behaved similarly on Percoll gradient so that we assume to have worked on comparable and homogenous mitochondrial fractions.

Oxidation of long-chain acyl moieties

The ability of isolated mitochondria from control and volume-overloaded hearts to oxidize palmitate is illustrated in Figs 2 and 3. It can be seen that, in L-carnitine supplemented mitochondria, the rate of palmitate conversion to short-chain acyls was about 5 times higher when 0.2 mM malate and [U-^{14}C]palmitate (instead of [1-^{14}C]palmitate) were used. However, even under these favourable conditions, the stimulatory effect of L-carnitine on the rate of formation of acid soluble

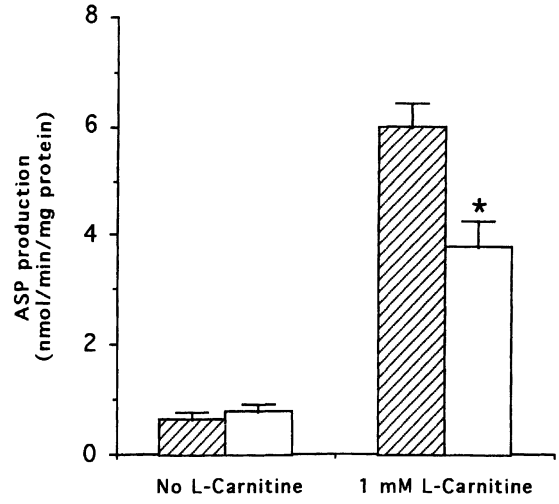

Fig. 2. Production rates of acid soluble products (ASP) from [U-^{14}C]-palmitate by isolated mitochondria from control (▨) and volume-overloaded (□) hearts. The mitochondria were incubated for 20 min in state 4 conditions in presence of 200 μM palmitate bound to albumin and 200 μM malate. The values are means ± S.E.M. for n = 10; *p < 0.05.

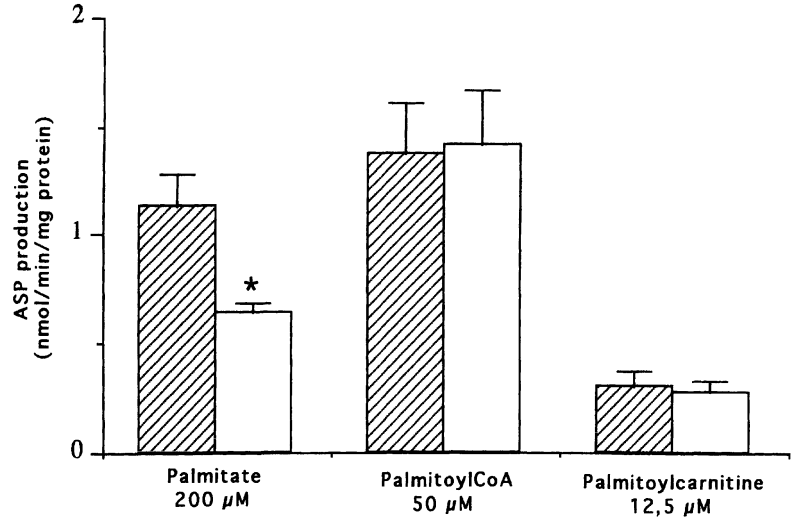

Fig. 3. Production rates of acid soluble products (ASP) from [1-^{14}C]palmitate, [1-^{14}C]palmitoyl-CoA and [1-^{14}C]palmitoylcarnitine respectively. The mitochondria were incubated in absence of both ADP and exogenous malate. Substrate concentrations were adjusted to obtain state 3 respiratory rates comparable to those recorded in presence of 10 mM glutamate/malate [1] ; (▨) control mitochondria, (□) mitochondria from volume-overloaded hearts. The values are means ± S.E.M. for n = 6; *p < 0.05.

products was significantly attenuated in the mitochondria from volume-overloaded hearts (Fig. 2). Figure 3 also shows the effects of chronic volume-overloading on the rates of the formation of acid soluble products from CoA and carnitine esters of [1-^{14}C]palmitate. It can be seen that, while the rate of palmitate oxidation by the mitochondria of hypertrophied hearts was reduced by about 40%, the relative rates of palmitoyl-CoA and palmitoylcarnitine oxidation remained comparable in both groups of mitochondria studied. The data presented in Fig. 3 strongly resemble the results of our polarographic studies [1] which indicated that, under state 3 conditions, the respiratory rates of palmitate supplied mitochondria from volume-overloaded hearts were reduced (from 187.7 ± 16.3 to 117.8 ± 13.4 natom O_2/min/mg). In contrast, the respective rates of state 3 respiration observed in presence of 10 μM palmitoylcarnitine were not significantly different (167.5 ± 12.7 instead of 176.4 ± 15.6 natom O_2/min/mg).

All these data suggested that the impaired palmitate oxidation by the mitochondria from volume-overloaded hearts relied essentially on alterations of enzyme(s) associated with outer mitochondrial membrane and involved respectively in palmitate activation and long-chain acyl transfer from CoA to carnitine [18, 37]. In our further work we tried to assess the saturation kinetics of the principal enzymes controlling these two steps.

Palmitate activation and transfer to carnitine

The kinetics of the palmitoyl-CoA synthase saturation by palmitate are illustrated in the upper panel of Fig. 4. After having been adjusted to Michaelis-Menten model by non-linear regression, the data collected from the mitochondria of control and volume-overloaded hearts gave quite similar V_{max} and K_m (Table 3) so that no difference between the two mitochondrial populations studied could be established.

The kinetics of saturation of carnitine palmitoyltransferase obtained at constant extramitochondrial palmitoyl-CoA/albumine ratio are illustrated in the middle panel of Fig. 4. As in the case of palmitoyl-CoA synthase, we did not see

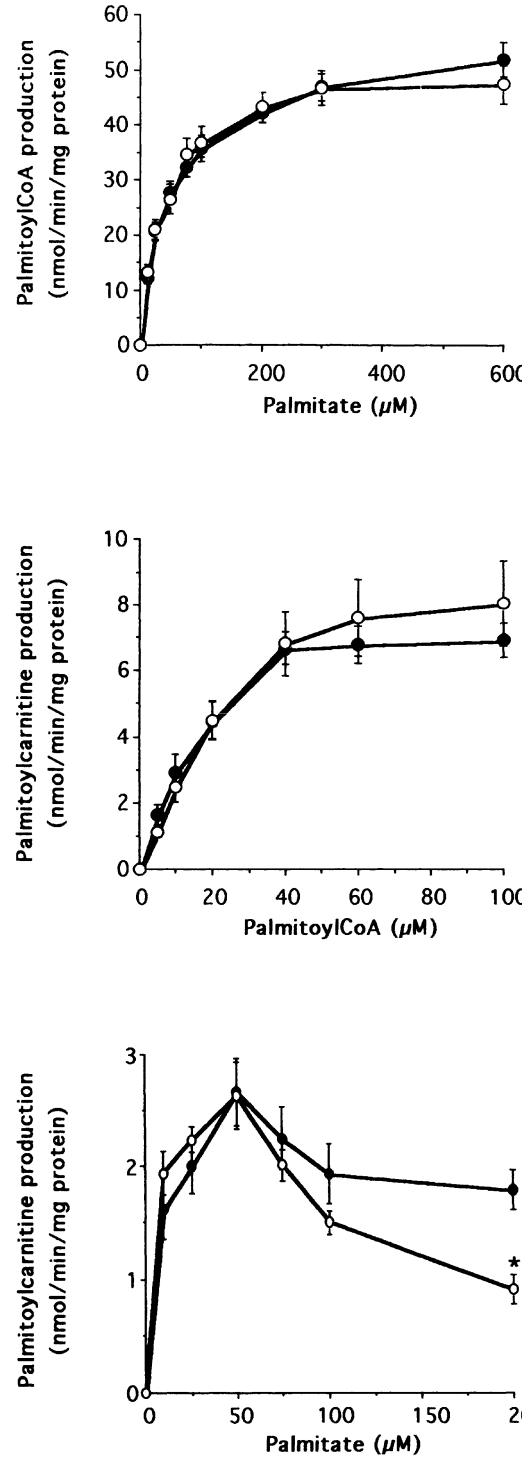

Fig. 4 (adjacent). Palmitate activation and palmitate transfer on L-carnitine in the mitochondria from control (●) and volume-overloaded (○) hearts. Upper panel: palmitoyl-CoA synthase activities expressed as the rates of production of [1-^{14}C]palmitoyl-CoA from [1-^{14}C]palmitate. Middle panel: carnitine palmitoyltransferase I activities expressed as production rates of [1-^{14}C]palmitoylcarnitine from [1-^{14}C]palmitoyl-CoA. Lower panel: coupled activities of palmitoyl-CoA synthase and carnitine palmitoyltransferase I expressed as production rates of [1-^{14}C]palmitoylcarnitine from [1-^{14}C]palmitate in presence of Mg-ATP, CoA and L-carnitine. The values are means ± S.E.M. for n = 6; *$p < 0.05$. The respective kinetic constants of palmitoyl-CoA synthase and carnitine palmitoyltransferase I are indicated in Table 3.

Table 3. Kinetic parameters of palmitoyl-CoA synthase and carnitine palmitoyltransferase I for their respective substrates

	Controls		Fistulae	
	K_m	V_{max}	K_m	V_{max}
Palmitoyl-CoA synthase				
ATP	1.33 ± 0.07	73.36 ± 1.95	1.51 ± 0.14	74.62 ± 3.57
CoA	32.17 ± 4.50	51.11 ± 1.77	29.54 ± 4.10	48.93 ± 1.61
Palmitate	38.54 ± 3.15	51.41 ± 1.12	46.32 ± 3.54	52.93 ± 1.84
Carnitine palmitoyltransferase I				
Carnitine	1.48 ± 0.31	15.67 ± 0.95	2.07 ± 0.57	16.61 ± 1.48
Palmitoyl-CoA	18.62 ± 4.27	8.77 ± 0.66	29.72 ± 6.55	11.02 ± 0.95

Values are means ± S.E.M. for n = 6. V_{max} are expressed in nmol/min/mg protein. K_m are mM for ATP and carnitine and μM for CoA, palmitate and palmitoyl-CoA.

any difference between the mitochondria from control and volume-overloaded hearts. Again, the kinetics obtained fitted well with Michaelis-Menten model and the values of V_{max} and K_m were not significantly different (Table 3). We also tested the sensitivity of carnitine palmitoyltransferase I to malonyl-CoA [11, 36]. In our hands, the amplitude of the inhibition observed in presence of 50 μM malonyl-CoA was not significantly different between mitochondria from control and volume-overloaded hearts (70 and 62% respectively).

The discrepancy between the altered long-chain fatty acid oxidation and the absence of changes in kinetic parameters of palmitoyl-CoA synthase or carnitine palmitoyltransferase prompted the studies of the prospective functional links between these two enzymes [17, 18, 37]. The mitochondria were supplied with palmitate and with all cofactors necessary for palmitate activation (Mg-ATP, CoA) and palmitoyl transfer from CoA to L-carnitine. The results obtained with this technique are presented in the lower panel of Fig. 4. It can be seen that, at palmitate concentrations above 50 μM, a substrate inhibition of coupled reaction took place in the mitochondria from both control and volume-overloaded hearts. However, at palmitate concentrations exceeding 100 μM, this inhibition was significantly stronger in the mitochondria from volume-overloaded hearts. When the respective kinetics were analyzed according to a substrate inhibition model [33], the following inhibition constants (K_{ss}) were obtained in the mitochondria from control and volume-overloaded hearts: 324.5 and 24.7 respectively. This amplification of substrate-induced inhibition of palmitoyl transfer from CoA to carnitine may contribute to impaired long-chain fatty acid oxidation by isolated mitochondria [1, this work]. It could also impair palmitate oxidation by volume-overloaded hearts [1, 21], especially under conditions characterized by high concentrations of fatty acids that we used in the present study.

Discussion

One major metabolic alteration in hypertrophied hearts is a decrease in long-chain fatty acid oxidation as expressed per g of tissue or per unit work [1, 2]. In contrast, the oxidation of short-chain fatty acids is not altered in mechanically overloaded hearts [1, 21]. This indicates that short-chain acyl dehydrogenases as well as the enzymes of TCA cycle and respiratory chain itself conserve their activities. It follows that a dysfunction of long-chain acyl dehydrogenases [13] or a kinetic limitation of the enzymes that control long-chain fatty acid activation [18] and long-chain fatty acid transfer from CoA to carnitine [36] could be considered. In this work, we confirm our earlier demonstration [1] that mitochondrial β-oxidation system is not affected since the rates of [^{14}C]-palmitoylcarnitine conversion into acid soluble products remained comparable between mitochondria from control and volume-overloaded hearts. On the other hand, the oxidation of palmitate was significantly slowed down despite the presence of saturating concentrations of all cofactors necessary. This could incriminate kinetic properties of mitochondrial palmitoyl-CoA synthase and carnitine palmitoyltransferase I. However, saturation kinetics of these two enzymes gave similar kinetic constants in both mitochondrial populations studied. In addition, the sensitivity of mitochondrial carnitine palmitoyltransferase I to malonyl-CoA was unchanged. This latter finding agrees with the predictions of Cook and Lappi [19] who suggested that, in diseased hearts, the modifications of carnitine palmitoyltransferase I sensitivity to malonyl-CoA do not occur. Rather, a change in carnitine palmitoyltransferase I regulation by cytosolic malonyl-CoA concentrations may be expected [38]. In this work, we did not assay malonyl-CoA in volume-overloaded hearts but we found that tissue contents of its precursor, i.e. acetyl-CoA [8], were significantly decreased. This finding argues against an excessive accumulation of malonyl-CoA in the hypertrophied hearts used in this study.

Another compound which can modulate carnitine palmitoyltransferase I activity is L-carnitine [11, 35]. In rat hearts, the K_m for L-carnitine is 5–10 higher than in other tissues reflecting in part higher carnitine concentrations in ventricular myocardium [38]. Therefore, it has been suggested that decreased intracellular concentrations of L-carnitine, as found in mechanically overloaded hearts [6, 9], could be rate limiting in the translocation of activated long-chain fatty acids from cytosol to mitochondria [1, 6]. This would contribute to impaired palmitate oxidation, thus decreasing long-chain fatty acid contribution to ATP production [2, 34]. However, for a limitation of fatty acyl transfer could account for decreased fatty acid oxidation, the intracellular concentrations of free carnitine should be lower than the respective K_m of rat heart carnitine palmitoyltransferase I [11]. The data

presented in this work suggest that this may be the case: while in control hearts the estimated intracellular carnitine concentrations (1.07 mM) were close to the apparent K_m of carnitine palmitoyltransferase I for L-carnitine (1.48 mM), in volume-overloaded hearts the estimated concentrations of free carnitine (0.62 mM) were lower than the respective K_m determined on the mitochondria isolated from these hearts (2.07 mM). However, it should be noted that, in our work, the saturation kinetics of carnitine palmitoyltransferase I were determined in presence of 100 μM palmitoyl-CoA which behaves as a competitive inhibitor to its cosubstrate carnitine [39]. Therefore, the values of the apparent K_m for carnitine presented in this work may be overestimated. After extrapolation to 0 palmitoyl-CoA, the apparent K_m value for carnitine may decrease to 250–450 μM [40] while *in situ*, the saturating concentration of L-carnitine has been estimated as 500 μM [41]. Therefore, the carnitine palmitoyltransferase I of mechanically overloaded hearts can be considered as carnitine limited only when tissue depletion of L-carnitine becomes more severe [41, 42].

In addition, it has been suggested [2, 38] that the inhibition of carnitine palmitoyltransferase I that occurs secondary to decreased tissue carnitine levels results in a depressed rate of fatty acid utilization only at low concentrations (0.4 mM) of exogenous fatty acids. Under these conditions [3], fatty acid oxidation supplied only 55% of total ATP produced in hypertrophied hearts as compared to 70% in control hearts. The ATP production that was lost due to depression of fatty acid oxidation was partially compensated by increased glycolytic rates [2]. When fatty acid concentrations were high (1.2 mM), this depression of fatty acid oxidation did not occur any longer [3]. The authors of these studies concluded that an increased supply of exogenous fatty acids may overcome the decrease in carnitine palmitoyltransferase I activity in hearts with a moderate cardiac hypertrophy. A similar situation was previously described in pressure overloaded hearts perfused with 0.4 mM palmitate but subjected to increasing workloads [2]. Under these conditions, a decrease in mitochondrial acetyl-CoA/ CoA ratio related to the acceleration of TCA cycle [35] resulted in a concomitant stimulation of 3-ketoacyl-CoA thiolase [12, 14] and a disinhibition of carnitine palmitoyltransferase I [43], thus increasing long-chain fatty acid oxidation.

A different situation prevailed in severely hypertrophied hearts used in this study [1]. In this model of cardiac hypertrophy, the oxidation of long-chain fatty acids accounted for 72% of ATP produced but it remained decreased when compared to control hearts (81%) despite the fact that high concentrations (1.2 mM) of exogenous palmitate have been used for the *in vitro* perfusions. In addition, the inhibition of long-chain fatty acid oxidation persisted even at high work loads [34]. This suggested that the increase in acetyl-CoA demand by TCA cycle failed to reactivate fatty acid utilization as well as their contribution to ATP production. Since our hearts were perfused in presence of both 1.2 mM palmitate and 11 mM glucose (+ insulin), the interference of glucose oxidation with fatty acid utilization [7, 41] could be considered. It has been suggested that, in some experimental conditions such as an excessive stimulation of glucose oxidation by dichloroacetate [8, 43], fatty acid oxidation may be inhibited by extra acetyl-CoA produced by pyruvate dehydrogenase complex. The acetyl-CoA produced in excess may inhibit directly 3-ketoacyl-CoA thiolase [12, 14] and, after having been exported out of the mitochondria, it can be converted by cytosolic acetyl-CoA carboxylase to malonyl-CoA, an inhibitor of overt carnitine palmitoyltransferase [11, 36]. In this particular case, the stimulation of glucose oxidation may account for a decrease in fatty acid oxidation [8, 14]. Similar competition between fatty acid and glucose oxidation was also described in severe carnitine deficiencies related to chronic administration of sodium pivalate [41, 42].

However, it should be noted that in mechanically overloaded hearts, the decrease in palmitate oxidation was not accompanied by any acceleration of glucose oxidation [2, 3]. Rather, a moderate carnitine depletion observed in these hearts resulted in a simultaneous inhibition of glucose and fatty acid oxidation [2, 38]. In addition, as described in this work, the impaired palmitate oxidation persisted even after the isolation of the mitochondria from volume-overloaded hearts. In these controlled conditions, we could eliminate any interference from other substrates as well as the lack of principal cofactors of lipid metabolism. We also demonstrated that, in contrast to palmitate, exogenous palmitoyl-CoA and palmitoylcarnitine were oxidized at comparable rates in both mitochondrial populations studied. At the same time, the kinetic properties of palmitoyl-CoA synthase and carnitine palmitoyltransferase I remained unchanged. These data suggested that, although these enzymes are not believed as kinetically coupled, an alteration of their functional cooperation [37] necessary for the oxidative utilization of the *in situ* generated palmitoyl-CoA may be considered (Fig. 5).

The data presented in this work clearly demonstrate that, in the mitochondria from volume-overloaded hearts, the utilization of the *in situ* generated palmitoyl-CoA may be inhibited by exogenous palmitate. However, we were unable to determine whether this substrate inhibition of coupled reaction was related to impaired transmembrane translocation of the *in situ* generated palmitoyl-CoA [18] or to the alterations of its transfer from palmitoyl-CoA synthase to carnitine palmitoyltransferase I [17, 37]. Moreover, this inhibition does not occur at low palmitate concentrations which raises a question of the physiological significance of our data. In this respect, the studies by Van der Vusse [44] and Hunneman [45] are of interest. According to these authors, tissue content of free fatty acids can be estimated as 300 nmol/g dry weight. If we consider a homogenous intracellular distribution and the

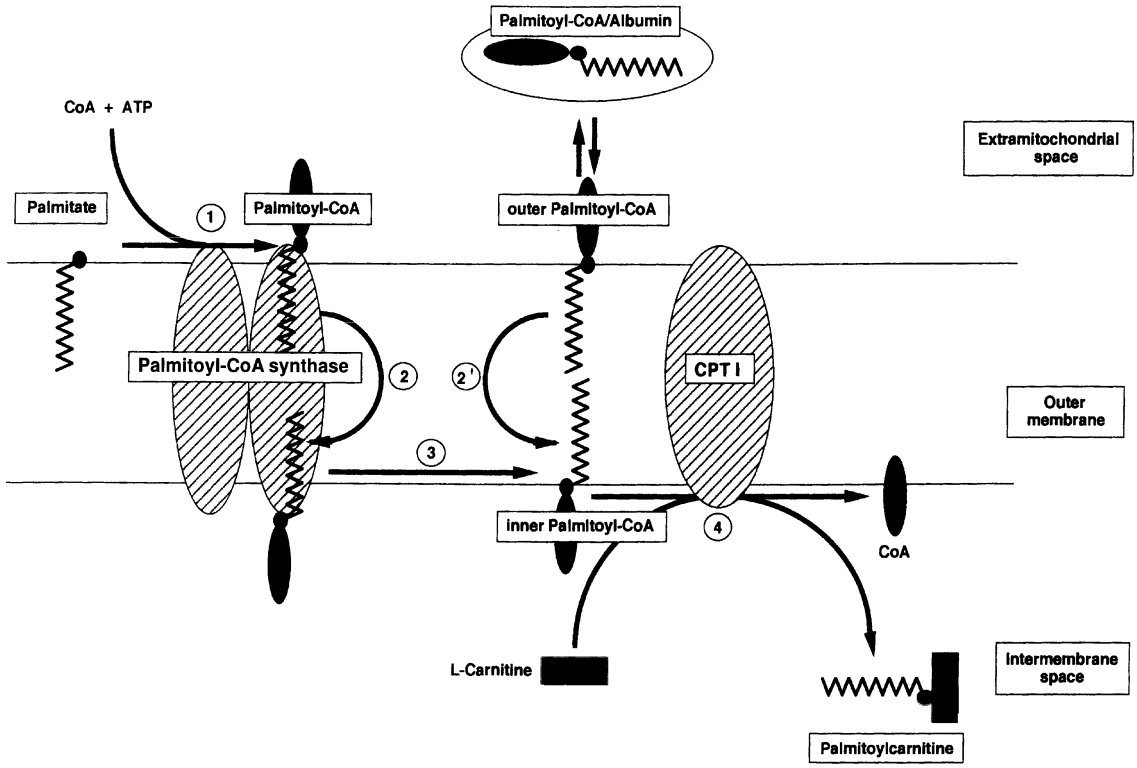

Fig. 5. Schematic representation of the interaction of the exogenous and the *in situ* generated palmitoyl-CoA with overt carnitine palmitoyltransferase (CPT I). The catalytic unit of this enzyme, inserted in the outer mitochondrial membrane, faces the intermembrane space [28]. Its principal function is to promote the transfer of long-chain fatty acyl groups from CoA to L-carnitine (step 4). Its substrate (palmitoyl-CoA) may originate from an extramitochondrial pool, e.g. palmitoyl-CoA/albumin complex [32], or it is generated *in situ* by palmitoyl-CoA synthase (step 1). While the transmembrane transport of the exogenous palmitoyl-CoA relies on a spontaneous flip-flop (step 2'), the *in situ* generated palmitoyl-CoA is believed to be translocated [18] by palmitoyl-CoA synthase (step 2) and then channeled [17, 37] to carnitine palmitoyltransferase I via the outer membrane (step 3). Our data suggest that the kinetics of reactions 1 and 4 as well as the transmembrane transport of exogenous palmitoyl-CoA (step 2') are unchanged in the mitochondria from volume-overloaded hearts. In contrast, the conversion of extramitochondrial palmitate to palmitoylcarnitine is significantly inhibited at palmitate concentrations exceeding 100 μM. These observations suggest that in the mitochondria from volume-overloaded hearts the functional links (steps 2 and 3) between palmitoyl-CoA synthase and carnitine palmitoyltransferase [17, 37] are deteriorated.

intracellular water space as 2.25 ml/g dry weight [21, 35], the intracellular concentrations of long-chain fatty acids (130–150 gmol/L) would be in the inhibitory range already at physiological concentrations of circulating fatty acids (0.5 mM). During the perfusions with high palmitate concentrations (1.2 mM), the prospective substrate inhibition of coupled reaction may be amplified.

The mechanisms of impaired cooperation between palmitoyl-CoA synthase and carnitine palmitoyltransferase I which reside on opposite sides of the outer mitochondrial membrane [28] remain still to be elucidated. It has been suggested that, at high concentrations, long-chain fatty acids and their CoA esters may behave as detergent agents [45]. This effect may be amplified under pathological conditions that have been shown to fragilize mitochondrial membranes [16]. This may be the case of chronic mechanical overload to the heart which was shown to induce qualitative and quantitative alterations in membrane phospholipides [15]. Such changes could modify kinetic properties of different membrane-associated enzymes [37] and, particularly, the functional cooperation between palmitoyl-CoA synthase and carnitine palmitoyltransferase I [17, 37]. Furthermore, in volume-overloaded hearts, the transmembrane channeling of the *in situ* generated palmitoyl-CoA from palmitoyl-CoA synthase to carnitine palmitoyl-transferase I [17, 18, 37] may be further slowed down by decreased tissue levels of L-carnitine [10].

It can be concluded that the impaired long-chain fatty acid utilization occurring in severely hypertrophied hearts may be

related to subtle alterations of mitochondrial membranes resulting in a breakdown of the functional links between palmitoyl-CoA synthase and carnitine palmitoyltransferase I. The cooperation between these two enzymes may be particularly deteriorated by high concentrations of circulating fatty acids and by decreased intracellular concentrations of L-carnitine, conditions promoting long-chain acyl-CoA accumulation in mitochondrial membranes.

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Effects of phosphodiesterase inhibitors on glucose utilization in isolated cardiac myocytes

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Abstract

The phosphodiesterase (PDE) inhibitor, enoximone, enhances the oxidation of fatty acids in cardiac myocytes. Since carbohydrate oxidation is tightly coupled and inversely related in cardiac tissue to fatty acid oxidation, this study was designed to investigate enoximone's effects on glucose metabolism in the heart. To determine if enoximone alters this reciprocal relationship, the effects of enoximone on [U-^{14}C]glucose and [2-^{14}C]pyruvate oxidation were determined in isolated cardiac myocytes. The effect of PDE inhibitors was also examined on pyruvate dehydrogenase complex (PDH) activity, a key component of oxidative glucose metabolism. Two PDE inhibitors, enoximone and milrinone, decreased PDH activity by 69 and 64%, respectively at 0.5 mM. This inhibition of PDH activity by enoximone was completely reversed after removing enoximone from the myocyte medium. PDH activity was unaffected by agents which alter cyclic nucleotide signaling: cGMP, dibutyryl cyclic AMP, and AMP. The effect of enoximone on [2-^{14}C]pyruvate oxidation was similar to that on PDH. Interestingly, the oxidation of glucose was decreased 35% by 0.5 mM enoximone. In isolated rat heart mitochondria (RHM), enoximone decreased PDH activity by 37%. These studies suggest that PDE inhibitors decrease carbohydrate utilization by inhibiting the PDH complex in the heart. The inhibition of PDH by PDE inhibitors appears unrelated to their effects on cAMP or cGMP. This inhibition of PDH by PDE inhibitors may occur, at least in part, secondary to stimulating fatty acid oxidation. (Mol Cell Biochem **180**: 129–135, 1998)

Key words: pyruvate dehydrogenase complex, enoximone, glucose oxidation, phosphodiesterase inhibitors

Introduction

Rat heart contains four phosphodiesterase (PDE) isoforms, PDE I, II, III and IV [1]. These isozymes exhibit different affinities toward intracellular Ca^{2+} and the cyclic nucleotides cAMP and cGMP [2]. Specific inhibitors of PDE were developed to increase cardiac contractile force theoretically through their effects on intracellular Ca^{2+} and cyclic nucleotides [3–5]. Although the physiological effects of these compounds are well established, their specific mechanism of action remains unclear. Recently an effect of PDE inhibitors on cardiac substrate utilization has been reported [6–8]. Enoximone, a specific PDE III inhibitor, increased fatty acid oxidation in isolated cardiac myocytes [8]. Surprisingly, enoximone exerts opposite effects on fatty acid oxidation in isolated heart mitochondria. In contrast to myocytes, enoximone inhibits palmitate oxidation by inactivating acyl-CoA synthetase [9]. Nonetheless in intact myocytes, stimulation of fatty acid oxidation remains the primary effect of enoximone.

Although the stimulation of fatty acid oxidation by enoximone is well established in the heart, its effect on carbohydrate utilization has not been determined. A reciprocal relationship between glucose and fatty acid utilization in skeletal and cardiac muscle was proposed by Randle more than three decades ago [10]. According to his hypothesis, glucose utilization in these tissues is inversely related to the rate of fatty acid oxidation. It has been suggested that this is due to inhibition of the pyruvate dehydrogenase complex (PDH) by end products of fatty acid β-oxidation such as acetyl-CoA and NADH [11]. In addition, increased fatty acid oxidation leads to acceleration of citrate production from the Citric Acid Cycle [12]. Citrate is an allosteric inhibitor of the phosphofructokinase (PFK), a key enzyme in the glycolytic

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pathway. This will ultimately lead to a decrease in glucose through this process (13).

This study was undertaken to determine if PDE inhibitors alter carbohydrate metabolism and if so, to investigate the mechanism by which this process might occur in the heart.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing 200–250 g, were obtained from Charles River Laboratory (Raleigh, NC, USA). Rats were allowed free access to standard diet and water *ad lib*. The animal protocol was approved by Duke University's Institutional Care and Animal Use Committee. Animals were sacrificed by decapitation.

Materials

[U-^{14}C]-D-glucose, [1-^{14}C]pyruvate, and [2-^{14}C]pyruvate, were purchased from New England Nuclear (Boston, MA, USA). Sigma was the source of bovine serum albumin (BSA, essentially fatty acid free). Enoximone and milrinone were a generous gift from Glaxo-Wellcome Inc. (RTP, NC). Joklik essential medium was purchased from Gibco Laboratories (NJ, USA). Collagenase type II was purchased from Worthington (NJ, USA).

Isolation of myocytes

Adult rat heart myocytes were isolated using the published method of Frangakis *et al.* [14]. Myocytes were isolated with Joklik essential medium containing 5.55 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The viability of myocytes isolated by this procedure was 80–90% as determined by trypan blue exclusion.

Metabolic studies using myocytes

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM HEPES (pH 7.4), were placed in a 25ml Erlenmeyer flask. Cells were preincubated with the desired concentrations of enoximone or milrinone for 10 min at 37°C. To this cell suspension was added 0.1 ml of a single labeled metabolic substrate yielding a final concentration of 2 mM [1-^{14}C]pyruvate (2×10^5 dpm), 2 mM [2-^{14}C]pyruvate (2×10^5 dpm], or 5.55 mM [U-^{14}C]-D-glucose (2×10^5 dpm). Some of these studies were performed in presence of 0.2 mM palmitate in the medium. Non-labeled palmitate (20 µl) was added to cell suspension prior to the addition of the radiolabeled substrate. The Erlenmeyer flask was then closed with a rubber septum containing a plastic center well. The incubation was continued during shaking at 37°C for 30 min. An injection of 0.4 ml of 1 M hyamine hydroxide was administered through the septum into the center wells to absorb the released CO_2, and the reaction was terminated by injecting 0.4 ml of 7% perchloric acid through the septum into the incubation medium. The flasks were then shaken continuously for 2 h at 37°C, at which time the plastic center well was removed, placed into a scintillation vial containing 10 ml of Scinti Verse BD, and counted in a liquid scintillation counter. Control experiments with $NaH^{14}CO_3$ added to the cell suspension showed that the release of $^{14}CO_2$ was complete 1 h after the addition of perchloric acid.

Stock solutions of substrates were prepared by dissolving glucose or pyruvate in a myocyte suspension buffer and by dissolving palmitic acid in a solution of defatted serum albumin in the cell suspension buffer. The molar ratio of palmitate to albumin was 2:1.

Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by the procedure of Chappel and Hansford [15]. The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM EGTA.

Determination of the PDH activity in rat heart mitochondria

These studies were performed in a reaction mixture contained in a final volume of 1.0 ml, 50 mM Tris-HCl (pH 7.4); 120 mM KCl; and 0.5 mM EDTA-K_2 (pH 7.4), 2 mM KP_i, and 0.1 mg/ml BSA, and 2 mM [1-^{14}C]pyruvate were placed in a 25-ml Erlenmeyer flask. Substrate oxidation was initiated by the addition of rat heart mitochondria (0.5–1 mg) which were preincubated with enoximone or without (control) for 10 min at room temperature. The PDH activity in mitochondria was determined by measuring the release of $^{14}CO_2$ from 2 mM [1-^{14}C]pyruvate.

Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Richmond, VA).

Analysis of acetylcarnitine by tandem mass spectrometry

Myocytes (2 mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgCl_2$, and 0.5 mM $CaCl_2$ were preincubated with L-carnitine (5 mM), or L-carnitine plus enoximone (0.5 mM)

for 10 min at 37°C. Myocytes were then incubated for an additional 30 min at 37°C after the addition of 2 mM pyruvate and 0.2 mM palmitate. At the end of this period, cell suspensions were centrifuged for 2 min. To 0.1 ml aliquots of the supernatant, 20 pmol of [$^2H_3$]acetylcarnitine was added as internal standard. The mixture was extracted with 0.8 ml methanol, centrifuged and clear supernatant was dried under nitrogen and immediately prepared for analysis of acetylcarnitine. The dried aliquots containing acetylcarnitine were incubated with 100 µl of 3 M HCl in n-methanol at 50°C for 15 min in a capped 1 ml glass vial. The esterifying agent was removed by evaporation under nitrogen and the derivatized sample was dissolved in 50 µl of methanol: glycerol (1:1; v/v) containing 1% octyl sodium sulfate (matrix). A QUATTRO tandem quadrupole mass spectrometer (Fisons-VG Instruments, Danvers, MA) equipped with a liquid secondary ionization source and a cesium ion gun was used for the analysis of acetylcarnitine. This method of analysis is based on the detection of a common fragment ion of acetylcarnitine methyl esters produced by collision-induced dissociation as previously described by Millington *et al.* [16]. Approximately 2 µl of sample matrix was analyzed and the data recorded and processed as previously described. The final spectra displayed the relative intensities of ions corresponding to the molecular weights of the individual acylcarnitine methyl esters. The concentrations of acetylcarnitine corresponding to m/z 218 were determined based on their intensities relative to the internal standard, labeled acetylcarnitine, which corresponds to m/z 221.

Statistical analysis

Data are presented as the mean ± S.D. of four separate experiments. Statistical significance was determined by using an unpaired *t*-test and analysis of variance. Neuman-Keuls analysis was used to determine statistical difference between groups ($p < 0.05$ was considered significant).

Results

Figure 1 shows the effect of increased concentrations of phosphodiesterase inhibitors on pyruvate dehydrogenase activity (PDH) as measured by quantifying the release of $^{14}CO_2$ from [1-^{14}C]pyruvate in isolated cardiac myocytes. Enoximone and milrinone decreased PDH activity in a concentration-dependent manner. The maximal inhibition of PDH with increasing the concentrations of enoximone and milrinone was similar so that at 0.5 mM, decreased PDH activity by 69 and 64%, respectively.

The effect of 0.5 mM enoximone on PDH activity in presence of varied concentrations of pyruvate is shown in Fig.

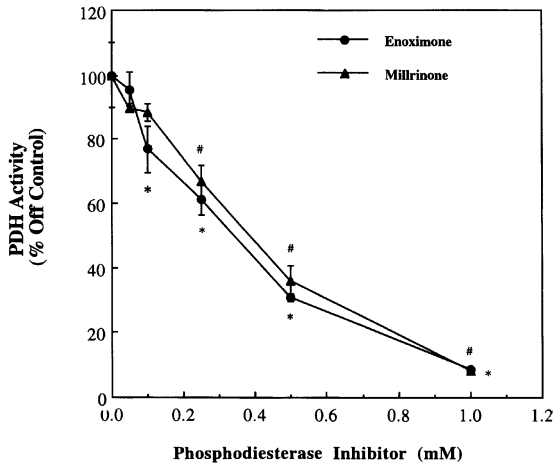

Fig. 1. Effect of increased concentrations of phosphodiesterase inhibitors on the PDH activity in isolated cardiac myocytes. These experiments were performed in presence of 0.2 mM palmitate in the incubation medium. Values are the mean ± S.D. of four separate experiments performed in triplicate. *' #, **' ##, and ***' ###, Indicate a $p < 0.05$, 0.01, and 0.001 of enoximone and millrinone vs control.

2. PDH activity was assayed in presence of different concentrations of pyruvate (0.04–5 mM) to determine the degree of inhibition was altered by the presence of exogenous PDH substrate. At the lower pyruvate concentrations (0.04–0.2 mM), enoximone decreased PDH activity approximately by 70%; whereas, PDH activity was decreased by 60% and 35% at 2 and 5 mM pyruvate, respectively. At no concentration was

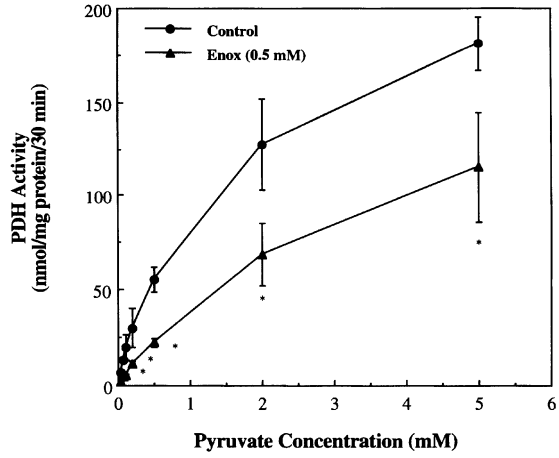

Fig. 2. The effect of enoximone on the PDH activity in presence of varied concentrations of pyruvate in cardiac myocytes. Values are the mean ± SD of four separate experiments performed in triplicate. *Indicate a $p <$ 0.05 of enoximone vs control.

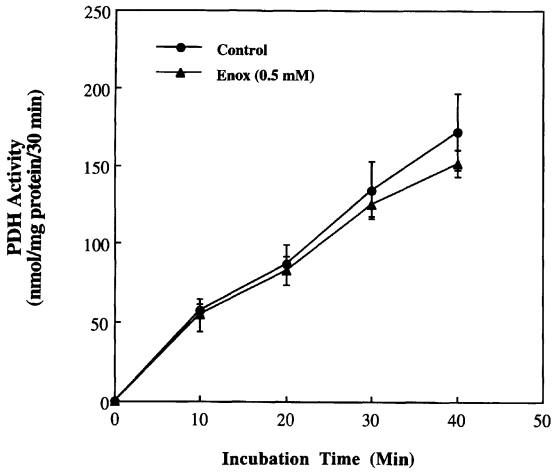

Fig. 3. The mechanism of inhibition of the PDH by enoximone in cardiac myocytes. Myocytes were preincubated with enoximone for 15 min at 37°C, then the medium was replaced with enoximone-free medium and the PDH activity was assayed in these cells. Values are the mean ± S.D. of four separate experiments performed in triplicate.

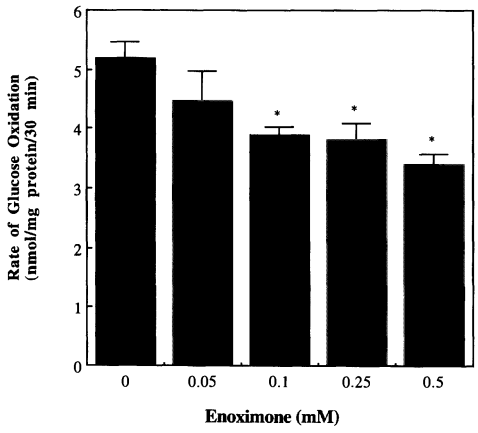

Fig. 4. The effect of enoximone on the oxidation of glucose in cardiac myocytes. For more experimental details see section under Materials and methods. Values are the mean ± S.D. of four separate experiments performed in triplicate. *Indicate a $p < 0.05$ of enoximone vs control.

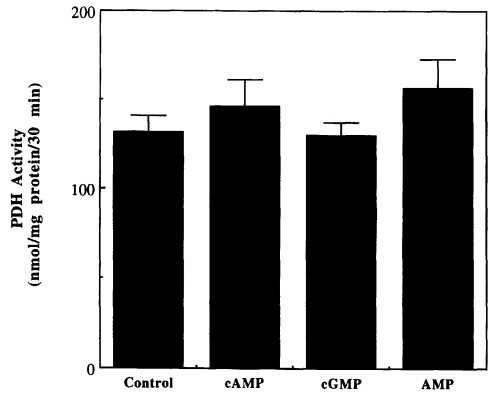

Fig. 5. The effect of cAMP, cGMP, and AMP on PDH activity in permeabilized cardiac myocytes. Cells were permeabilized with 5 μM digitonin. Values are the mean ± S.D. of four separate experiments performed in triplicate.

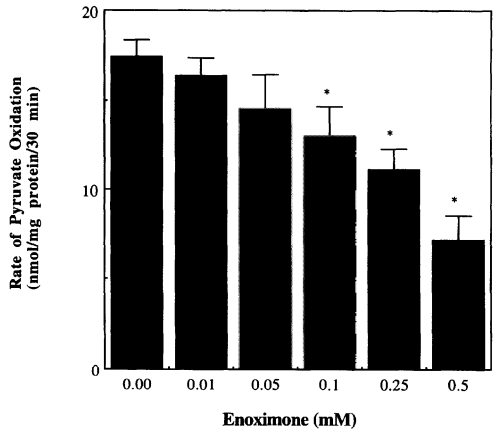

Fig. 6. The effect of enoximone on the oxidation of [2-^{14}C]pyruvate in isolated cardiac myocytes. For more experimental details see section under Materials and methods. Values are the mean ± S.D. of four separate experiments performed in triplicate. *Indicate a $p < 0.05$ of enoximone vs. control.

excess pyruvate able to overcome the effect of enoximone on PDH activity.

Figure 3 depicts the reversibility of PDH complex inhibition after enoximone exposure in isolated cardiac myocytes. Cells were preincubated with 0.5 mM enoximone for 15 min at 37°C, then the medium was removed and cells were washed twice and incubated with enoximone-free medium and PDH activity was determined. As shown in Fig. 3, the inhibition of PDH activity was completely reversed when enoximone was removed from incubation medium.

The effect of enoximone on [U-^{14}C]glucose oxidation which reflects glucose utilization beyond the PDH step is shown in Fig. 4. Glucose oxidation was significantly decreased (25–35%) by enoximone at 0.1–0.5 mM. However, the decrease in glucose oxidation by enoximone was much less than that of PDH.

Figure 5 shows the effect of cAMP, cGMP, or AMP on PDH activity in permeabilized myocytes. This experiment was designed to determine if the decrease in PDH activity

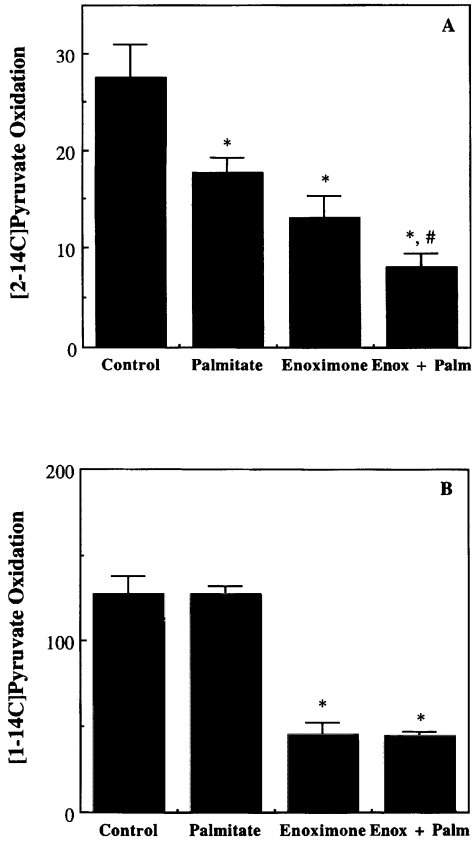

Fig. 7. The effect of enoximone on $^{14}CO_2$ release from [1-^{14}C]- and [2-^{14}C]pyruvate in presence and absence of fatty acids. For more experimental details see section under Materials and methods. Values are the mean ± S.D. of four separate experiments. *Indicate a $p < 0.05$ of the effect of palm, enox, enox + palm vs control; #Indicate a $p < 0.05$ of the effect of enox + palm vs. cont + palm or cont + enox.

by enoximone was mimicked by cyclic nucleotides. After permeabilization of myocytes with 5 μM digitonin, cAMP (50 μM), cGMP (50 μM), or AMP (50 μM) had no effect on PDH activity.

The effect of enoximone on [2-^{14}C]pyruvate oxidation in isolated myocytes is shown in Fig. 6. The oxidation of [2-^{14}C]pyruvate is an index of pyruvate-derived acetyl-CoA oxidation through the Krebs cycle. The oxidation of [2-^{14}C]pyruvate was decreased 60% in the presence of 0.5 mM enoximone similar to the effect seen on PDH.

The effect of enoximone in presence of exogenous palmitate on the PDH activity (Fig. 7a) and subsequent oxidation of acetyl-CoA through the Krebs cycle in isolated cardiac myocytes is shown in Fig. 7b. As demonstrated, exogenous palmitate had no effect on the PDH activity, but significantly decreased the acetyl-CoA flux through the

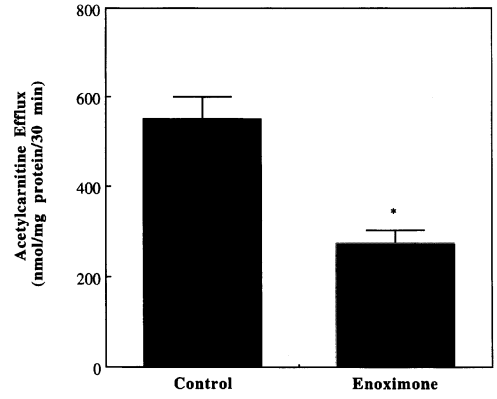

Fig. 8. The effect of enoximone on the efflux of acetylcarnitine in cardiac myocytes. For more experimental details see section under Materials and methods. Values are the mean ± S.D. of four separate experiments. *Indicate a $p < 0.05$ of enoximone vs. control.

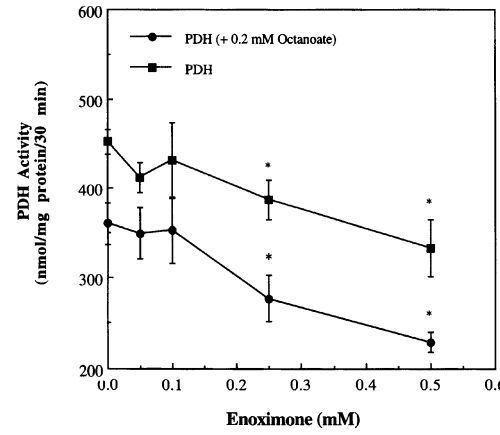

Fig. 9. The effect of enoximone on the PDH activity in isolated rat heart mitochondria. For more experimental details see section under Materials and methods. Values are the mean ± S.D. of four separate experiments. *Indicate a $p < 0.05$ of enoximone vs. control.

Krebs cycle in presence and absence of enoximone.

Figure 8 shows the effect of enoximone on acetylcarnitine efflux in isolated cardiac myocytes. The rate of acetylcarnitine accumulation in the medium is an index of the mitochondrial acetyl-CoA level which is derived from pyruvate metabolism. The rate of acetylcarnitine accumulation in the absence of pyruvate is negligible (data not shown). Enoximone at 0.5 mM significantly decreased pyruvate-derived acetylcarnitine by 50%.

Figure 9 shows the effect of enoximone on PDH activity in isolated rat heart mitochondria (RHM). PDH activity was significantly decreased by 0.25 and 0.5 mM enoximone.

However, the inhibition of PDH in mitochondria was less than in isolated intact myocytes (36 vs. 69%). Also, the inhibition of PDH in mitochondria in absence of octanoate was much higher than in presence of octanoate (0.2 mM).

Discussion

Previous studies from this laboratory have demonstrated that the phosphodiesterase inhibitor, enoximone stimulates fatty acid (palmitate) oxidation in isolated cardiac myocytes [6–8]. According to Randle's hypothesis, 'the glucose-fatty acid cycle,' glucose utilization should be decreased secondary to stimulation of fatty acid oxidation in skeletal and cardiac tissues [11]. Findings from the present study are consistent with this hypothesis, in that glucose oxidation and PDH activity are decreased by enoximone. The data are consistent with the idea that inhibition of PDH activity by enoximone occurs secondary to increased fatty acid utilization whereby end products of fatty acid β-oxidation such as acetyl-CoA and NADH feed back to inhibit PDH. These compounds have been previously shown to decrease PDH activity in cardiac tissue [17]. However, the possibility of inhibiting the PDH complex by the end product of carbohydrate metabolism (acetyl-CoA) cannot be eliminated. The acetyl-CoA produced from β-oxidation is tightly coupled to the Krebs cycle in the heart [18]. Thus, its increase, by enoximone, would likely decrease carbohydrate-generated acetyl-CoA oxidation through the Krebs cycle. This effect would then lead to the accumulation of carbohydrate generated acetyl-CoA and hence a decrease in PDH activity. The further decrease of acetyl-CoA produced from pyruvate through the Krebs cycle in presence of exogenous palmitate in isolated myocytes and the further decrease in PDH activity in presence of octanoate in isolated rat heart mitochondria supports this hypothesis. However, the data demonstrating that exogenous palmitate had no effect on the PDH activity in isolated myocytes suggests that in the intact heart the situation may be more complex, perhaps reflecting the effect of enoximone on both exogenous and endogenous fatty acid oxidation.

The reversal of the enoximone-induced inhibition of PDH after removal of this compound from incubation medium suggests that the inhibition of the PDH activity by enoximone occurs by end-product inhibition rather than by post-translational modification (or covalent modification of the PDH) of the complex. It should be also noted that inhibition of PDH by enoximone can not be fully accounted for by its effect on fatty acid oxidation because PDH activity is still decreased, albeit to a lesser degree, by enoximone when palmitate is removed from the incubation medium. However, it is possible that the decreased PDH activity seen in the absence of palmitate reflects oxidation of endogenous fatty acids produced within the heart from endogenous triacylglycerols. In theory these endogenous fatty acids could maximally regulate the PDH in intact myocytes. Indeed, fatty acids derived from endogenous stores can account for 50% of the energy requirements in the isolated working heart in the absence of exogenous fatty acids [19, 20]. In this study, the similar pattern of inhibition of acetyl-CoA flux through the Krebs cycle and the PDH inhibition by enoximone suggests that PDH is the primary inhibitory target of enoximone. This is supported by the observation of decreased acetylcarnitine flux by enoximone in this study. Acetylcarnitine is produced from mitochondrial acetyl-CoA by carnitine:acetylcarnitine transferase [21]. A recent study from this laboratory revealed that the acetyl-CoA produced from pyruvate metabolism is much more accessible to mitochondrial L-carnitine than β-oxidation-derived acetyl-CoA [22].

Another interesting finding from the present study is that the accumulation of acetylcarnitine in the medium when cells preincubated with L-carnitine and pyruvate reflected pyruvate flux through the PDH. Data from this study showed that the inhibition of pyruvate flux through the PDH by enoximone was paralleled to the decrease of acetylcarnitine accumulation in the medium. Since these experiments were performed in presence of high concentration of L-carnitine and only a single metabolic substrate, further studies are needed to determine if this correlation is valid under all physiological conditions. The pyruvate dehydrogenase enzyme (PDH) is a key enzyme for oxidative carbohydrate metabolism and plays important role in controlling carbohydrate-fatty acid cross talk in the myocardium [23]. It is of great significance to have a simple non-invasive assay to measure the enzyme activity.

Findings from this study and other studies indicate that enoximone shifts cardiac metabolism in favor of fatty acids [7–9]. Recently it has been shown that agents such as dichloroacetate (DCA), and fatty acid oxidation inhibitors which shift cardiac metabolism in favor of carbohydrate protect the myocardium against ischemic injury [24–27]. These agents reduce toxic intermediates of fatty acid oxidation and/or enhance anaerobic glycolysis during ischemia. Therefore, further studies are needed to evaluate effects PDE inhibitors in ischemic heart diseases.

The lack of inhibition of the PDH activity by cAMP or cGMP suggests that enoximone is not acting through its role on these second messengers. This is consistent with the recent study by Kelso *et al.* [28] and the study by Katano and Endoh [29] which demonstrated that PDE III inhibitors such as enoximone and milrinone at 0.5 mM, the same concentration used in this study, did not increase intracellular cAMP in isolated cardiac myocytes [28, 29]. Furthermore, the inhibition of the PDH activity in isolated rat heart

mitochondria in this study suggests that this effect occurs independent of cyclic nucleotides produced in the cytosol.

In conclusion, we report the inhibition of the PDH activity and glucose utilization by enoximone in cardiac myocytes. This process appears to be independent of enoximone's cAMP regulatory effect and it occurs, at least in part, secondary to stimulating fatty acid oxidation in the heart.

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Energy metabolism and mechanical recovery after cardioplegia in moderately hypertrophied rats

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Abstract

It is well established that severe hypertrophy induces metabolic and structural changes in the heart which result in enhanced susceptibility to ischemic damage during cardioplegic arrest while much less is known about the effect of cardioplegic arrest on moderately hypertrophied hearts. The aim of this study was to elucidate the differences in myocardial high energy phosphate metabolism and in functional recovery after cardioplegic arrest and ischemia in mildly hypertrophied hearts, before any metabolic alterations could be shown under baseline conditions.

Cardiac hypertrophy was induced in rats by constriction of the abdominal aorta resulting in 20% increase in heart weight/body weight ratio (hypertrophy group) while sham operated animals served as control. In both groups, isolated hearts were perfused under normoxic conditions for 40 min followed by infusion of St.Thomas' Hospital No. 1 cardioplegia and 90 min ischemia at 25°C with infusions of cardioplegia every 30 min. The changes in ATP, phosphocreatine (PCr) and inorganic phosphate (Pi) were followed by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Systolic and diastolic function was assessed with an intraventricular balloon before and after ischemia.

Baseline concentrations of PCr, ATP and Pi as well as coronary flow and cardiac function were not different between the two groups. However, after cardioplegic arrest PCr concentration increased to 61.8 ± 4.9 μmol/g dry wt in the control group and to 46.3 ± 2.8 μmol/g in hypertrophied hearts. Subsequently PCr, pH and ATP decreased gradually, concomitant with an accumulation of Pi in both groups. PCr was transiently restored during each infusion of cardioplegic solution while Pi decreased. PCr decreased faster after cardioplegic infusions in hypertrophied hearts. The most significant difference was observed during reperfusion: PCr recovered to its pre-ischemic levels within 2 min following restoration of coronary flow in the control group while similar recovery was observed after 4 min in the hypertrophied hearts. A greater deterioration of diastolic function was observed in hypertrophied hearts.

Moderate hypertrophy, despite absence of metabolic changes under baseline conditions could lead to enhanced functional deterioration after cardioplegic arrest and ischemia. Impaired energy metabolism resulting in accelerated high energy phosphate depletion during ischemia and delayed recovery of energy equilibrium after cardioplegic arrest observed in hypertrophied hearts could be one of the underlying mechanisms. (Mol Cell Biochem **180**: 137–143, 1998)

Key words: myocardial protection, hypertrophy, phosphocreatine, cardioplegia

Introduction

Hypertrophy produces a number of morphological, metabolic and physiological changes in the heart which could result in increased susceptibility to ischemic damage [1–5]. Among these modifications the most important are: decrease in capillary density, cell enlargement, increase in both extracellular (collagen) and intracellular protein synthesis, decreased phosphocreatine content as well as increased glycolytic capacity, altered calcium handling and contraction-

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relaxation coupling [3, 6–10]. Enhanced susceptibility to ischemic damage is thought to be related to the functional and/or anatomic alterations of the coronary vascular bed rather than to changes in high energy phosphate concentrations [11] but other studies demonstrate accelerated turnover of high energy phosphates in the hypertrophied hearts [5] which indicate that high energy phosphate metabolism could be involved.

Maintenance of an adequate balance in high energy phosphate metabolism plays an important role in cardioprotection during cardiac surgery [12]. However, studies related to this subject have been limited typically to starting and final time points due to limitation of destructive extraction procedures prior to metabolic analysis. Nuclear magnetic resonance spectroscopy (NMR) offers the advantage of sequential non-invasive evaluation of metabolic changes, allowing one to follow not only the final metabolic status but also dynamic changes throughout the experiment [13, 14]. The profile of metabolic changes in the ischemic heart following repeated infusions of cardioplegic solution and the role of these changes in mechanical recovery has not been well characterized under pathological conditions such as various degrees of cardiac hypertrophy.

In this study we evaluated the effect of mild cardiac hypertrophy on the changes in phosphocreatine, ATP, inorganic phosphate content and pH throughout a cardioplegic arrest/ischemia/reperfusion experimental protocol. In addition to metabolic status, mechanical recovery of systolic and diastolic function was evaluated in the same hearts.

Materials and methods

Animals and aortic banding procedure

All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH publication no. 85-23, revised 1985), and the European Convention on Animal Care. Male Wistar rats weighing 200–250 g were subjected to constriction of the abdominal aorta according to a modified procedure previously described [15]. Briefly, in the hypertrophied group (n = 8) following induction of anaesthesia, the abdominal cavity was opened and a suture was placed around the abdominal aorta between left and right renal arteries. To maintain a constant diameter of the constriction, a 0.8 mm blunt needle was placed inside the band at the time of tightening which was withdrawn thereafter. Sham operated animals (n = 10) were subjected to a similar procedure without constriction of the aorta. Animals were maintained for 6 weeks after surgery before commencing the experiments. Details of the morphological changes induced by aortic banding are given in Table 1. All hearts in the hypertrophied group were excluded if heart dry weight (mg)/body weight (g) ratio was below 0.6 or above 0.8.

Table 1. Heart weight in relation to body weight after aortic banding

	Aortic banded	Control
Weight of rat (g)	498* ± 21	559 ± 12
Dry weight of heart (mg)	341 ± 20	309 ± 9
Heart wt/body wt ratio (mg/g)	0.683** ± 0.021	0.552 ± 0.013

Values represent the mean ± S.E.M.. *$p < 0.05$, **$p < 0.001$ vs. control.

Heart perfusion

Rats were anaesthetized with pentobarbital (60 mg/kg body weight) and heparinized with 200 IU sodium heparin via the femoral vein. The hearts were rapidly excised, placed in ice-cold perfusion buffer, immediately attached to a Langendorff perfusion system and perfused with filtered (0.45 μm pore size) Krebs-Henseleit buffer solution at a constant pressure of 85 mmHg at 37°C as described previously [16]. The buffer solution contained: 118 mM NaCl, 4.7 mM KCl, 1.2 $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 11 mM glucose, 1.4 mM $CaCl_2$ and was continuously gassed with a 95%O_2/5%CO_2 mixture.

Functional assessment

Assessment of mechanical function was performed using a balloon catheter inserted into the left ventricle to determine systolic pressure and end-diastolic pressure – volume relations [17]. The balloon was inflated with differing volumes of water in 25 μl steps from 0–150 μl. Left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were recorded at each loading of the balloon. Coronary flow was measured by timed collection of the coronary effluent.

^{31}P NMR and other metabolic determinations

The changes in myocardial ATP, phosphocreatine (PCr) and intracellular inorganic phosphate (Pi) were followed using ^{31}P NMR (Bruker AMX-400 wide bore vertical system, ^{31}P frequency 161.9 MHz). Fully relaxed spectra were acquired at 20 min of normoxic perfusion (36 scans, 90° angle and 15 sec interpulse delay). Subsequently, saturated spectra (86 or 240 scans, 60° angle, 1 sec interpulse delay) were collected throughout the experiment. An initial ATP concentration of 23 μmol/g dry wt, as measured by high-performance liquid

chromatography (HPLC) in both normal (n = 5) and hypertrophied (n = 3) hearts, was taken for calibration of the NMR data. Calculations of the concentrations in the saturated spectra were corrected using saturation factors obtained from repeated fully relaxed and saturated spectra acquired during baseline conditions. Differences in the saturation factor obtained at 37°C and 25°C were found to be less than 5% in the solution of ATP, PCr and inorganic phosphate with intracellular concentrations of inorganic ions. Intracellular pH was calculated from the shift of intracellular inorganic phosphate peak in relation to PCr position.

Tissue extracts for HPLC analysis were prepared from freeze-dried hearts using 0.6 M perchloric acid (25 µl/mg dry tissue). The extracts were then centrifuged (13 000 g for 3 min at 4°C) and the supernatant was neutralized with 2 M KOH. Analysis of nucleotides, nucleosides and bases was performed using a reversed-phase high performance liquid chromatography method (HPLC) described previously [18]. The equipment used was a Merck-Hitachi chromatograph.

Experimental protocol

The experimental protocol is shown in Fig. 1. After an initial 40 min of normoxic perfusion required for optimization of the magnet and collection of initial spectra, baseline left ventricular function was evaluated using a balloon catheter as described before. Subsequently, hearts were arrested by infusion of St. Thomas' Hospital cardioplegic solution No. 1 (Martindale Pharmaceuticals, UK) at a constant pressure of 70 mmHg at 25°C for 4 min. Hearts immersed in cardioplegic solution were maintained at 25°C with the aid of the temperature control unit of the NMR probe. Cardioplegic fluid infusion was repeated every 30 min. After a total of 90 min of cardioplegic arrest, hearts were reperfused with Krebs buffer at 37°C. After 40 min of reperfusion, mechanical function was evaluated. At the end of the perfusion protocol, hearts were freeze-clamped for analysis of nucleotide concentrations. Five additional normal hearts and three hypertrophied hearts were freeze-clamped after 20 min of normoxic perfusion without ischemia to determine initial metabolite concentrations.

Statistics

All values are presented as means plus standard error of the mean (S.E.M.). Statistical comparison between the control and hypertrophied group of the metabolic NMR data and mechanical function parameters was performed using unpaired Student's *t*-test with Bonferroni correction for repeated measures [19]. Coronary flow data were analysed using two way analysis of variance (ANOVA) and mophological changes induced by hypertrophy were compared using unpaired Student's *t*-test. A value of $p < 0.05$ was considered as a significant difference.

Results

The changes of phosphate metabolite concentrations throughout the protocol in the control and hypertrophied groups are presented in Fig. 2. In controls there was an initial increase of 30% in PCr concentration and a total depletion of Pi during the first infusion of cardioplegic fluid. After the infusion, there was a gradual decrease in PCr concomitant with accumulation of Pi. Subsequently, PCr was rapidly restored after each infusion of cardioplegic solution while the Pi signal completely disappeared. However, depletion of PCr was more rapid after the second and the third infusions and increases in Pi during the ischemic intervals were more pronounced. ATP concentration gradually decreased throughout cardioplegic arrest.

The initial values of PCr, ATP and Pi in hypertrophied

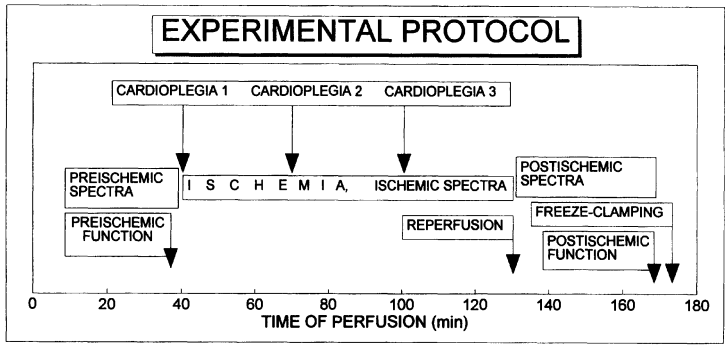

Fig. 1. Experimental protocol.

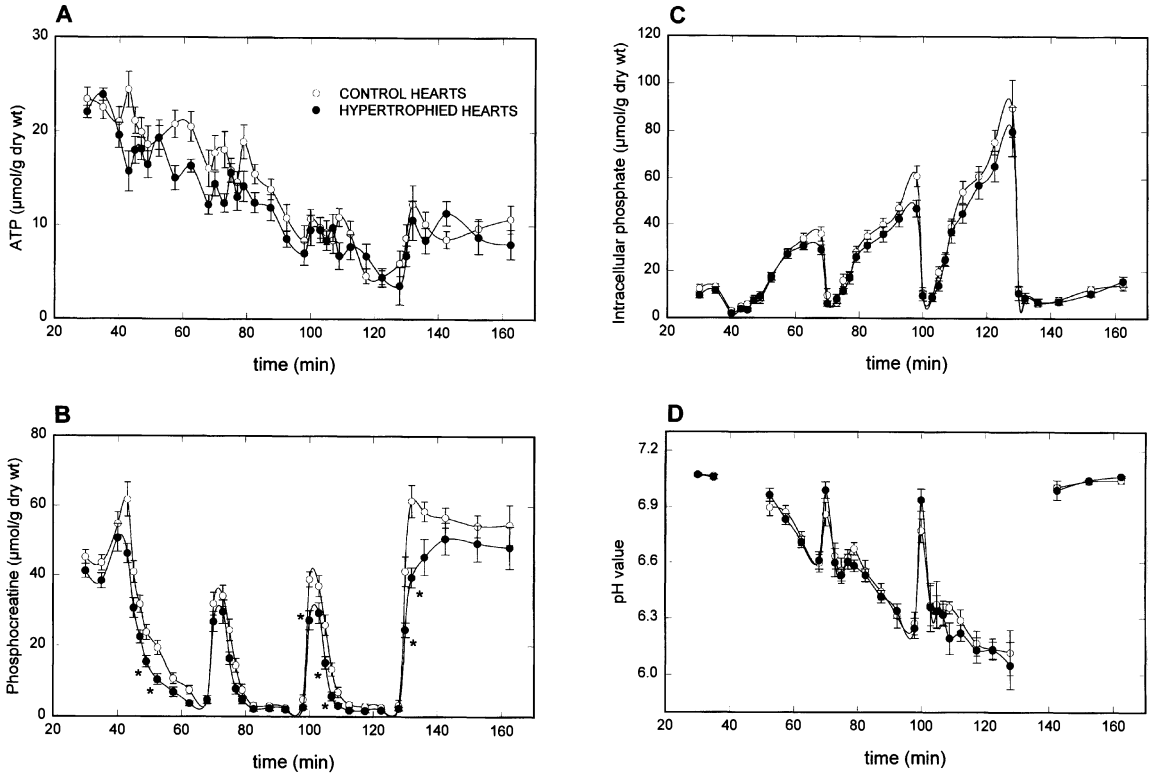

Fig. 2. Changes of (A) ATP, (B) phosphocreatine, (C) intracellular inorganic phosphate and (D) intracellular pH in the normal and mildly hypertrophied rat hearts subjected to cardioplegic arrest, ischemia at 25°C with repeated infusions of cardioplegic solution and reperfusion. Values represents the mean ± S.E.M. *$p < 0.05$ vs. control.

hearts were similar to the control group. The profile of metabolic changes throughout the experiment was also generally similar to the control group. However, several important differences were observed. The increase in PCr during the first cardioplegic infusion was slightly smaller than in control hearts, the decrease of PCr concentration after cardioplegic infusion was faster and, especially, the recovery of PCr after ischemia was delayed in hypertrophied hearts.

Table 2 shows coronary flow in hypertrophied and control hearts before reperfusion and at different times during reperfusion. No significant differences were observed, although coronary flow was slightly lower in hypertrophied hearts after 15 min of reperfusion. Table 3 represents nucleotide concentrations in the control and hypertrophied hearts, evaluated by HPLC at the end of the protocols. There were no significant differences between the two groups.

Figures 3A and 3B represent the relationship between peak systolic (LVSP) and end diastolic (LVEDP) pressures vs. intraventricular balloon volumes. Before ischemia there was a tendency for higher values of LVSP in hypertrophied hearts. Similarly, the LVEDP curve tended to increase more steeply

Table 2. Coronary flow in hypertrophied and control hearts before ischemia and at different time of reperfusion

	Aortic banded	Control
	ml/min/g dry wt	
Before ischemia	51.3 ± 2.9	56.6 ± 3.3
20 min reperfusion	36.4 ± 2.1*	44.9 ± 3.3
40 min reperfusion	34.5 ± 2.0*	39.3 ± 3.8*

Values represents the mean ± S.E.M. *$p < 0.05$ vs. before ischemia.

Table 3. Adenine nucleotide content evaluated in freeze-clamped hearts at the end of experiment by HPLC

	Aortic banded	Control
	µmol/g dry wt	
ATP	11.79 ± 1.04	11.56 ± 1.01
ADP	4.00 ± 0.32	3.53 ± 0.32
AMP	0.75 ± 0.11	0.69 ± 0.17
NAD	5.08 ± 0.75	5.07 ± 0.61

Values represents the mean ± S.E.M.

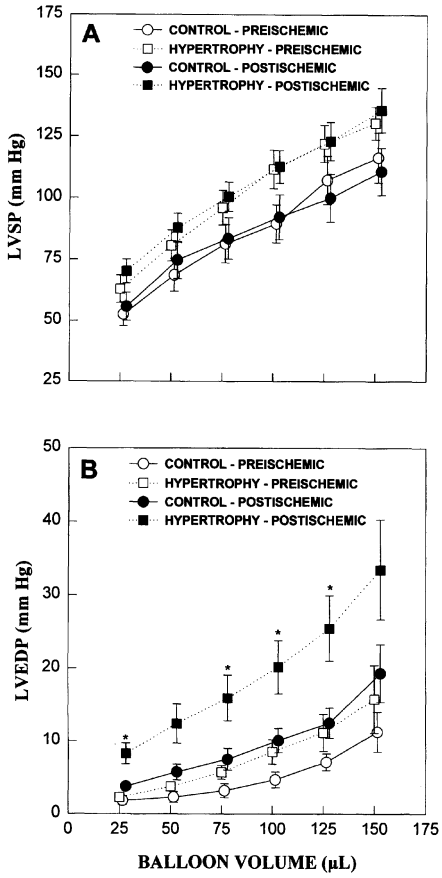

Fig. 3. Mechanical function before and after cardioplegic arrest and ischemia. (A) Relation of left ventricular systolic pressure (LVSP) and (B) relation of left ventricular end diastolic pressure (LVEDP) vs. balloon volume before ischemia and after 40 min of reperfusion. Values represents the mean ± S.E.M. *$p < 0.05$ vs. control.

in this group. After ischemia and 40 min of reperfusion, there was a greater degree of functional deterioration in hypertrophied hearts. Although there was no change of the systolic function represented by the LVSP curve, the diastolic function (LVEDP) in the hypertrophied hearts had deteriorated to a significantly greater extent than in controls.

Discussion

This study demonstrates that even moderate hypertrophy which does not induce metabolic changes under baseline conditions could affect high energy phosphate metabolism and its response to cardioplegic arrest and ischemia. In the hypertrophied hearts, faster decrease in PCr during ischemia after cardioplegic infusion and especially delayed recovery of PCr concentration during reperfusion was shown, which could play an important role in impaired recovery of diastolic function in the hypertrophied hearts.

Slight reduction in PCr concentration after cardioplegic infusion in the hypertrophied hearts studied here may be related to emerging depletion of the creatine pool. Decrease in the creatine metabolites resulting in lower PCr levels has been repeatedly reported in different models of hypertrophy [3, 20] including hypertrophied human hearts [21]. However, there are several reports showing that hypertrophy has no effect on PCr or creatine pool [22–24]. Substantial transmural gradients in high energy phosphate concentrations may contribute to the discrepancies in these data [5]. Our results demonstrated no significant difference in baseline PCr concentration in the hypertrophied heart which may be related to the relatively low degree of hypertrophy we studied. Differences in PCr concentrations could also be related to the perfusion conditions since variations in the perfusion pressure are known to affect the function and metabolic status of the hypertrophied heart to a much greater extent than in the normal heart [2]. We used relatively high perfusion pressures which could minimize the differences under baseline conditions. A smaller increase in PCr concentration after cardioplegic infusion indicate that energy reserves in the form of high energy phosphate bonds before ischemia were lower in hypertrophied hearts. This in turn could lead to accelerated and more pronounced deterioration of all energy dependent metabolic processes during ischemia.

The lower rate of PCr restoration after ischemia is particularly interesting and implies that either pathways of high energy phosphate generation are impaired in hypertrophied hearts or there is increased energy utilization. Since there is evidence that the glycolytic pathway is accelerated in hypertrophy [7], a lower capacity for mitochondrial oxidative phosphorylation is a more likely explanation. Alterations in structure and metabolism of mitochondria or decrease of their numbers, as well as increased oxygen diffusion distances due to decreased capillary density, decreased cell surface/cell volume ratio or increased extracellular matrix could be observed in hypertrophy [6, 25, 26] and may contribute to the impaired PCr recovery. However, these morphological changes may not be sufficient in mildly hypertrophied hearts we studied to cause such a prominent difference in the rate of metabolic recovery. Consequently other factors at the level of regulation of mitochondrial function could also contribute to this phenomenon. The alteration in energy metabolism in the hypertrophied hearts highlighted in this study correlated with impaired diastolic function. This is in agreement with previous studies which showed that myocardial relaxation is an active process which depends on intact high energy phosphate metabolism [27, 28].

An important potential problem associated with this study

is that cardiac hypertrophy may induce an increase in vascular resistance. Increasing perfusion pressure in the hypertrophied hearts has been normally used to overcome this problem [29]. However, we have recently shown that perfusion and administration of cardioplegia at elevated pressures may induce endothelial damage [30]. To avoid differences in endothelial dysfunction caused by perfusion under different pressures, hearts in both groups were perfused at similar but elevated pressures so that baseline coronary flow expressed in relation to heart weight was not different between the groups. Furthermore, similar PCr/ATP ratio under baseline conditions indicate that hearts in both groups were adequately oxygenated.

This study has identified severe abnormalities of high energy phosphate metabolism in mildly hypertrophied hearts following cardioplegic arrest and ischemia which were demonstrated in the absence of metabolic changes under baseline conditions. This impairment of energy metabolism was associated with altered diastolic function.

Acknowledgements

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Role of nucleoside transport and purine release in a rabbit model of myocardial stunning

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Abstract

Previously, we have demonstrated the role of nucleoside transport and purine release in post-ischemic reperfusion injury (myocardial stunning) in several canine models of ischemia. Since rabbits are deficient of xanthine oxidase, it is not known whether selective blockade of purine release is beneficial in a rabbit model of coronary artery occlusion and reperfusion (stunning). Therefore, we determined the hemodynamic and metabolic correlates in response to myocardial stunning in the presence or absence of selective nucleoside transport blocker (p-nitrobenzylthioinosine, NBMPR) and adenosine deaminase inhibitor (erythro-9-(2-hydroxy-3-nonyl)adenine, EHNA).

Sixty adult anaesthetized rabbits were surgically prepared for hemodynamic measurements. After stabilization period, the left anterior descending coronary artery was occluded for 15 min and reperfused for 30 min. Transmural myocardial biopsies were obtained from the ischemic LAD area and from the non-ischemic posterior (circumflex, CFX) segment of the myocardium.

Rabbits (n = 60) were randomly assigned to either the control or the EHNA/NBMPR-treated group (n = 30 each). Each group was further divided to either functional or metabolic groups (n = 15 each subgroup). Each animal received intravenously 30 ml of either a vehicle solution or 100 M EHNA and 25 M NBMPR 10 min before ischemia.

Although administration of EHNA/NBMPR did not affect the heart rate, it did cause mild hypotension (about 20–30%). Fifteen minutes of LAD occlusion resulted in significant ATP depletion and concomitant accumulation of nucleosides in both groups ($p < 0.05$ vs. baseline and non-ischemic CFX segment). AMP was higher in the LAD compared to the CFX segment. Significant accumulation of adenosine was observed in the treated group compared to the control group.

It is concluded that EHNA/NBMPR induced site specific entrapment of adenosine of nucleoside transport in the rabbit heart, *in vivo*. (Mol Cell Biochem **180**: 145–151, 1998)

Key words: ischemic-reperfusion, nucleoside transport, myocardial stunning

Introduction

Myocardial ischemia triggers rapid degradation of ATP leading to accumulation of diffusible adenine nucleosides (adenosine and inosine) and nucleopurines (hypoxanthine and xanthine) [1, 2]. Inosine is the major metabolise of ATP degradation during ischemia [3, 4]. Upon reperfusion, inosine is rapidly transported via the nucleoside transport protein into the interstitial space and taken up by the endothelial cells where it is further degraded to hypoxanthine. In the presence of molecular oxygen, hypoxanthine is oxidized to xanthine and superoxide free radical. Oxidation of nucleopurines is catalyzed by endothelial xanthine oxidase. Similarly, xanthine is oxidized to uric acid and free radicals. Theoretically, 4 moles of superoxide radicals will be produced for each mole of ATP metabolized to uric acid.

Oxygen-derived free radicals have been implicated in the pathogenesis of post-ischemic reperfusion-mediated injury [5, 6] including ventricular arrhythmias [7], myocardial stunning [8] and infarction [9]. The source and species of

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oxygen radicals remains to controversial. In addition, loss of precursors during post-ischemic reperfusion delays myocardial ATP recovery via the salvage synthetic pathway and the heart solely depends on the *de novo* pathway to replete ATP [10, 11]. Several attempts have been made to enhance myocardial ATP replenishment during reperfusion [12, 13]. None of these strategies have lead to full recovery of ATP within 60 min of reperfusion [14–16]. Previously, we demonstrated that selective pharmacological blockade of nucleoside transport and inhibition of adenosine deaminase resulted in trapping of endogenous adenosine during ischemia and reperfusion and recovery of myocardial ATP and ventricular function in canine models of global myocardial stunning [17–22]. These agents provided protection of the myocardium against oxygen-derived free radical-mediated reperfusion injury. The role of myocardial hypoxanthine and xanthine in post-ischemic reperfusion injury has been demonstrated in our laboratory [18]. The role of outward flux of adenosine via adenine nucleoside transport in relation to myocardial bioenergetics has not been elucidated using specific blockers of nucleoside transport protein.

Since rabbit hearts are known to be deficient of xanthine oxidase, it is anticipated that selective nucleoside trapping, theoretically, should not attenuate reperfusion injury mediated by purine-derived free radicals dependent upon xanthine oxidase for formation. Therefore, we determined whether selective blockade of nucleoside transporter by p-nitrobenzylthioinosine (NBMPR) and inhibition of adenosine deaminase by erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) during ischemia and reperfusion attenuates postischemic dysfunction (stunning) and improves hemodynamic recovery in a rabbit model of coronary artery occlusion.

Materials and methods

Sixty adult New-Zealand rabbits of either sex (2–3 kg) were randomly assigned to two groups (n = 30 each): the control group receiving 30 ml of the vehicle solution and the EHNA/NBMPR-group where animal were treated with 30 ml of solution containing 100 μM EHNA and 25 μM NBMPR. Under the same experimental protocol, each group rabbits were further divided into two subgroups (n = 15 each): one group of animals was assigned for hemodynamic analysis and a separate group was assigned for metabolic assessments. Rabbits were premedicated with ketamine/HCl (8 mg/kg, i.m.) and anesthetized with sodium pentobarbital (20 mg/kg i.v.). After tracheotomy rabbits were ventilated with 95% oxygen and 5% carbon dioxide using a Harvard 607A ventilator. The right jugular vein was cannulated for intravenous fluid delivery. Heparin (1000 unit/kg) was administered intravenously. The left common carotid artery was cannulated and connected to a pressure transducer to monitor systemic blood pressure. A median sternotomy was performed and the heart suspended in a pericardial sling. Heart rate, mean, systolic and diastolic pressures were monitored throughout the experiment. After stabilization, rabbits received either normal saline (30 ml) (control group) or saline containing 100 μM EHNA and 25 μM NBMPR. Myocardial biopsies and hemodynamic parameters were obtained before and after drug administration to determine the effect of these drugs before ischemia. The proximal left anterior descending (LAD) coronary artery was ligated using an adjustable snare for 15 min at 37°C. At the end of regional ischemia, myocardial biopsies were taken from the ischemic LAD and non ischemic circumflex region of the left ventricle. Tissue samples were extracted with ice-cooled trichloroacetic acid and neutralized as previously described [23, 24]. Adenine nucleotides, nucleosides and purine bases were determined using HPLC. Protein determination was performed as described by Lowry [25]. Data are presented as mean ± S.E.M. Sequential measurements were compared with repeated measures analysis of variance with SAS software. Differences were considered significant if p value was less than 0.05.

Results

Hemodynamic parameters

The effect of EHNA/NBMPR on heart rate before, during, and after LAD coronary artery occlusion is depicted in Fig. 1A. Intravenous infusion of EHNA/NBMPR did not significantly alter heart rate when compared to the untreated control group (p = NS) before, during acute regional ischemia or during reperfusion. Intravenous infusion of 30 ml of 100 μM EHNA and 25 μM NBMPR before ischemia significantly reduced mean arterial pressure compared with the control group ($p < 0.05$, Fig. 1B). Systolic and diastolic arterial pressure were also reduced during ischemia and reperfusion suggesting a decreased vascular resistance after administration of drugs (Figs 1C and 1D, respectively). In a separate group (n = 5), intravenous infusion of phenylephrine (0.01 μg/min) as a drip prevented the pressure reduction induced by EHNA/NBMPR prior to ischemia (Fig. 1D). Administration of phenylephrine did not, however, alter the pattern of myocardial ATP degradation nor affect the EHNA/NBMPR-induced intramyocardial entrapment of adenine nucleosides during ischemia. In isolated rabbit hearts, intracoronary infusion of EHNA/NBMPR did not affect intraventricular contractility assessed using an isovolumic intraventricular

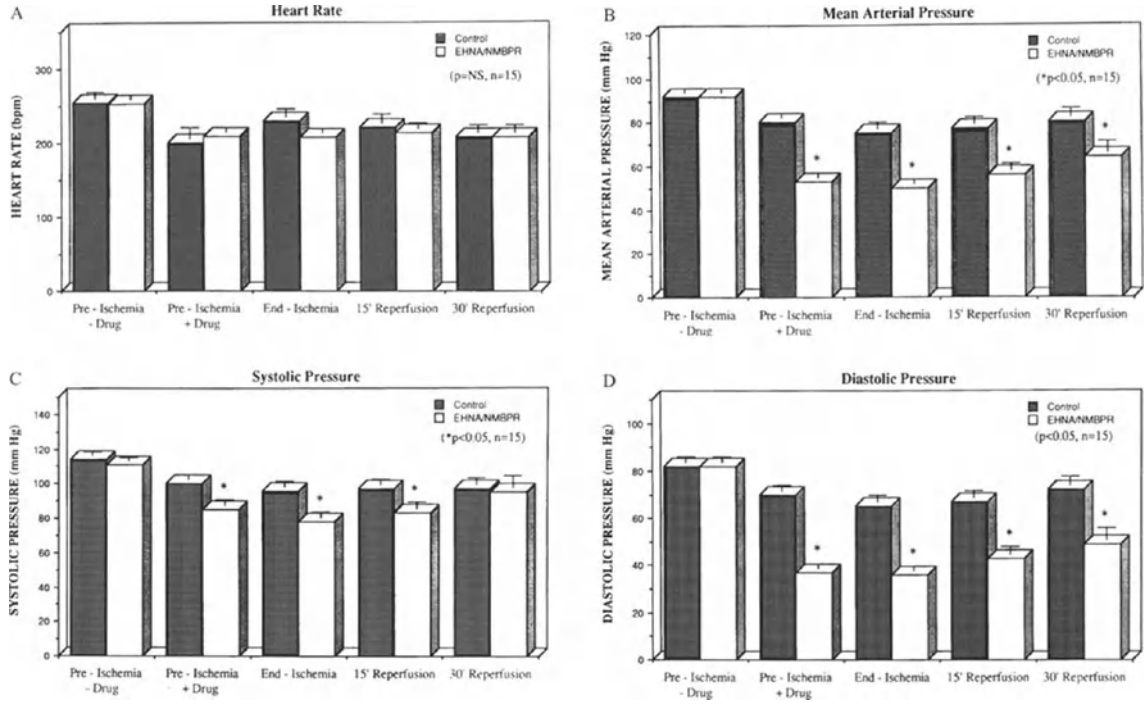

Fig. 1. Effects of EHNA/NBMPR on the heart rate (A), mean arterial pressure (B), systolic (C) and diastolic pressures (D). Results are presented as mean ± S.E.M. Thirty rabbits were used in each group in such a way that each experiment was terminated at two different points of the study. Therefore, we considered the total number of animals in each complete study is 15 rabbits. Statistical analysis demonstrated no significant differences between groups with respect to the heart rate.

balloon (unpublished data). The hypotensive effects also were abolished by reducing the concentration of EHNA/NBMPR in an intact rabbit model.

Myocardial adenine nucleotide pool metabolism

Myocardial ATP at baseline was similar in both groups before ischemia and after drug administration (25.0 ± 1.0 vs. 25.3 ± 0.9 nmol/mg protein, respectively) (Figs 2A-I and 2A-II). Occlusion of the LAD for 15 min resulted in significant loss of ATP in the ischemic region of both groups (control group = 10.5 ± 0.6 vs. EHNA/NBMPR group = 12.2 ± 0.9 nmol/mg protein) compared to baseline ($p < 0.05$). ATP levels at the end of ischemia were greater in the EHNA/NBMPR-treated group compared to the control group ($p < 0.05$, Fig. 2A-I). After 15 min of reperfusion, the EHNA/NBMPR-treated group had more ATP (15.9 ± 1.4 nmol/mg protein) than the control group (12.2 ± 0.9 nmol/mg protein) ($p < 0.05$). No significant differences were found between groups at the end of 30 min of reperfusion. Furthermore, there were no significant differences between groups or time in myocardial ATP levels in the non-ischemic region of the left ventricle (Fig. 2A-II). Myocardial ADP levels were similar in both groups before and after ischemia and during reperfusion in the LAD and CFX segments of the left ventricle (Figs 2B-I and 2B-II). There were no significant differences in myocardial ADP levels between groups throughout the experiment. Similar observations were made with respect to the level of myocardial AMP (Figs 2C-I, 2C-II, 2D-I and 2D-II), except that a slight decrease in myocardial AMP in the treated group was observed at the end of ischemic period.

The most dramatic differences between the EHNA/NBMPR-treated and the control groups were found in myocardial adenosine (Fig. 3A) and inosine (Fig. 3B) during ischemia and reperfusion in the ischemic LAD segment and non-ischemic regions. Myocardial adenosine levels at pre-ischemia were not detectable. However, significant accumulation of adenosine occurred at the end of 15 min of LAD occlusion in both groups compared to pre-ischemic levels ($p < 0.05$). At the end of ischemia, the EHNA/NBMPR-treated group accumulated more adenosine (6.7

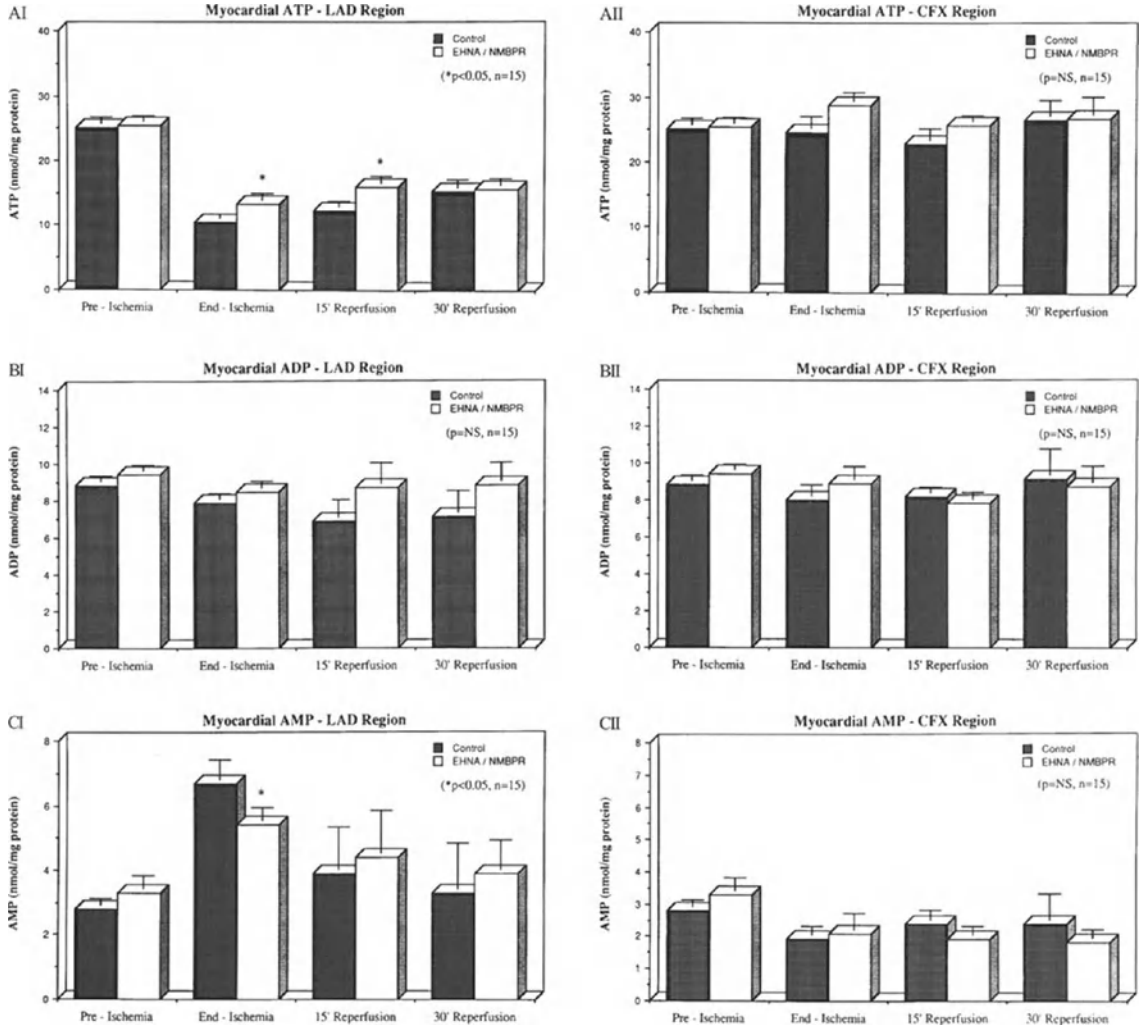

Fig. 2. Effect of ischemia and reperfusion on myocardial (A) ATP, (B) ADP, (C) AMP levels in the presence and the absence of EHNA/NBMPR in ischemic (LAD Region, I) and nonischemic (CFX Region, II) segment of the left ventricle. Statistical analysis demonstrated significant differences between groups with respect ATP and AMP in the LAD region.

± 1.2 nmol/mg protein) than that of the control group (2.5 ± 0.4 nmol/mg protein) ($p < 0.05$). After 15–30 min of reperfusion, myocardial adenosine was greater in the previously ischemic myocardium (20 fold) in the EHNA/NBMPR group than in the control group ($p < 0.05$). In the non-ischemic CFX myocardium, adenosine levels were not detectable before, during or after LAD occlusion (Data not shown). Fig. 3B illustrates inosine in the LAD region following ischemia and reperfusion. At the end of 15 min LAD occlusion, the control group accumulated significantly greater levels of inosine (12 fold) than in EHNA/NBMPR-treated group ($p < 0.05$).

Discussion

Results from the present study demonstrate the effect of specific nucleoside trapping during acute regional myocardial ischemia in an *in vivo* rabbit model chosen for its deficiency of xanthine oxidase. EHNA was effective in

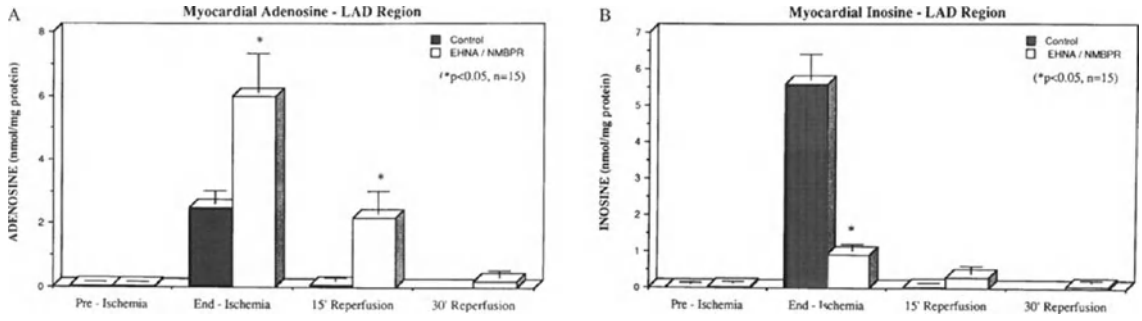

Fig. 3. Effect of ischemia and reperfusion on myocardial adenosine (A) and inosine (B) levels in the presence and the absence of EHNA/NBMPR in ischemic (LAD region) segment of the left ventricle. Statistical analysis demonstrated significant differences between groups with respect to adenosine and inosine in the ischemic zone. However, there were no significant difference between groups with respect to adenosine and inosine levels in the and non-ischemic CFX region (data not shown).

inhibiting adenosine deaminase activity while NBMPR was potent in selectively blocking adenosine and inosine transport before, during and after LAD occlusion in rabbit myocardium *in vivo*. These results are consistent with results obtained in canine models of global ischemia and reperfusion [17, 18, 20–22]. Myocardial ATP was greater in ischemic myocardium pretreated with EHNA/NBMPR.

Unlike dogs, administration of saline solution containing 100 μM EHNA and 25 μM NBMPR in rabbits induced significant hypotension (Figs 1B–1D) without a significant chronotropic effect (Fig. 1A). These observations may be related to the vasodilatory action of accumulated adenosine as a result of intravenous EHNA/NBMPR infusion and modulation of adenosine metabolism. These effects were counteracted by phenylephrine drip. Although phenylephrine prevented EHNA/NBMPR-induced hypotension, it did not alter the pharmacological effects of EHNA and NBMPR in entrapping adenine nucleosides during ischemia and reperfusion. In separate experiments, when the concentration of EHNA/NBMPR was reduced to one half of that used in the present study, drug administration did not cause hypotension or chronotropic effects.

It is interesting to note that tissue AMP levels were high in both groups at the end of the ischemic period. Accumulation of AMP during ischemia is a unique feature of the adult rat and rabbit myocardium and is not seen in the myocardium of dogs and pigs. Age- and species-related differences in 5′-nucleotidase were previously reported [23–25]. As a result of AMP accumulation in ischemic myocardium, ATP recovery was noted in both groups. This is also true for global myocardial ischemia in adult rats [22]. Upon reperfusion, intracellular AMP is rapidly salvaged for ATP resynthesis.

Myocardial ischemia induced rapid depletion of ATP and concomitant rise in adenine nucleosides. In the absence of EHNA/NBMPR, inosine was the major metabolise accumulated in the myocardium at the end of 15 min of LAD occlusion. Pretreatment with EHNA/NBMPR resulted in significant entrapment of adenosine, rather than inosine, at

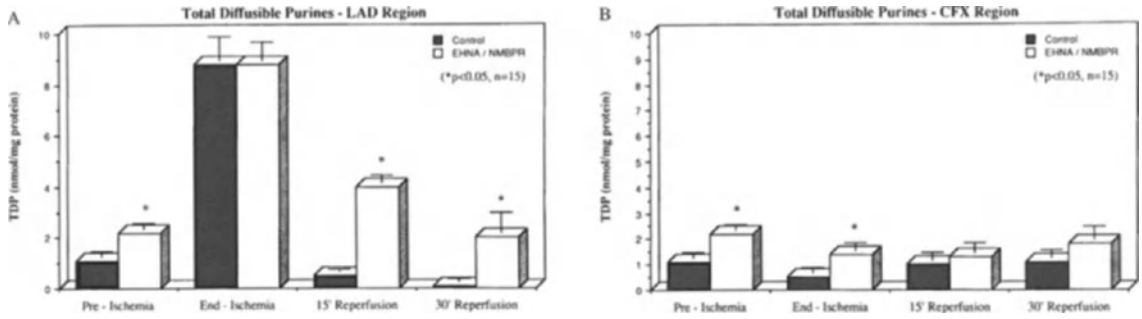

Fig. 4. Effect of ischemia and reperfusion on myocardial total diffusible purines (adenosine+inosine+hypoxanthine and xanthine) levels in the presence and the absence of EHNA/NBMPR in ischemic (LAD Region, I) and non-ischemic (CFX Region, II) segment of the left ventricle. Statistical analysis demonstrated significant differences between groups with respect to total purines in the ischemic zone but not in the non-ischemic areas during reperfusion.

the end of LAD occlusion. Similar results were obtained in a canine model of myocardial global ischemia [17, 22]. ATP levels were greater in the EHNA/NBMPR-treated group at the end of 15 min of LAD ligation compared to control group (Fig. 2A). These results could be explained by at least two possible mechanisms. First, the reduced arterial pressure may have lowered the cardiac energy demand of the drug-treated group. Since similar metabolic data were obtained in a separate group of rabbits intravenously infused with phenylephrine as well as EHNA/NBMPR, this mechanism is less likely to explain the difference in myocardial ATP at the end of LAD ligation. The second, and most likely, mechanism is that intramyocardial entrapment of adenosine reduced the rate of ATP depletion during ischemia by feedback inhibition. Similar observations have been previously reported in other laboratories when adenosine (100 μM) was continuously infused in dog [20] or isolated rat hearts [21, 22] prior to global ischemia. In the present study, repletion of myocardial ATP was noted in both control and drug-treated groups (Fig. 2A) within 30 min of reperfusion.

The entrapped adenosine in the EHNA/NBMPR treated group allows formation of AMP, as catalyzed by adenosine kinase and early ATP replenishment within 15 min of reperfusion. However, no significant differences were detected at the end of 30 min of reperfusion between groups (Fig. 2A). Adenosine may have other cardioprotective effects in addition to repletion of myocardial ATP. Nucleoside trapping may have prevented the formation of purine-derived free-radical substrates during reperfusion. It has been shown that adenosine attenuates catecholamine responsiveness [26, 27], calcium-dependent slow action potentials [28], release of cAMP and responsiveness to histamine [29, 30], and inhibits phosphodiesterase [31]. Therefore, site-specific entrapment of adenosine may have multiple cardioprotective actions.

Results obtained from the present study reveal the important role of the adenine nucleoside transport mechanism in regulation of metabolic flux of adenosine and inosine during ischemia and reperfusion. Inosine generated during ischemia is the only precursor of hypoxanthine and xanthine during post-ischemic reperfusion. The present study provides an inexpensive model to assess the pharmacological efficacy of nucleoside transport inhibitors in an *in vivo* model of acute myocardial ischemia where the non-ischemic segment of the left ventricle acts as a internal control. This model allows simultaneous monitoring of hemodynamic effects of nucleoside transport inhibitors and their pharmacological actions.

Selective blockade of adenosine and inosine transport has a potential cardioprotective effect against alleged oxygen-derived free radical-mediated reperfusion injury, at least via the reaction catalyzed by myocardial and/or endothelial xanthine oxidase. Human nucleoside transport protein has been pharmacologically identified to be similar to that of the canine, swine and rabbit models. It is concluded that modulation of adenosine metabolism and transport may have very important clinical relevance.

Acknowledgement

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Ischemic preconditioning in rat heart: No correlation between glycogen content and return of function

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Abstract

We tested the hypothesis that glycogen levels at the beginning of ischemia affect lactate production during ischemia and postischemic contractile function.

Isolated working rat hearts were perfused at physiological workload with bicarbonate buffer containing glucose (10 mmol/L). Hearts were subjected to four different preconditioning protocols, and cardiac function was assessed on reperfusion. Ischemic preconditioning was induced by either one cycle of 5 min ischemia followed by 5, 10, or 20 min of reperfusion (PC5/5, PC5/10, PC5/20), or three cycles of 5 min ischemia followed by 5 min of reperfusion (PC3 × 5/5). All hearts were subjected to 15 min total, global ischemia, followed by 30 min of reperfusion. We measured lactate release, timed the return of aortic flow, compared postischemic to preischemic power, and determined tissue metabolites at selected time points.

Compared with preischemic function, cardiac power during reperfusion improved in groups PC5/10 and PC5/20, but was not different from control in groups PC5/5 and PC3 × 5/5. There was no correlation between preischemic glycogen levels and recovery of function during reperfusion. There was also no correlation between glycogen breakdown (or resynthesis) and recovery of function. Lactate accumulation during ischemia was lowest in group PC5/20 and highest in the group with three cycles of preconditioning (PC3 × 5/5). Lactate release during reperfusion was significantly higher in the groups with low recovery of power than in the groups with high recovery of power.

In glucose-perfused rat heart recovery of function is independent from both pre- and postischemic myocardial glycogen content over a wide range of glycogen levels. The ability to utilize lactate during reperfusion is an indicator for postischemic return of contractile function. (Mol Cell Biochem **180**: 153–161, 1998)

Key words: isolated working rat heart, lactate release, cardiac work

Introduction

Ischemic preconditioning of the heart [1] depletes glycogen, reduces lactate accumulation and lessens intracellular acidosis during subsequent ischemia [2–4]. These observations are consistent with those of Neely and Grotyohann [5] who have reported improved ischemia tolerance of rat hearts subjected to ischemia and reperfusion after depletion of glycogen during the preischemic period. Several investigators have proposed that the depletion of glycogen before ischemia may be causally related to the mechanism(s) of ischemic preconditioning [3, 4]. However, the evidence in support of this hypothesis is inconsistent. We have reported earlier that raising glycogen levels of the heart *in vivo* lessens the effects of ischemia *in vitro* [6–8] and can be as efficient as preconditioning in improving postischemic contractile function [9]. We have proposed that myocardial glycogen is an important factor preserving function and energy metabolism on reperfusion [6]. These observations have been challenged by investigators who have observed a relationship between preischemic glycogen depletion and protection against ischemic damage by preconditioning [10]. However, these investigators, like our

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laboratory [11, 12], have not been able to observe improved ischemia tolerance by preischemic glycogen depletion without ischemic preconditioning.

In order to resolve the issue whether the myocardial glycogen content affects postischemic contractile function in preconditioned hearts, we used the isolated working rat heart model and tested several protocols of ischemic preconditioning. We also measured lactate release during reperfusion and determined the tissue content of glucose-6-phosphate, lactate, and citrate at selected time points. We found that postischemic recovery of contractile function was independent from myocardial glycogen content but that the degree of return of function after ischemia was inversely related to lactate release during reperfusion.

Materials and methods

Animals

Male Sprague-Dawley rats (275–300 g, n = 79) were obtained from Harlan (Houston, TX, USA) and fed *ad libitum* on Purina lab chow. The use of animals and the experimental protocol were approved by the Animal Welfare Committee of the University of Texas Houston Health Science Center.

Materials

All chemicals were obtained from Fisher Scientific, Lexington, MA, USA or Sigma Chemical Co., St. Louis, MO, USA. All enzymes and cofactors were obtained from Boehringer Mannheim, Indianapolis, IN, USA or Sigma Chemical Co., St. Lois, MO, USA.

Working heart preparation

The preparation has been described in detail earlier [13]. Briefly, rats were anesthetized with sodium pentobarbital (5 mg/100 g body wt. i.p.). After injection of heparin (200 IU) into the inferior vena cava, the heart was rapidly removed and placed in ice cold Krebs-Henseleit bicarbonate buffer. The aorta was freed of excess tissue and mounted on a stainless steel cannula of the perfusion apparatus. A brief period of retrograde reperfusion (less than 5 min) with oxygenated buffer containing glucose (10 mmol/L) was necessary to wash out any blood from the heart and to perform the left atrial cannulation. Hearts were then perfused as working hearts at 37°C with recirculating Krebs-Henseleit buffer (200 ml) containing 1% defatted bovine serum albumin, Cohn fraction V, and glucose (10 mmol/L) as substrate. The buffer contained 2.5 mmol/L calcium. The perfusate was gassed with 95% O_2 – 5% CO_2.

All experiments were carried out at standard workload (15 cm H_2O preload and 100 cm H_2O afterload). Aortic flow and coronary flow were measured every 5 min by timing the rise of the fluid meniscus in a calibrated glass cylinder. Cardiac output was calculated as the sum of aortic and coronary flow. Heart rate and aortic pressures were continuously measured with a Hewlett-Packard transducer and recording system (Hewlett Packard, Waltham, MA, USA).

Perfusion protocols

Control hearts were perfused for 25 min, followed by 15 min of total, global, normothermic ischemia and 30 min of reperfusion. In the experimental groups, ischemic preconditioning (PC) was achieved after 15 min of perfusion by either one cycle of 5 min ischemia and 5, 10, or 20 min of reperfusion (groups PC5/5, PC5/10, and PC5/20), or by three cycles of 5 min ischemia with 5 min of reperfusion (PC3 × 5/5). As in the control group, all hearts were subjected to 15 min ischemia followed by 30 min of reperfusion. Pacing of the hearts was not required since the heart rate returned rapidly to preischemic values upon reperfusion after ischemia. In order to determine tissue metabolite contents, hearts were freeze-clamped immediately at three time points: after preconditioning-reperfusion (prior to sustained ischemia), after sustained ischemia (prior to reperfusion), and at the end of the experiments.

Perfusate samples

Samples of the coronary effluent (1 ml) were withdrawn every 10 min. In addition, we collected the first 5 ml of coronary effluent on reperfusion (in aliquots of 1 ml). Samples were stored on ice until assayed. Lactate was measured in a glucose/lactate analyzer (2300 STAT, YSI Inc., Yellow Springs, Ohio, USA). Myocardial lactate release was calculated by the appearance of lactate in the perfusion medium using a modification of the procedure described earlier [13]. The difference in the perfusate concentrations of lactate at the beginning of ischemia and at the end of reperfusion were multiplied with the amount of perfusate (ml) remaining. The lactate content of all previously taken samples was added [13]. Lactate release was expressed as μmol per g wet weight.

Tissue extraction and metabolites

At the end of the perfusion, hearts were freeze-clamped between aluminium blocks cooled to the temperature of liquid nitrogen [14]. The frozen tissue was ground under liquid nitrogen and extracted with 6% perchloric acid. The tissue extracts were neutralized and assayed immediately for glucose-6-phosphate (G6P), lactate, and citrate as described by Bergmeyer [15]. Glycogen was determined by hot KOH digestion and ethanol precipitation followed by incubation with amyloglucosidase as described by Walaas and Walaas [16]. A small portion of the pulverized tissue was dried in an oven (70°C) to constant weight, and the wet-to-dry weight ratio was calculated. Tissue metabolite data are presented as micromoles per g dry weight (μmol/g dry), and glycogen as micromoles glucose per g dry weight. Ischemic lactate accumulation was calculated by subtracting the tissue lactate content at the beginning of ischemia from the tissue lactate content at the end of ischemia.

Assessment of contractile performance

Mean aortic pressure (cm H_2O) was calculated as [systolic + (diastolic pressure × 2)]/3. Heart rate was measured as beats per min and cardiac output as milliliters per min. We did not normalize the cardiac output for heart weight because all hearts were of similar size (approximately 1.1 g wet weight).

Hydraulic power (mW) was determined as [mean aortic pressure (cm H_2O) × cardiac output (ml/min)]/612 as described earlier [9]. Kinetic power was not calculated because of the inherent difficulty in its estimation and because the amount of kinetic work is small compared to hydraulic work (i.e. less than 10% of total cardiac work) [13].

At the beginning of the reperfusion period, the afterload fell below the preset level of 100 cm H_2O and aortic flow ceased since we did not use any retroperfusion (Langendorff-mode). Recovery of cardiac performance was assessed in two ways: first, we measured the time from the onset of reperfusion to the resumption of aortic flow at preischemic mean aortic pressure and called it recovery time. Secondly, we compared pre- and postischemic cardiac power. Preischemic cardiac power was measured under steady state conditions during the last 10 min of the preischemic perfusion period (i.e. before preconditioning-ischemia in the experimental groups). Postischemic power was obtained as a mean of the postischemic cardiac power measurements at 5, 10, 20, and 30 min after the onset of reperfusion and expressed in % of preischemic power in the same group.

Statistical analysis

All data are presented as mean ± S.E.M. A single factor analysis of variance (ANOVA) with *post hoc* multicomparison analysis (Newmann-Keuls test) was used for data comparison. Differences were considered statistically significant when $p < 0.05$.

Results

Recovery of contractile function

Table 1 shows recovery time, cardiac power before ischemia (baseline power), and recovery of power during reperfusion. The recovery time was significantly shortened by ischemic preconditioning when 5 min preconditioning ischemia was followed by 10 or 20 min of reperfusion (PC5/10, PC5/20). These groups also recovered cardiac power almost completely during the remaining reperfusion period ($p < 0.001$ compared to control). Unexpectedly, when the preconditioning reperfusion time was only 5 min (PC5/5), the recovery time after 15 min ischemia was significantly longer than in the control group ($p < 0.05$), and at the same time postischemic power remained depressed. Increasing the number of preconditioning stimuli by using three cycles of 5 min ischemia with 5 min of reperfusion (PC3 × 5/5) shortened the recovery time but had no beneficial effect on return of postischemic function (ns. compared to control). The differences in preischemic power were not statistically different among groups. After preconditioning ischemia cardiac power returned quickly (between 20 sec and 1 min) to preischemic values in all experimental groups (data not shown).

Glycogen

Table 2 shows the tissue glycogen content before sustained ischemia, after sustained ischemia and at the end of reperfusion. Preischemic glycogen was highest in the control group (104 ± 7.5 μmol/g dry). One cycle of ischemic preconditioning resulted in maximal reduction of 21% of glycogen relative to control (PC5/10, 21.9 μmol/g dry reduction). Three cycles resulted in the lowest preischemic glycogen levels of all groups (PC3 × 5/5, 57.3 ± 8.1 μmol/g dry, 45% reduction compared to control). During ischemia, glycogen further decreased in all groups. The amount of glycogen breakdown was greatest in the PC5/5 group (49 μmol/g dry) and smallest in the PC3 × 5/5 group (23.9 μmol/g dry). In both groups, recovery of postischemic power was not different from control. Glycogen at the end of ischemia was highest in the control group (71.6 ± 2.5 μmol/heart) and lowest in the

Table 1. Recovery of contractile function after 15 min of ischemia

Group	Recovery time (sec)	Baseline power (mW)	Recovery of power (% baseline)
Control	320 ± 10	8.52 ± 0.58	72.1 ± 3.8
PC5/10	180 ± 15**	9.50 ± 0.43	91.8 ± 3.9***
PC5/20	193 ± 32**	6.81 ± 0.47	104 ± 4.4***
PC5/5	506 ± 77**	7.18 ± 0.69	68.6 ± 3.9
PC3 × 5/5	244 ± 78	9.36 ± 0.82	65.3 ± 5.8

Values are mean ± S.E.M. n = 5–9 for each group, *p < 0.05, **p < 0.01, ***p < 0.001 compared to control. Recovery time was measured from the onset of reperfusion to the resumption of aortic flow. Recovery of power was determined as the mean of all postischemic cardiac power measurements and is expressed as % of preischemic power in the same group (Baseline power).

PC3 × 5/5 group (33.9 ± 3.8 µmol/heart). Both groups exhibited a similar return of postischemic function. At the end of reperfusion, the groups involving ischemic preconditioning showed evidence of net glycogen resynthesis, whereas no glycogen resynthesis was observed in the control group.

When the mean postischemic recovery of contractile function was plotted against the preischemic glycogen content there was no correlation between the two parameters (Fig. 1, correlation coefficient r = 0.354, ns). There was also no correlation between functional recovery and glycogen levels at the end of ischemia or at the end of reperfusion (data not presented).

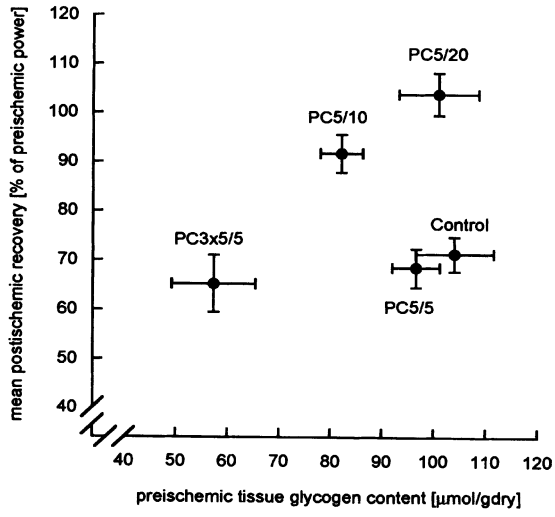

Fig. 1. Relationship between the preischemic myocardial glycogen content and postischemic return of function in the control and the four preconditioned groups. Please refer to text for experimental protocols. Data (means ± S.E.M.) were obtained from two sets of experiments for each group (n = 4–9). Glycogen at the beginning of ischemia is independent from postischemic return of power, expressed as % of preischemic power. The correlation coefficient is r = 0.354 (ns).

Lactate release

Figure 2 shows lactate concentrations in the perfusate from a representative group (PC5/5). Note the difference in lactate level during the first 5 min of reperfusion. There was no correlation between lactate accumulation during ischemia and lactate washout in any of the groups.

Figure 3 shows the amount of lactate released from the hearts during reperfusion as calculated from the appearance of lactate in the perfusate. Myocardial lactate production before ischemia was minimal and did not differ among groups

Table 2. Glycogen

Group	Before ischemia	End of ischemia	End of reperfusion
Control	104 ± 7.5	71.6 ± 12.5[b]	77.8 ± 3.2[b]
PC5/10	82.1 ± 4.1*	52.8 ± 9.1[b]	79.9 ± 4.7[+]
PC5/20	101 ± 7.8	63.0 ± 7.1[a]	84.1 ± 7.2
PC5/5	96.6 ± 4.6	47.6 ± 7.5[b]	80.7 ± 8.0[+]
PC3 × 5/5	57.3 ± 8.1*	33.9 ± 3.8[a]	56.4 ± 2.4[+]

Values are mean ± S.E.M. n = 4–9 in each group. Units are µmol glucose/g dry weight. *p < 0.05 compared to control, [+]p < 0.05 compared to end of ischemia, [a]p < 0.05, [b]p< 0.01 compared to before ischemia.

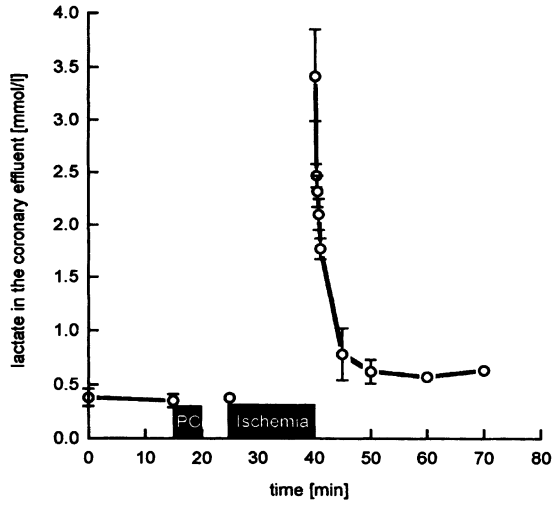

Fig. 2. Lactate concentration in the coronary effluent during the perfusion period of group PC5/5. The first 5 points after ischemia represent the first 5 ml of coronary effluent after the beginning of reperfusion. PC, ischemic preconditioning. Data are mean ± S.E.M. n = 9.

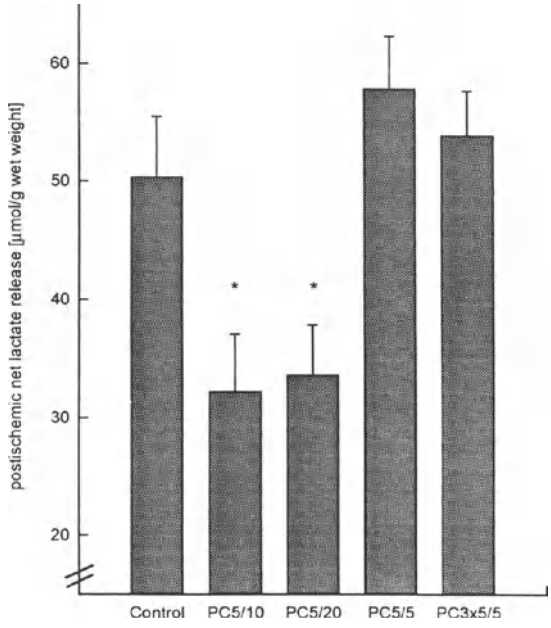

Fig. 3. Myocardial net lactate release during the reperfusion period as calculated from the appearance and disappearance of lactate in the perfusate. Please refer to text for experimental protocols. Note that the groups with good functional recovery (PC5/10, PC5/20) release less lactate during reperfusion than the groups with poor recovery (control, PC5/5, PC3 × 5/5). Data are mean ± S.E.M. *p < 0.05.

(data not shown). Lactate release was significantly reduced in the groups with improved recovery (PC5/10, PC5/20). In groups PC5/5 and PC3 × 5/5 (poor recovery), lactate release was similar to that released in the control group. Figure 4 shows an inverse relationship between mean postischemic power and postischemic lactate release (correlation coefficient r = –0.655, p < 0.001).

Metabolites

Table 3 shows tissue contents of glucose-6-phosphate (G6P), lactate, and citrate before sustained ischemia, after sustained ischemia, and at the end of reperfusion. We measured these metabolites because they are allosteric regulators of several of the following key enzymes of the glycolytic pathway: hexokinase, glyceraldehyde 3-phosphate dehydrogenase, and phosphofructokinase. There were no statistically significant differences among groups in metabolite content before ischemia. As expected, with sustained ischemia lactate rose significantly in all groups. Lactate accumulation (postischemic – preischemic lactate content) was highest in group PC3 × 5/5 (98.8 µmol/g dry), which showed poor recovery of function, and in group PC5/10 (94.6 µmol/g dry), which showed good recovery of contractile function. It was lowest in group PC5/20 (59.0 µmol/g dry). G6P was elevated at the end of ischemia in group PC5/10 and in the control group. With reperfusion,

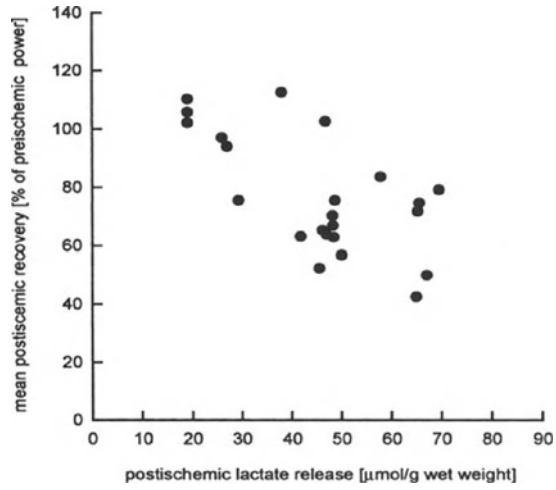

Fig. 4. Relationship between the postischemic myocardial lactate release and postischemic return of function in 23 experiments (n = 4–6 in each group). Postischemic return of power was expressed as % of preischemic power. Lactate release was calculated from the appearance and disappearance of lactate in the perfusate. The correlation coefficient is r = –0.655 (p < 0.001).

Table 3. Tissue content of glucose-6-phosphate (G6P), lactate, and citrate at different time points during the experiments

Group	Metabolite	Before ischemia	After ischemia	End of reperfusion
	G6P	1.44 ± 0.35	2.67 ± 0.24[a]	1.12 ± 0.12
Control	Lactate	4.94 ± 0.44	83.1 ± 15.4[b]	8.38 ± 1.56
	Citrate	1.87 ± 0.04	1.63 ± 0.22	2.59 ± 0.28
	G6P	0.72 ± 0.09	2.09 ± 0.24[b]	1.08 ± 0.11
PC5/10	Lactate	8.42 ± 1.26	103 ± 3.86[b]	6.84 ± 1.02
	Citrate	1.09 ± 0.17	0.72 ± 0.10	2.53 ± 0.15[b]
	G6P	0.93 ± 0.11	0.27 ± 0.12[b,**]	0.64 ± 0.07
PC5/20	Lactate	18.1 ± 3.63	77.1 ± 11.90[b]	5.23 ± 1.00[b]
	Citrate	n.d.	0.31 ± 0.07	1.77 ± 0.17
	G6P	1.05 ± 0.11	0.98 ± 0.27**	1.14 ± 0.16
PC5/5	Lactate	11.6 ± 4.32	98.8 ± 12.80[b]	8.72 ± 1.24
	Citrate	n.d.	0.85 ± 0.09	2.29 ± 0.20
	G6P	0.62 ± 0.14	0.39 ± 0.12**	0.44 ± 0.13
PC3 × 5/5	Lactate	10.2 ± 1.42	110 ± 13.50[b]	6.63 ± 1.73
	Citrate	0.78 ± 0.23	0.39 ± 0.09	0.64 ± 0.17**

Values are mean ± S.E.M. n = 4–9 in each group. Units are µmol/g dry weight. [a]p < 0.05, [b]p < 0.01 compared to before ischemia, **p < 0.01 compared to control, n.d. metabolites not determined.

G6P and lactate levels returned to preischemic values. Citrate levels were elevated at the end of reperfusion compared to those at the end of ischemia. This elevation suggests increased carboxylation and oxidation of pyruvate (lactate) during reperfusion. However, there was no correlation between any of the metabolites measured and postischemic return of function.

Discussion

The present experiments were performed to assess whether glycogen modulates the response to ischemic preconditioning in rat heart. They suggest that the phenomenon of ischemic preconditioning is neither related to pre- or postischemic glycogen content, nor were they related to the tissue contents of G6P, lactate, or citrate. Instead, we found that the efficacy of ischemic preconditioning in the isolated working rat heart is not an 'all-or-none' phenomenon with respect to recovery. We found that important features associated with the return of function in our model are the preconditioning reperfusion time and the heart's ability to oxidize lactate during reperfusion.

Cardiac function and metabolism

We based our analysis of ischemic damage on two functional parameters, recovery time and postischemic power. This approach is meaningful, because the contractile function of the isolated working rat heart is the same as cardiac function *in vivo* (pressure*volume work), albeit under controlled environmental conditions. We are aware that there may be a discordance between the assessment of ischemic damage by infarct size and the assessment by functional recovery [4]. Our data are however, in line with results from other studies that also used return of steady state cardiac function [3, 17–19] to assess ischemic damage and cardiac protection with ischemic preconditioning. We are not certain whether the recovery time, representing a period of contractile dysfunction and high metabolic activity [9, 19, 20], can be compared directly to the clinically observed 'stunned myocardium' [21, 22]. Irrespective of the terminology, this parameter is not only easy to measure but also of possible clinical relevance with respect to the recovery of the arrested heart after controlled ischemia, e.g. hypothermic ischemic arrest during cardiac surgery.

In our model, it is difficult to rule out the possibility that the improvement of contractile function with ischemic preconditioning reflects a reduction in stunning present during the entire reperfusion period rather than a salvage of damaged myocardial cells. However, we demonstrated earlier [9] preserved structural integrity of isolated mitochondria with ischemic preconditioning (5 min ischemia, 10 min reperfusion) in the same preparation, indicating actual cell salvage by ischemic preconditioning.

It is not obvious to us why there was a bimodal effect of preconditioning, and why the preconditioning protocols with 5 min of reperfusion (PC5/5 and PC3 × 5/5) did not result in improved postischemic return of function. However, we are not the first to observe a lack of protection with ischemic preconditioning using protocols that have been shown to be protective, albeit in a different animal model and protocol [17, 23, 24]. The lack of protection with three cycles of preconditioning was surprising since Liu and Downey [25] had advanced the concept that this lack was due to a higher threshold of ischemic preconditioning in rat heart and could be overcome by increasing preconditioning stimuli, although in an open chest and not in an isolated heart model. Minhaz *et al.* [26] reported that a delay of perfusion due to the preparation of the heart could lead to unintentional preconditioning. Although we cannot exclude an influence of the preparation procedure on our results, it would be the same on every heart and therefore not responsible for the differences among our groups. The influence of the preparation procedure may be responsible for different results between two investigators with the same model.

Glycogen and recovery of function

We reported earlier a protective role of high glycogen levels at the beginning of ischemia on postischemic return of function [6–8] and suggested that glycogen would provide substrate readily available for anaerobic energy production from glycolysis. Accelerated glycolysis will lead to a higher lactate accumulation which in turn has been implicated as a cause for intracellular acidosis [27]. We reasoned that glycogen, when present in high concentration, may protect the heart from the loss of energy rich phosphates and adenine nucleotides [6]. Other investigators suggested exactly the opposite and demonstrated a protective effect of preischemic glycogen depletion against ischemic damage with [2–4, 23] or without [5, 28] ischemic preconditioning. It was suggested that preischemic glycogen depletion is related to the mechanism of ischemic preconditioning [4]. The same investigators also demonstrated that prolonging the preconditioning reperfusion time, after several (2–4) cycles of ischemic preconditioning (which caused an almost complete depletion of glycogen) led to repletion of glycogen stores and resulted in loss of protection. In a recent study, Cross *et al.* [29] suggested that a high glycogen content can be both beneficial and detrimental. The beneficial effects are thought to be effective in prolonging ischemia tolerance as long as ischemia is ended before complete depletion of the glycogen stores. For that period of time, ATP can be continuously

generated. If ischemia goes beyond depletion of the glycogen stores, ATP production is thought to cease. Subsequently, the stronger acidosis causes damage to the myocardium. This hypothesis would explain the differences between the results of our study and the study by Wolfe *et al.* [4], where preischemic glycogen was almost completely depleted. However, Cross *et al.* [29] advanced their hypothesis on the basis of experiments with low-flow ischemia and did not consider total ischemia. In any case, the lack of correlation between the preischemic glycogen content and the post-ischemic return of cardiac function presented in this study and the results from the study by Cross *et al.* [29] exclude the possibility of preischemic glycogen depletion being involved in the mechanism of ischemic preconditioning. These conclusions were also drawn by Asimakis [30] when he reevaluated results of an earlier study from his laboratory [3].

Our data further suggest that a high glycogen content before ischemia is also not a prerequisite for good return of functional recovery (Table 2, group PC5/10). This observation is in keeping with our earlier demonstration [9] that the preischemic glycogen level does not affect the amount of glycogen breakdown during ischemia. We therefore conclude that the glycogen content of the heart is not related to cardiac function after ischemia over a wide range of glycogen levels. A possible role of accelerated glycogen turnover [31] needs to be investigated.

Lactate and return of function

Our results strongly suggest that lactate accumulation during ischemia is not detrimental to the heart and is inconsequential for the return of postischemic contractile function. Lactate accumulation was highest in groups PC3 × 5/5 (98.8 μ/g dry, poor recovery of function) and PC5/10 (94.6 μmol/g dry, good recovery of function). It was higher in groups PC5/10, PC5/5 and PC3 × 5/5 compared to control. The increased lactate accumulation with ischemia in the hearts of most of the groups involving preconditioning was an unexpected finding since most studies on ischemic preconditioning demonstrate decreased lactate accumulation during ischemia [2–4]. Lactate accumulation has been shown to accompany intracellular acidosis [27, 32]. It is not clear whether the preconditioned groups in this study displayed more severe acidosis than the control group because lactate accumulation alone does not give rise to acidosis [33], and the intracellular pH was not measured.

With reperfusion, lactate is either oxidized [7, 11], or washed out in the perfusate. We found an inverse relationship between the postischemic net lactate release, as measured from the appearance of lactate in the perfusate during the entire reperfusion time, and the return of contractile function (Fig. 3). It may be argued that this effect is due to less lactate production during or after ischemia or due to a reduction of glycolysis during reperfusion in the hearts with a better return of function which would be consistent with the hypotheses advanced by Neely *et al.* [5] and Wolfe *et al.* [4]. This possibility, however, is unlikely for two reasons: first, lactate production during ischemia, calculated as the difference between post- and preischemic tissue lactate content, was greater in group PC5/10, which showed a good functional recovery and less lactate release during reperfusion compared to control, and secondly, glucose was the only substrate during reperfusion next to lactate. If lactate oxidation is not increased during reperfusion glycolysis is necessary to maintain energy production. Thus, our results suggest that endogenously produced lactate during ischemia is used as a fuel for respiration during reperfusion in hearts recovering full contractile function, and that increased postischemic lactate release is the result of impaired oxidative metabolism in hearts with poor recovery of function. The decreased postischemic lactate release in hearts with better postischemic function can also be explained by an increased energy demand which is met by endogenously produced lactate. Therefore, metabolism of lactate and its first product of oxidation (pyruvate) may play a crucial role for postischemic contractile function, although the exact metabolic fate of pyruvate remains to be investigated. Pyruvate dehydrogenase activation with subsequent oxidation of pyruvate [34] as well as anaplerotic pyruvate carboxylation via malic enzyme [11, 35] have both been suggested as mechanisms that improve postischemic recovery.

Other studies on the mechanism of ischemic preconditioning advanced a number of additional hypotheses. These hypotheses include the activation of signal transduction cascades by an ischemic by-product during preconditioning-reperfusion (e.g. adenosine) which may result in the opening of ion channels [36], or translocation and activation of protein kinase C [37]. The final effect of these cascades are not yet known, but it is reasonable to assume that their actions include the regulation of metabolic pathways such as glycogen breakdown/synthesis or the regulation of pyruvate dehydrogenase and malic enzyme. A full discussion of these signalling pathways is beyond the scope of the present study.

Limitations of the study

Any extrapolation from the present results to the heart *in vivo* has to take into account that our studies were performed with only one substrate (glucose) and in the absence of insulin or catecholamines. Thus, the restraining effects of competing fatty acid substrates on myocardial glucose metabolism, and the modulating effects of hormones on myocardial glucose uptake and glycogen turnover were not present.

In some groups (control, PC5/10, PC3 × 5/5), the lactate

accumulation during ischemia was greater than the stoichiometrically equal amount of glycogen breakdown. It is most likely that exogenous glucose taken up from the perfusate, which was present in the coronary circulation during ischemia, accounts for the difference. This is a plausible argument as the following calculation shows. At a glucose concentration of 10 mmol/L, an extracellular fluid space of 0.36 ml/g wet [38], and a wet/dry weight ratio of 5.5 the amount of glucose available from extracellular sources would exceed 20 μmol/g dry. However, we can only speculate about this assumption, since free glucose in the freeze-clamped hearts was not measured.

Conclusions

The phenomenon of ischemic preconditioning can be reproduced in the isolated working rat heart. While the preconditioning protocol has effected the return of function, the latter is not related to the tissue content of key metabolites of glucose metabolism (glycogen, glucose-6-phosphate, lactate, and citrate). Specifically, the recovery of function is independent from myocardial glycogen content over a wide range of glycogen levels. The ability to oxidize lactate during the reperfusion period correlates best with postischemic recovery of contractile function. The results also strongly suggest that other than metabolic mechanisms underlie the phenomenon of ischemic preconditioning.

Acknowledgements

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The liver isoform of carnitine palmitoyltransferase I is activated in neonatal rat cardiac myocytes by hypoxia

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Abstract

Fatty acids are the preferred substrate of ischemic, reperfused myocardium and may account for the decreased cardiac efficiency during aerobic recovery. Neonatal cardiac myocytes in culture respond to hypoxia/serum- and glucose-free medium by a slow decline in ATP which reverses upon oxygenation. This model was employed to examine whether carnitine palmitoyltransferase I (CPT-I) modulates high rates of β-oxidation following oxygen deprivation. After 5 h of hypoxia, ATP levels decline to 30% control values and CPT- I activity is significantly stimulated in hypoxic myocytes with no alteration in cellular carnitine content or in the release of the mitochondrial matrix marker, citrate synthase. This stimulation was attributed to an increase in the affinity of hypoxic CPT-I for carnitine, suggesting that the liver CPT-I isoform is more dominant following hypoxia. However, there was no alteration in hypoxic CPT-I inhibition by malonyl-CoA. DNP-etomoxiryl-CoA, a specific inhibitor of the liver CPT-I isoform, uncovered identical Michaelis kinetics of the *muscle* isoform in control and hypoxic myocytes with activation of the *liver* isoform. Northern blotting did not reveal any change in the relative abundance of mRNA for the liver vs. the muscle CPT-I isoforms. The tyrosine phosphatase inhibitor, pervanadate, reversed the hypoxia-induced activation of CPT-I and returned the affinity of cardiac CPT-I for carnitine to control. Reoxygenation was also associated with a return of CPT-I activity to control levels. The data demonstrate that CPT-I is activated upon ATP depletion. Lower enzyme activities are present in control and reoxygenated cells where ATP is abundant or when phosphatases are inhibited. This is the first suggestion that phosphorylation may modulate the activity of the liver CPT-I isoform in heart. (Mol Cell Biochem **180**: 163–170, 1998)

Key words: carnitine palmitoyltransferase 1, DNP-etomoxiryl-CoA, malonyl-CoA, CPT-I isoforms, hypoxia, reoxygenation, pervanadate, mRNA

Introduction

Augmentation of plasma fatty acids in the coronary circulation impairs cardiac performance. Moreover, the deleterious effects of fatty acids on cardiac contractility are even more pronounced following reperfusion of the ischemic heart. In porcine models of ischemia and reperfusion, there is a rapid return of fatty acid oxidation with a postischemic depression in energy production from glucose metabolism [1]. Agents which inhibit the first step in fatty acid oxidation (the malonyl-CoA sensitive carnitine palmitoyltransferase I, CPT-I) stimulate glucose metabolism by decreasing fatty acid oxidation, and augment mechanical recovery of the postischemic heart [2]. It has been suggested that normal control of fatty acid oxidation is changed in the postischemic heart so that the high rates of fatty acid oxidation observed are uncoupled from cardiac work [3]. Lopaschuk and coworkers have also demonstrated that β-oxidation is activated in the postischemic heart perfused with fatty acids by a decrease in the levels of malonyl-CoA relative to control levels [4]. Furthermore, the activity of CPT-I and its sensitivity to malonyl-CoA appear unchanged in mitochondria isolated from the ischemic rat heart under these conditions [4], suggesting that the primary control of cardiac

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CPT-I activity by malonyl-CoA is unaffected by ischemia and reperfusion.

Recently, evidence for two separate isoforms of CPT-I, i.e. the liver and the muscle isoforms, explains the differential inhibition of the liver and muscle enzymes by malonyl-CoA (i.e. I_{50} = 3 μM and 30 nM, respectively). We have demonstrated a low affinity component of malonyl-CoA inhibition in neonatal cardiac myocyte CPT-I (I_{50} = 0.8 μM) which is intermediate between the I_{50} for the muscle and liver isoforms [5]. It has been shown that cardiac muscle contains *both* the liver and muscle isoforms which explains the intermediate kinetic parameters measured for the cardiac enzyme [6A]. Low levels of the liver CPT-I isoform in the adult heart may allow fatty acid oxidation to continue at some basal level even though malonyl-CoA may rise to the inhibitory range for the muscle CPT-I. We examined the possibility that the continued expression of the liver isoform in cardiac muscle may also be relevant to recovery from ischemia. If liver CPT-I activity predominates during re-oxygenation, the choice of fatty acid rather than glucose as a substrate may arise from the greater affinity of the liver enzyme for its substrate, carnitine, and for this enzyme's markedly diminished sensitivity towards inhibition by malonyl-CoA. This may be particularly relevant to the neonatal heart where under normal conditions the liver isoform contributes up to 50% of the total CPT-I activity [7]. Therefore, we examined the effects of prolonged hypoxia and substrate deprivation on CPT-I activity in the neonatal cardiac myocyte and the potential mechanisms responsible for the *increases* in malonyl-CoA sensitive CPT-I activity observed following hypoxia. Subsequently, a reversal in enzyme activation occurs upon re-oxygenation, along with cellular ATP repletion. This is the first suggestion that cardiac CPT-I may be modulated *in situ* by covalent modification which reflects the phosphorylation status of the cardiac myocyte.

Materials and methods

Cell culture model for CPT assay

Neonatal rat cardiac myocytes were isolated and cultured as previously described by this laboratory [8]. The cells were washed for 10 min at 37°C with Dulbecco's modified Eagle's medium (DMEM) containing 40 mM Hepes [4-(2-hydroxyethyl)piperazine-1-(2-ethanesulfonic acid)]. The cells were permeabilized by incubation for 10 min at 37°C in 0.5 ml of medium 'J' [9] which contains 40 mM Hepes, pH 7.2, 140 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′tetraacetate (EGTA), 0.566 mM $CaCl_2$, 5 mM ATP, 6 μg/ml oligomycin and 5 μM NaN_3 to which digitonin (5 μM final concentration) was added. This concentration of digitonin provides maximal expression of malonyl-CoA-sensitive CPT-I [8]. After removal of the permeabilization medium, medium J containing 1% bovine serum albumin and either 30 μM palmitoyl-CoA or 50 μM decanoyl-CoA ± malonyl-CoA was added to a final volume of 0.5 ml. The CPT-I reaction was started by addition of either a *range* of `1-[^{14}C]carnitine concentrations from 0.04–0.4 mM (specific activity = 4000 dpm/nmole) or a single concentration as indicated. CPT-II was measured by addition of Triton X-100 (0.1%) to the cells in medium J, a condition which fully expresses CPT-II and inactivates CPT-I [8].

Inhibition of liver CPT-I with DNP-etomoxir

DNP-etomoxir, a specific inhibitor of the liver CPT-I isoform [7] was a gift of Dr. Wayne Brouillette of the Department of Chemistry at the University of Alabama at Birmingham. The myocytes were treated with this inhibitor following washing and permeabilization as follows. The permeabilized cells were incubated in a medium containing 100 mM Tris-HCl, pH 7.2, 125 mM KCl, 6.5 mM $MgCl_2$, 6 mM ATP, 0.35 mM GSH, 50 μM CoASH, 0.6% fatty acid-free BSA and 60 μM DNP-etomoxir for 30 min at 22°C to allow conversion of the DNP-etomoxir into DNP-etomoxiryl-CoA [7]. The neonatal myocytes were then washed with 0.9% NaCl and 0.4% BSA for 5 min at 37°C, and the CPT-I activity measured as previously described [10].

Effects of hypoxia on CPT-I in neonatal cardiac myocytes

To evaluate the effects of hypoxia on CPT-I activity in the neonatal rat cardiac myocytes, cells are cultured in 35 mm dishes and washed one time with glucose- and glutamine-free DMEM which had been degassed and equilibrated with 95% N_2/5% CO_2. At the end of the hypoxic incubation, the cells are permeabilized as described above and CPT is measured immediately. In separate experiments, citrate synthase is measured in digitonin-permeabilized control and hypoxic cells ± 0.1% Triton X-100 as previously described by this laboratory [10]. ATP levels in control, hypoxic and reoxygenated cells were determined using bioluminescence as described by the Sigma Technical Bulletin BSCA-1. Following the addition of luciferase, the amount of light emitted was measured immediately using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). In experiments where the cells were treated with pervanadate, 100 mM pervanadate was prepared by incubating 100 mM sodium orthovanadate with 0.34% H_2O_2 at 30°C for 15 min,

followed by 0.2 mg catalase addition for another 20 min incubation [11]. The cells cultured in 35 mm dishes were washed once with glucose- and glutamine-free DMEM which had been degassed and equilibrated with 95% N_2 and 5% CO_2. The myocytes were then incubated for 5 h at 37°C under control conditions with and without 50 μM pervanadate or in hypoxic chambers with 2 ml of the deoxygenated, substrate-free medium ± 50 μM pervanadate. At the end of the 5 h, CPT-I activity was measured as described above. Cellular protein was measured by the Lowry method [12].

RNA isolation and Northern blot analysis

RNA was isolated from the cultured neonatal rat cardiac myocytes by using the Ultraspec™ RNA isolation system (Biotex Laboratories, Houston, Texas, USA). Total RNA and RNA purity were quantitated spectrophotometrically using absorbance at 260 and 260/280 nm ratios. For Northern blotting, 10 μg of total cellular RNA was electrophoresed in 1% agarose containing 0.6% formaldehyde at 45 V for 3 h. Fractionated RNA was transferred to a Duralon-UV nylon membrane (Stratagene, La Jolla, CA, USA) for 24 h. Following prehybridization and hybridization with the labelled cDNA probes for either muscle CPT-I or for liver CPT-I [13], the blots were visualized by autoradiography following storage at –70° for 24–72 h. The relative amounts of each mRNA were determined by densitometric scanning in the linear response range. The level of each mRNA for CPT-I was corrected for the amount of 28S RNA.

Statistical treatment

All experiments were carried out in duplicate on three or more separate cultures on neonatal cardiac myocytes. The results are expressed at the mean ± S.E.M. and Student's *t*-test for paired and nonpaired variates was employed to make comparisons between two groups.

Results

Hypoxic incubation of neonatal cardiac myocytes in glucose-free DMEM for up to 5 h produces a steady decline in ATP levels from 24.4 ± 0.48 to 7.9 ± 0.43 nmoles/mg cellular protein (Fig. 1). Total cellular protein does not change from control levels even after 5 h of hypoxic incubation [14]. After the onset of reoxygenation, return of ATP is not immediate, so that ATP levels remain depressed even after 3 h of recovery (Fig. 1). However, following 18 h of reoxygenation, the ATP content of the cardiac myocytes

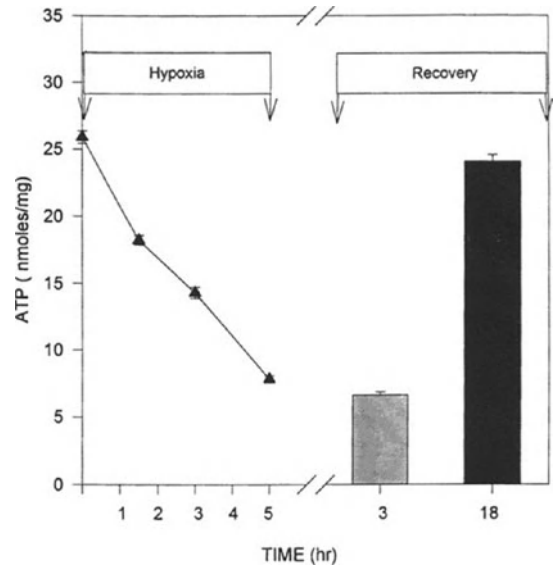

Fig. 1. Cardiac myocyte ATP levels during hypoxia and reoxygenation. ATP levels in the neonatal cardiac myocytes were measured using bioluminescence as described in Materials and methods. The time course of ATP depletion is indicated by ▲, where n = 5 different cultures ± S.E.M. (within symbol). Reoxygenation data are represented as histograms where □ = 3 h of reoxygenation (n = 3 different cultures ± S.E.M.) and ■ = 18 h of reoxygenation (n = 3 different cultures ± S.E.M.

has returned to levels which are not significantly different from control (Fig. 1).

Accurate separation of CPT-I from CPT-II activity is assessed by (1) the malonyl-CoA sensitivity of the activity measured, with CPT-I being almost completely inhibited by 100 μM malonyl-CoA, and (2) minimization of the expression of mitochondrial CPT-II as judged by the degree of latency of the mitochondrial matrix enzyme, citrate synthase. This issue is particularly important when hypoxia is employed as a metabolic perturbation since membrane disruption is often characteristic of ischemic myocytes in contracture [15]. The CPT-I activity presented in Table 1 for both the control and hypoxic enzymes is inhibited 95% by 100 μM malonyl-CoA [control: 5.3 ± 0.06 nmoles/20 min/mg (–malonyl-CoA) vs. 0.241 ± 0.02 (+malonyl-CoA); hypoxic: 7.1 ± 0.12 nmoles/20 min/ mg (–malonyl-CoA) vs. 0.334 ± 0.02 (+malonyl-CoA)]. CPT-II activity under the same conditions is completely *insensitive* to this concentration of malonyl-CoA [10]. The absence of any significant expression of citrate synthase in the digitonin-permeabilized control and hypoxic myocytes confirms the comparable latency of the mitochondrial matrix compartments, even after 5 h of hypoxia (Table 1). Maintenance of

myocyte carnitine levels in the cytosol during the control and hypoxic incubations (in the absence of carnitine addition to the DMEM medium) attests to the preservation of sarcolemmal integrity, as does the complete return of cellular ATP (Table 1 and Fig. 1).

To investigate the mechanism by which CPT-I activity is increased in the hypoxic myocytes, it seemed possible that either more CPT protein was present (an unlikely possibility since mitochondrial protein synthesis is depressed during anoxia [16]) or that existing CPT-I was activated. A double reciprocal plot of CPT-I activity versus palmitoyl- or decanoyl-CoA, revealed no change in the affinity of the enzyme for its acyl-CoA substrate (data not shown). However, when carnitine was varied as substrate, CPT-I now demonstrated an *increase* in its affinity for carnitine from a K_m of 0.25 mM under control conditions to a K_m of 0.14 mM after 5 h of hypoxia (Fig. 2). Since the affinity of the liver CPT-I is 0.03 μM compared to 0.5 mM for the muscle isoform [7], these data suggested that activity of the liver isoform had become more prominent in the hypoxic myocytes. For this to occur, the hypoxic cells could be more enriched in the *content* of the liver isoform compared to the muscle isoform of CPT-I, e.g. if the muscle isoform is more

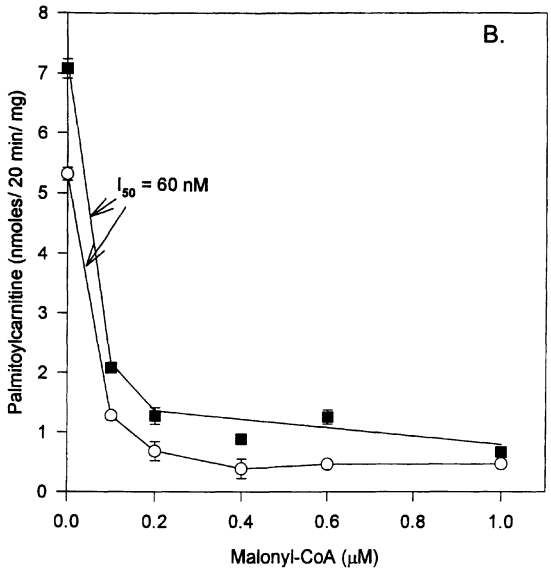

Fig. 2. Carnitine palmitoyltransferase kinetics in control and hypoxic neonatal cardiac myocytes. Double reciprocal plot analysis was carried out on CPT-I activity measured after 5 h incubation under control, oxygenated conditions (●) vs. 5 h incubation under hypoxic conditions (▲). The results were fitted to the best line using linear regression analysis where $r \geq 0.99$ and the data represent the average of 6 different cultures (control) and 3 different cultures (hypoxic) ± S.E.M. Some of the error bars lie within the symbols.

sensitive to degradation or inactivation. This expectation should be reflected by a *decrease* in the relative affinity of the total hypoxic isoform population for malonyl-CoA. Under conditions of low carnitine, expression of the liver isoform is favored (87% of the liver CPT-I V_{max}; 29% of the muscle V_{max} at 0.2 mM carnitine); at higher carnitine concentrations (0.8 mM), the muscle isoform becomes more active (96% of the liver isoform V_{max}; 62% of the muscle V_{max}). However, the I_{50} values for malonyl-CoA are not significantly different between control and hypoxic conditions (Figs 3A and 3B). This finding suggests that the proportion of liver compared to muscle CPT-I in the neonatal cardiac myocytes is unchanged by hypoxia and that, instead, the *activity* of the enzyme(s) is enhanced resulting from a change in its affinity for carnitine.

To investigate the latter possibility, the inhibitor, DNP-etomoxir, which is specific for the liver isoform of CPT-I with little or no effect on the muscle isoform [6B], was used to determine which enzyme protein was modified by hypoxia. Under conditions of control and hypoxic incubation, the total activity of cardiac myocyte is increased by 5 h of hypoxia while the levels of activity of the muscle isoform (i.e. activity measured in the presence of DNP-etomoxir) are unchanged (Fig. 4). The efficacy of the inhibition is shown in the inset to Fig. 4 where hypoxia has no effect on the muscle enzyme over a range of carnitine concentrations. The K_m for the muscle isoform (inset) was calculated to be 0.53 mM, a value which is consistent with the K_m previously reported for muscle CPT-I, i.e. 0.5 mM [7]. Therefore, the increased activity of CPT-I in hypoxia could be entirely attributed to a change in liver CPT-I activity (Fig. 4).

Since it has been reported that mitochondrial stress stabilizes nuclear encoded mRNA transcripts [17], it was possible that hypoxia could trigger a switch in the relative abundance of CPT-I mRNA which would affect mitochondrial isoform expression upon reoxygenation of the hypoxic cells. Northern blotting was carried out on RNA isolated from control and hypoxic myocytes and the mRNA content of the liver and muscle isoforms was compared (Fig. 5). When muscle and liver CPT-I message abundance was expressed relative to the 28S RNA content of control and hypoxic myocytes, no significant change was observed between the mRNA abundance of the two isoforms (Fig. 5). Therefore, it appears that the pattern of isoform expression is unaltered by hypoxia either at the transcriptional level or by changes in the relative rates of mRNA turnover.

The pathway by which liver CPT-I is activated by hypoxia should reflect a transient physiological response to a change in the cellular environment. A predominant mechanism of covalent modification of cellular proteins is that of phosphorylation. Prior studies have suggested that CPT-II may be affected by phosphorylation events in hepatocytes in culture [18]. In hypoxic cardiac myocytes, our laboratory

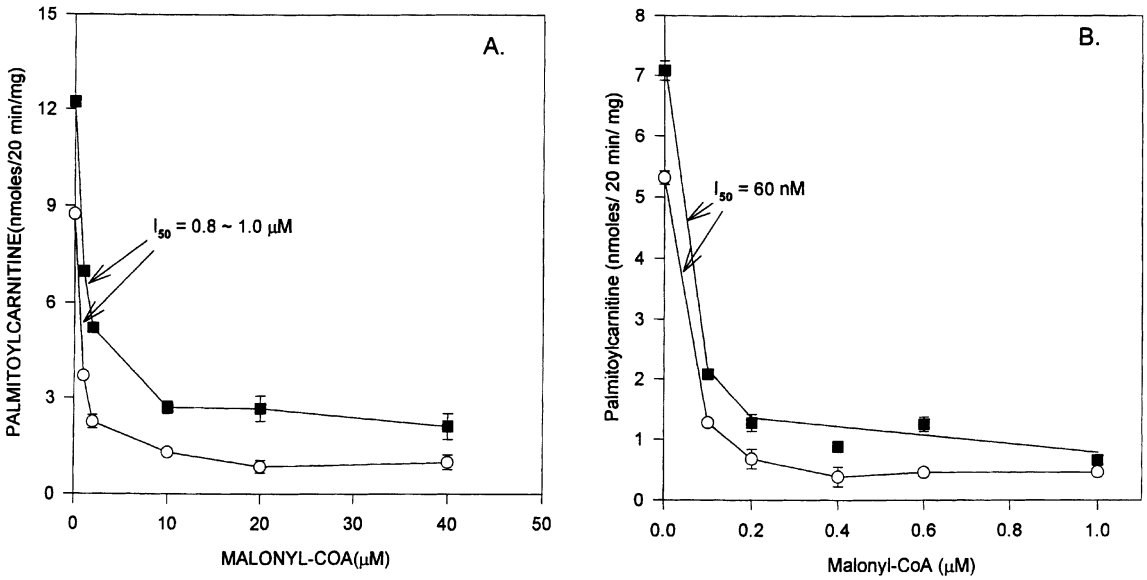

Fig. 3. Malonyl-CoA inhibition of carnitine palmitoyltransferase-I in control and hypoxic neonatal cardiac myocytes. (A) CPT-I activity was measured in the absence and presence of malonyl-CoA concentrations ranging from 1–40 μM in the presence of 30 μM palmitoyl-CoA and 0.8 mM l-carnitine, where ○ = CPT-I activity from control, normoxic myocytes (n = 3 different cultures, mean ± S.E.M.) and ■ = CPT-I activity from myocytes after 5 h of hypoxia (n = 3 different cultures, mean S.E.M.). The I_{50} is the concentration of malonyl-CoA which inhibits CPT-I activity by 50%. (B) CPT-I activity was measured in the absence and presence of malonyl-CoA concentrations ranging from 1–40 μM in the presence of 30 μM palmitoyl-CoA and 0.2 mM l-carnitine, where ○ = CPT-I activity from control, normoxic myocytes (n = 3 different cultures, mean ± S.E.M.) and ■ = CPT-I activity from myocytes after 5 h of hypoxia (n = 3 different cultures, mean ± S.E.M.). The I_{50} is the concentration of malonyl-CoA which inhibits CPT-I activity by 50%.

[14] has previously reported a pattern of diminished phosphorylation of acetyl-CoA carboxylase, a protein involved in fatty acid metabolism which is a known substrate for AMP kinase [4]. Therefore, it seemed possible that hypoxia was activating phosphatases which dephosphorylate cellular protein substrates. The specific and potent phosphatase inhibitor, pervanadate, is known to target phosphotyrosine phosphatase [19]. When incubated with control cardiac myocytes for 5 h, 50 μM pervanadate had no effect on the level of total CPT-I activity (Fig. 6). However, when CPT-I was activated by 5 h of hypoxia, pervanadate, present throughout the hypoxic incubation, completely abolished this activation, returning CPT-I activity to control levels (Fig. 6) and restoring the shift in K_m for carnitine to the value measured in control cells (Fig. 6, inset). Moreover, 18 h of reoxygenation not only returned ATP to control levels, but also *decreased* the activity of CPT-I from 20.28 ± 0.62 to 11.18 ± 0.52 nmoles/20 min/mg (control CPT-I = 11.02 ± 0.39 nmol/20 min/mg). The data are consistent with a role for the phosphorylation status of the neonatal cardiac myocyte in the suppression of the activity of the liver CPT-I isoform, possibly by phosphorylation of a tyrosine at or near the catalytic domaine.

Discussion

The present data address the interesting demonstration that CPT-I activity in cardiac muscle is composed of two isoforms, the liver and the muscle proteins, which are expressed as two different gene products [20]. The importance of the liver isoform to cardiac metabolism is speculative but it has been reasonably assumed that the lower affinity of the liver CPT-I for the inhibitor malonyl-CoA (vs. the low I_{50} of the muscle CPT-I) allows β-oxidation to continue at some low rate in the face of high prevailing malonyl-CoA concentrations. The activation of cardiac CPT-I by hypoxia also supports the general observation of high rates of β-oxidation in adult heart during early periods of ischemic reflow [2, 3]. However, during reperfusion of adult heart following ischemia, no increases in CPT-I activity or in the I_{50} of the enzyme for malonyl-CoA were observed [4]. The differences between these studies and our data may relate to the adult rat heart model vs the neonatal rat cardiac myocytes in culture. It is known that the liver isoform constitutes only 3% of the total CPT-I protein in the adult vs. 25% in the neonatal rat heart [7]. It is noteworthy that in the neonatal heart, where carnitine concentrations are low,

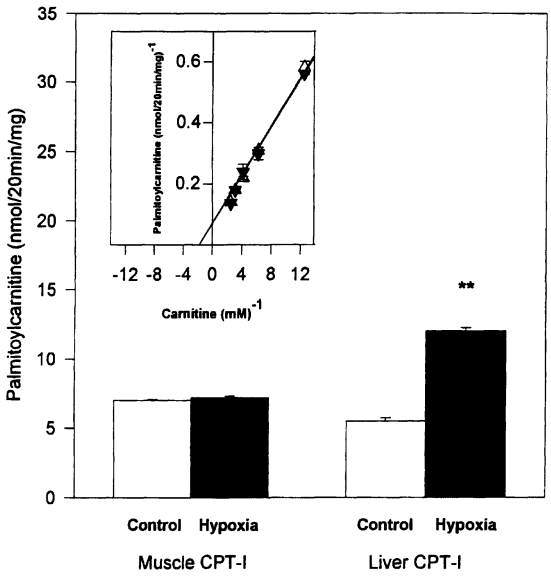

Fig. 4. Hypoxia increases activity of the liver but not the muscle isoform of CPT-I. Cardiac myocytes were incubated under control, normoxic and hypoxic conditions for 5 h as described under Materials and methods. After permeabilization, DNP etomoxir was converted to DNP-etomoxiryl-CoA (Brown *et al.* 1995), and CPT-I activity was measured in the absence (□, *total* CPT-I, n = 4 ± S.E.M.) and the presence (■, *muscle* CPT-I, n = 4 ± S.E.M.) of etomoxiryl-CoA. The activity of the liver isoform was obtained by subtracting the activity of the muscle isoform from the total activity ($p < 0.01$). Inset: Double reciprocal plot of the cardiac myocyte CPT-I measured in the presence of DNP-etomoxiryl-CoA at varying l-carnitine concentrations (▼, n = 3 different cultures ± S.E.M.).

liver CPT-I *activity* accounts for up to 60% of the total fatty acid oxidation in the newborn heart [7]. Thus it is likely that the decreased contribution of the liver CPT-I isoform to total CPT-I activity in adult heart may make changes in this isoform difficult to detect, particularly without separation of the relative activities contributing to the total activity measured. Our results agree with the former studies in that there is no apparent change in the inhibitory sensitivity of the heart CPT-I for malonyl-CoA [4]. The different I_{50} values observed in the presence of 0.2 mM vs. 0.8 mM carnitine agree with other conclusions that carnitine may decrease the effectiveness of malonyl-CoA to regulate CPT-I [21]. Since the low carnitine concentration is less favorable for activation of the muscle CPT-I which demonstrates a low K_I for malonyl-CoA, the direction of the K_I shift is unexpected. This observation is of interest with respect to the high concentrations of malonyl-CoA measured in heart muscle vs. the low I_{50} for malonyl-CoA of the predominating muscle CPT-I isoform. It is conceivable that, at the carnitine levels in adult heart which range from 1.5–2.5 mM [22], malonyl-CoA is a far less effective inhibitor of the muscle CPT-I than would be anticipated from the I_{50} measurements.

The suggestion that the liver isoform is responsive to hypoxia in the heart cell and that the muscle isoform is unaffected is consistent with the known plasticity of the liver CPT-I both in its activity and in the modulatory effects of diet and diabetes on its sensitivity to malonyl-CoA [23]. Harano *et al.* [18] have suggested that CPT may be regulated in the short term by isolated rat hepatocytes through a phosphorylation-dephosphorylation mechanism. However, in the latter study the immunoprecipitated CPT likely represented either CPT-II or a mixture of the entire CPT complex so that a possible role for covalent modification of CPT-I has not been firmly established. We observed a small (11%) change in CPT-II activity with hypoxia which may reflect these earlier observations [18]. Guzmán and Geelan [24] have reported modulation of CPT-I activity and fatty acid oxidation by a variety of agonists and growth factors using digitonin-permeabilized hepatocytes. These authors suggested that enhanced phosphorylation of CPT-I may lead to a *stimulation* of CPT-I activity and a resultant activation of β-oxidation [25]. Okadaic acid, an inhibitor

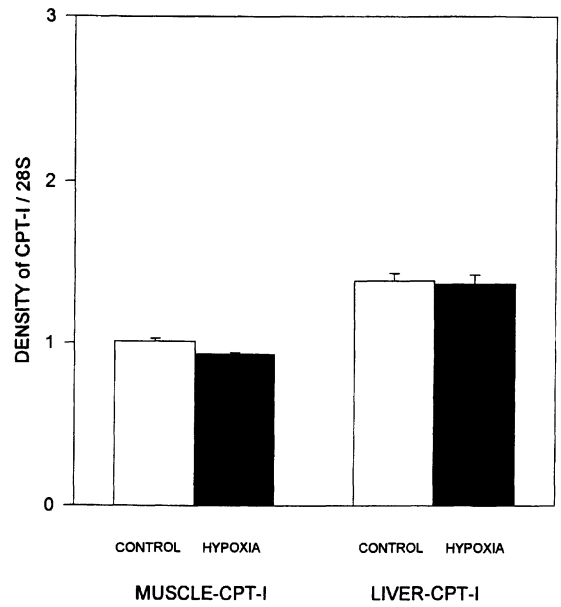

Fig. 5. Northern Blotting of CPT-I isoform mRNA after control and hypoxic incubation. RNA was isolated from control and hypoxic (5 h) neonatal cardiac myocytes in culture. For Northern blotting, the fractionated RNA was hybridized to cDNA probes for the liver and muscle isoforms of CPT-I, and the relative amounts of each mRNA were visualized by densitometric scanning. Control RNA (□) and RNA from hypoxic myocytes (■) were corrected for the amount of 28S ribosomal RNA in the extracts, where n = 3 determinations.

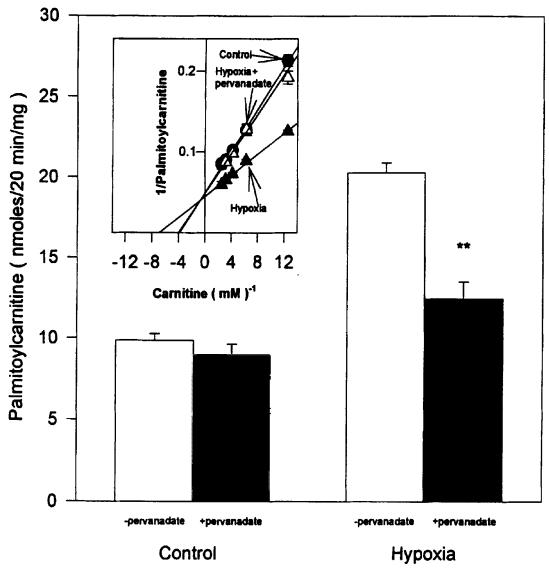

Fig. 6. Pervanadate inhibits the hypoxia-induced increase in CPT-I by inhibiting the liver isoform of CPT-I. Pervanadate was present (60 μM) during 5 h of normoxic and hypoxic incubation. Following permeabilization of the cardiac myocytes, CPT-I activity was measured in the control and hypoxic cells which had been incubated in the absence (□, n = 3 different cultures ± S.E.M.) and presence (■, n = 3 ± S.E.M.) of pervanadate ($p < 0.01$). Inset: The double reciprocal plot of total CPT-I activity measured in control cells (●, n = 6 different cultures ± S.E.M.), in control cells in the presence of pervanadate (○, n =3 different cultures ± S.E.M.) and in hypoxic cells in the absence of pervanadate (▲, n = 3 ± S.E.M.).

of serine/threonine phosphatases [26], was representative of the maximal stimulatory effect of a variety of agonists. If multiple phosphorylation sites are present on the liver isoform of CPT-I, as has been revealed for the hepatic acetyl-CoA carboxylase [27], a complex pattern of regulation may account for the action of various kinases on this enzyme, including inhibition of catalytic activity by phosphotyrosine. Inhibitory effects of phosphotyrosine on src kinase activity is one example of multiple modulatory effects of phosphorylation/ dephosphorylation events [28]. Little is known concerning phosphatase induction during ischemia although specific dephosphorylation events have been observed or have been predicted [14, 29, 30]. During transient ischemia in the brain, induction of CL100 protein tyrosine phosphatase mRNA and protein levels takes place with suggested implications in the onset of neuronal damage [31]. The role of protein phosphatases in hypoxic/ ischemic heart is a potentially important area in the maintainance and recovery of cardiac myocyte shape, function and metabolism. While it is possible that pervanadate may have secondary effects unrelated to phosphorylation which alter the kinetics of CPT-I, we have obtained preliminary data which demonstrates that following immunoprecipitation of neonatal myocyte protein using antiphosphotyrosine antibodies, a band at 88 kD is recovered which is immunoreactive against an internal recombinant peptide for amino acids 316–430 specific for the liver CPTo (CPT-I, courtesy of Dr. Shri Pande, Montreal Canada). This recent information supports our suggestion that the kinetic data reflect a role for phosphorylation in the control of activity of the liver isoform of CPT-I in cardiac myocytes.

In summary, a dramatic activation of CPT-I is observed in hypoxic myocytes which is independent of any change in the content of either the mRNA for the muscle or liver isoform or in the activity of the muscle-specific CPT-I. Our results suggest that the activity of the hypoxic myocyte CPT-I reflects changes in ATP levels in the cardiac myocytes, so that high ATP is associated with lower CPT-I activity. The site of this activation is the liver isoform of CPT-I where phosphorylation-dependent modulation of CPT activity appears to play a role in this process.

Acknowledgements

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Human myocardial ATP content and *in vivo* contractile function

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Abstract

The study was designed to characterize the relationship between the metabolite content of human cardiac muscle and *in vivo* cardiac function. ATP, total adenine nucleotides, and NAD were quantified in human myocardial biopsies using high performance liquid chromatography. Right ventricular endomyocardial biopsies were obtained from 43 patients with dilated cardiomyopathy, 6 with restrictive cardiomyopathy, 10 with normal systolic and diastolic function, and from 24 cold preserved human donor hearts. Transmural samples of failing right and left ventricular free walls were obtained during cardiac transplantation surgery in 8 patients. ATP, total adenine nucleotides, and NAD were similar in the cold-preserved donor hearts and in right ventricular endomyocardial biopsies from the 10 individuals with normal systolic and diastolic function. In contrast, these values were significantly depressed in tissue samples from patients with dilated or restrictive cardiomyopathy. There was a significant correlation between ATP and pulmonary capillary wedge pressures but not ejection fractions. Declines in the sizes of myocardial ATP, adenine nucleotide, and pyridine nucleotide pools in the human myocardium are associated primarily with diastolic but not systolic dysfunction. (Mol Cell Biochem **180**: 171–177, 1998)

Key words: ATP, NAD, adenine nucleotides, dilated cardiomyopathy, endomyocardial biopsy, diastolic dysfunction

Introduction

There has long been speculation that altered energy metabolism plays a role in human non-ischemic dilated cardiomyopathy ([4, 16, 17] for reviews). Primary metabolic defects, such as those associated with abnormal carnitine transport [37], are well known for their ability to produce potentially reversible cardiac failure. In post-viral dilated cardiomyopathy, circulating antibodies against the mitochondrial adenine nucleotide translocase [25] are thought to interfere with energy metabolism [24, 26]. With other forms of hypertrophy and failure, altered hemodynamics may lead to chronic subendocardial ischemia in the presence or absence of coronary artery disease [15, 30, 35].

Nevertheless, there are conflicting data on the energy status of the failing human myocardium. Some, but not all, *in vivo* ^{31}P-NMR studies have show declines in phosphocreatine (PCr)/ATP ratios (reviewed in [4]). Such declines may be unrelated to the declines in PCr/ATP typically seen in myocardial ischemia, however, as total creatine appears to be decreased in the failing myocardium 14, 31]. The status of the ATP and total adenine nucleotide pool is also controversial. Early attempts to demonstrate ATP depletion in surgical specimens produced mixed results [5, 15]. Conflicting data have also been obtained with endomyocardial biopsies of awake subjects. An early study from our laboratory showed significant declines in the ATP content of right ventricular endomyocardial biopsies from failing human myocardium [35] with a significant correlation between ATP depletion and diastolic dysfunction. However, a very similar study by another laboratory found no differences between the metabolite content of biopsies from normal and failing human hearts [4].

The present study reinvestigates the issue of the ATP

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content of human myocardium. Sample sizes are larger than in previous studies; a subset of patients with pure diastolic dysfunction has been included; two sources of normal myocardial tissue were used; and samples were analyzed by high performance liquid chromatography (HPLC) so that ATP breakdown products and NAD could be quantified.

Materials and methods

Myocardial biopsies

Tissue samples were obtained from: (1) patients undergoing diagnostic right ventricular endomyocardial biopsy; (2) cold preserved donor hearts just before implantation; and (3) end-stage failing ventricles of patients undergoing cardiac transplantation surgery. Studies of transplant recipients were confined to those without significant coronary artery disease.

All patients undergoing right ventricular endomyocardial biopsy were being evaluated for unexplained systolic and/or diastolic ventricular dysfunction, symptoms of congestive heart failure, unexplained angina, or to obtain control data before chemotherapy as part of a protocol to assess anthracycline cardiotoxicity. Informed consent was obtained in each instance in compliance with institutional review policies for human subject research. Significant coronary artery disease (luminal stenosis $\geq 50\%$), valvular heart disease and pericardial disease were excluded in all patients with diagnostic left and right heart catheterization and coronary angiography. Right heart catheterization and right ventricular endomyocardial biopsy [32] were performed using standard techniques (right internal jugular approach). Tissue samples were frozen in liquid nitrogen less than 15 sec after closing of the bioptome jaws and stored at –80°C until analyzed.

Individuals with normal systolic and diastolic function

Ten patients had normal left ventricular ejection fractions (≥ 0.55), and normal left ventricular end diastolic pressures (≤ 13 mm Hg).

Dilated cardiomyopathy

Forty-three patients were diagnosed as having dilated cardiomyopathy. Mean left ventricular ejection fraction (± S.D.) was 0.28 ± 0.11, pulmonary artery wedge pressure was 18 ± 9 mm Hg, and cardiac index was 2.7 ± 0.7 L/min/m^2. There were 29 men and 14 women in this group: mean age was 43 years (range 26–74 years).

Restrictive cardiomyopathy

Six patients had restrictive cardiomyopathy. Mean left ventricular ejection fraction was 0.53 ± 0.05 and pulmonary artery wedge pressure was 24 ± 7 mm Hg. There were 5 women and 1 man: mean age was 60 years (range 28–77 years).

Cold-preserved donor hearts

The cold-preserved donor hearts were those described previously in [28]. At the time of harvest, all hearts were cardiopleged via the aortic stump with a chilled solution containing 55 mM glucose, 1.1 mM mannitol, 22.4 mM sodium bicarbonate and 30 mM KCl and stored in an ice bath. Immediately prior to implantation, samples were taken from the right interventricular septal surface using a cardiac bioptome and immediately frozen in liquid nitrogen. All samples were obtained within 5 h of harvest.

End stage failure

Open chest samples of the left and right ventricular free wall ('tru-cut' needle) were obtained during cardiac transplantation surgery in 8 patients with end-stage non-ischemic heart failure. The specimens were immediately frozen in liquid nitrogen, and samples of the LV free wall were quickly divided into epicardial, midwall and endocardial sections.

Nucleotide assays

Analysis of acid-soluble nucleotides was carried out as previously described [11]. In brief, frozen tissue samples were homogenized with perchloric acid using a Biomedix microhomogenizer system (Biomedix, Middlesex, UK). The neutralized acid extract was analyzed by HPLC and the denatured protein pellet was acetone-washed, dissolved in 1 N NaOH, and analyzed for non-collagen protein by the method of Lowry *et al.* [19] using bovine serum albumin as the standard. Nucleotide data are given in nanomoles/milligram total protein (nmol/mg protein), means ± S.E.M.

Statistical analyses

Measurements on right ventricular endomyocardial biopsies from awake subjects were separate experiments from those on the explanted (donor or recipient) hearts. The two data sets were therefore analyzed separately. Scheffe's procedure and the STATPAK PC program (Merrill Dow Publishing Company, Columbus, Ohio, USA) were used for both sets

of multiple comparisons (ANOVA). Differences were considered statistically significant at a p value < 0.05.

Results

Table 1 summarizes the ATP, NAD and total adenine nucleotide content of biopsies from the several groups of hearts. Note that the values for endomyocardial biopsies taken from patients with normal systolic and diastolic function and from the chilled, cardiopleged hearts of normal organ donors do not differ. These values are quite in line with what we and others have reported for rapidly aspirated, snap-frozen transmural biopsies of normal swine and canine hearts [8, 20]. They also agree with ^{31}P-NMR data giving a normal *in vivo* human myocardial ATP concentration of ~7 μmol per g wet weight [4], assuming a conversion factor of 0.16 mg Lowry protein per mg wet weight, and with the early data of Jones *et al.* on the metabolite content of normal human myocardium [15]. These values are higher, however, than those reported by Chidsey *et al.* [5] and Regitz and Fleck [23] for normal human myocardium.

Second, ATP and total adenine nucleotides are significantly lower in the endomyocardial biopsies from patients with dilated or restrictive cardiomyopathy ($p < 0.001$). Third, mean ATP and total adenine nucleotide values for transmural biopsies of hearts in end stage failure are low but show no statistically significant transmural gradients. NAD values paralleled those for the adenylates. NAD was similar for the endomyocardial biopsies from individuals with normal systolic and diastolic function and from normal donor hearts whereas the NAD content of endomyocardial biopsies from cardiomyopathic hearts was significantly depressed.

There is a highly significant correlation between total adenine nucleotides and the NAD content of myocardial biopsies (Fig. 1). It should be noted that in cardiac muscle, values for NAD are a good measure of the total intracellular pyridine nucleotide pool. The phosphorylated form of NAD, NADP, is present in relatively low concentrations, and levels of reduced pyridine nucleotides (i.e. NADH and NADPH) are low as well. We have not given values for NADH because reduced pyridine nucleotides are unstable in the presence of acid and are thus destroyed by our extraction process. Nevertheless, the acid-degradation products of NADH are resolved by HPLC, and their sum can be used to estimate the amount of NADH originally present [10]. In 62 individual biopsies where NADH was quantified by this procedure, the NAD-to-NADH ratios were 7.6 ± 3.3 (mean ± standard deviation) and there was no discernible correlation between this ratio and ATP or total adenine nucleotide content.

When data from all endomyocardial biopsy subjects were analyzed, there was a significant negative correlation between pulmonary capillary wedge pressure and ATP (Fig. 2) but there was no correlation between ejection fraction and ATP ($r = 0.009$, data not shown).

Table 1. Metabolite data for human myocardial biopsies

	ATP	EAN	NAD
RV EBX			
normal EBX (n = 10)	40.1 ± 1.9	52.2 ± 2.8	6.40 ± 0.40
normal donor (n = 24)	38.2 ± 2.2	47.4 ± 2.5	5.89 ± 0.20
DCM-EBX (n = 43)	24.4 ± 1.3*	34.7 ± 1.5*	4.54 ± 0.17*
RCM-EBX (n = 6)	12.0 ± 3.3*	19.2 ± 4.8*	2.46 ± 0.69*
	* $p < 0.001$ vs. normals		
Transmural (n = 8)			
RV	18.8 ± 3.0	27.1 ± 3.6	3.28 ± 0.31
LV epi	14.4 ± 3.6	24.7 ± 5.1	3.71 ± 0.59
mid	17.4 ± 3.6	28.4 ± 4.1	3.51 ± 0.61
endo	17.9 ± 4.3	28.0 ± 5.9	3.46 ± 0.71

All data are mean ± S.E.M., nmol/mg biopsy protein. Normal EBX, endomyocardial biopsies from individuals with normal systolic and diastolic function. DCM-EBX, endomyocardial biopsies from individuals with dilated cardiomyopathy. RCM-EBX, endomyocardial biopsies from individuals with restrictive cardiomyopathies.

Discussion

Methodological considerations

It is clear from Table 1 that samples of ventricular myocardium from patients with dilated or restrictive cardiomyopathy have, on average, less than normal amounts of ATP, total adenine nucleotides, and NAD. One could argue that extensive fibrosis might be responsible for the apparent net loss of tissue metabolites and that nucleotide concentrations in viable myocytes are unaltered. However, nucleotide values have been normalized to total NaOH-soluble protein, as analyzed by the method of Lowry *et al.* [19]. Connective tissue is only sparingly soluble in NaOH and the Lowry method is generally assumed to measure non-collagen protein. Moreover, a previous quantitative study of human hearts with end-stage dilated cardiomyopathy found only 15 ± 6% fibrosis in endocardial samples from the right side of the interventricular septum [34]; in the present study, the adenine and pyridine nucleotide pools were depressed in most instances by more than 30%.

Another concern could be the delay between tissue sampling and metabolic arrest in liquid nitrogen, which averaged ~10 sec. This might be expected to alter phosphocreatine and possibly the ATP/ADP ratios. However, ATP/ADP ratios for the samples of normal myocardium were well

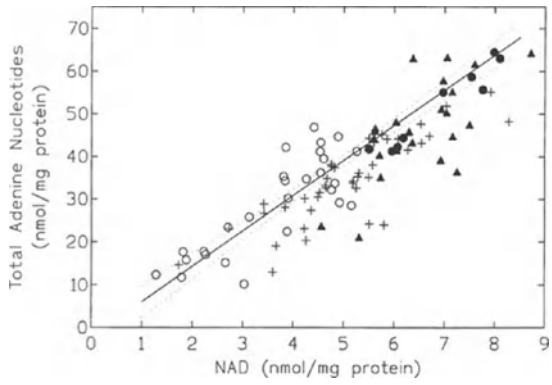

Fig. 1. Graph showing individual NAD and total adenine nucleotide values for tissue samples described in Table 1. Open circles depict data for transmural biopsies from transplant recipients; closed triangles represent samples from cold-preserved normal donor hearts; closed circles are for right ventricular biopsies from normal controls; and the plusses are for right ventricular endomyocardial biopsies from patients with dilated cardiomyopathy. The 95% confidence limits for the regression line are shown as dotted curves. The correlation coefficient was 0.874, $p < 0.0001$.

within the range typically described in carefully-done studies of experimental animal hearts. Thus, any significant decline in high energy phosphates most likely affected just the phosphocreatine pool, which has a strong buffering effect on ATP/ADP ratios. Nevertheless, it must be borne in mind that in the setting of low *in vivo* phosphocreatine, as might be seen in failing myocardium [6], more appreciable declines in ATP/ADP ratios might have occurred during sampling. Thus we have also emphasized the size of the total adenine nucleotide pool. Since each biopsy is a closed system, there can be no change in total adenine nucleotides

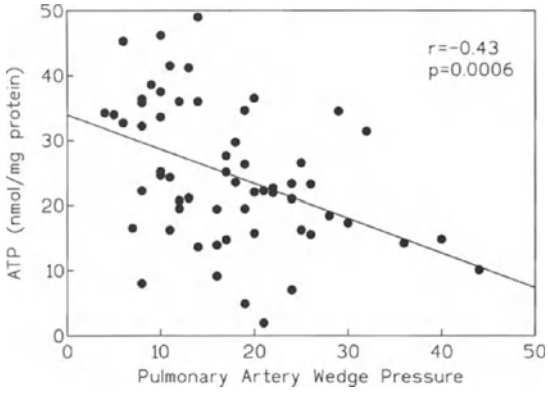

Fig. 2. Graph showing the correlation between pulmonary artery wedge pressure and ATP content of endomyocardial biopsies.

plus their degradation products. The latter are easily identified and quantified in the HPLC chromatograms [11]. In fact, very few biopsies contained more than 2 nmol/mg AMP, adenosine, inosine, or hypoxanthine.

It should be noted that a recent study by Regitz and Fleck [23] failed to detect differences in ATP or total adenine nucleotide concentrations in endomyocardial biopsies from 'normal' and cardiomyopathy patients. It is interesting that their mean values for ATP (23 nmol/mg) and total adenylates (39 nmol/mg) in samples from patients with dilated cardiomyopathy were nearly identical to those shown by us in Table 1. The major difference between the two studies involves the normal values. In the study by Regitz and Fleck, 'normal' adenine nucleotide profiles were obtained in 14 biopsies from patients in whom cardiac disease was suspected, but coronary, valvular, hypertensive, or myocardial disease was excluded. Our study obtained data from cold-preserved normal donor hearts as well as from cancer patients with normal ejection fractions and left ventricular end diastolic pressures.

Correlation between ATP and cardiac performance

In dilated cardiomyopathy, both systolic and diastolic function may be impaired. For the cardiomyopathy study group, mean ventricular ejection fractions were low while end diastolic pressures were high. Whether depressed ejection fractions or elevated end diastolic pressures can be attributed to a paucity of ATP is not entirely clear, however. With respect to ejection fractions, there can be a significant positive correlation with myocardial ATP [2], but this is not always the case [29]. Clearly, if the failing heart were inadequately perfused, one might envision a situation comparable to myocardial hibernation, where contractile function is somehow down-regulated to match substrate and oxygen delivery [21, 22]. In the hibernating myocardium, however, total high energy phosphates can be close to normal [9]. On the other hand, failing ventricles could resemble the stunned myocardium, but while the post-ischemic stunned myocardium has low ATP, its contractile deficit appears attributable to reperfusion-generated free radical damage to the myofibrils and/or sarcoplasmic reticulum [3, 7]. Myocardial hibernation and/or stunning are probably not the cause of low ejection fractions in patients with truly non-ischemic cardiomyopathies. We specifically excluded patients that had coronary artery disease with coronary angiography. Accordingly, we would argue that low ATP and a low ejection fraction need not be causally related, and a positive correlation between the two could merely reflect the fact that ATP and ejection fraction are both lower in failing than non-failing hearts.

The data from patients with primary diastolic dysfunction lend support to the above argument [29] As a group, these

individuals had normal ejection fractions but lower mean ATP, total adenine nucleotides, and NAD in their right ventricular endomyocardial biopsies than even those individuals with markedly depressed ejection fractions. A number of studies have shown good correlations between myocardial ATP and end diastolic pressures [29–33], and it has been argued on theoretical grounds that relaxation should be more sensitive than contraction to energy status [16]. ATP has a relaxing effect on cardiac myofibrils, and studies with permeabilized rat myocytes have shown that varying MgATP over the 1–10 mM range (estimates of normal cytosolic MgATP range from 5–10 mM) greatly alters myofibrillar calcium sensitivity. That is, at each controlled level of free calcium, when the concentration of MgATP is decreased there is an increase in unloaded myofibriliar shortening [1]. Calcium sequestration by the sarcoplasmic reticulum is also quite sensitive to ATP and to ATP-to-ADP ratios, and these nucleotide effects are readily observed in permeabilized myocytes with physiologically relevant concentrations of ATP [12, 38]. Thus, the rate at which the sarcoplasmic reticulum removes calcium from the myofibrillar space during diastole should decline when ATP falls and/or ADP rises. This along with an increased myofibrillar calcium sensitivity should lead to decreased diastolic compliance.

Regulation of intracellular nucleotide content

A great deal of what is known about myocardial nucleotide metabolism has been derived from studies of acute ischemia or hypoxia. With a major reduction in oxygen delivery, ATP falls and ADP and AMP increase. AMP is then degraded to inosine monophosphate or adenosine, both of which can be converted to inosine and hypoxanthine. Adenosine, inosine, and hypoxanthine are all able to diffuse across the sarcolemma into the extracellular space and their efflux leads to a net reduction in the size of the intracellular purine pool. Thus, when oxygen delivery is restored, ATP cannot rebound fully and immediately to preischemic levels. Over time, however, adenosine and hypoxanthine can be taken up by the myocytes and used to replenish intracellular adenine nucleotides.

In the absence of ischemia, there is a far more gradual but continuous degradation of adenine nucleotides, which is closely matched by nucleotide synthesis through the *de novo* and salvage pathways. The steady state pool size – or set point – is thus governed by the balance between these opposing reactions. In dilated cardiomyopathy, this balance may be altered, resulting in a decline in total adenine nucleotides. However, it is becoming increasingly apparent that the failing myocardium can be quite heterogeneous, and it could be that some myocytes contain relatively normal amounts of adenine nucleotides whereas others have degenerated to the point where they are severely depleted.

NAD and total pyridine nucleotides

As mentioned above, transient episodes of myocardial ischemia could explain the low ATP and adenine nucleotide content of some failing human hearts. Post-ischemic recovery of ATP and total adenine nucleotides is a slow process [8], one which might not keep pace with intermittent ischemic episodes. An ischemic basis for the loss of NAD seems less likely, however. Mild ischemic insults have not typically been associated with a pronounced loss of total pyridine nucleotides. And while the pyridine nucleotides are known to decline following experimental coronary artery occlusion, this process occurs on a much slower time scale and is thought to result primarily from inhibition of energy-dependent NAD synthesis in the face of continuous degradation via NAD glycohydrolase activity [18].

Interestingly, experimental cardiac hypertrophy produced by excess epinephrine [27] or thyroxine [13] also leads to a significant decline in myocardial NAD, when the data are expressed in terms of tissue mass. If the data were expressed per myocyte, however, NAD might not be depressed. It may therefore be quite relevant that a critical step in the synthesis of NAD, i.e. the conversion of nicotinate mononucleotide to nicotinate adenine dinucleotide (deamido NAD) occurs in the nucleus [27]. If nuclear NAD production were to remain constant while the myocytes increased in size, one might predict that intracellular NAD concentrations would decline.

It should also be noted that while the bulk of myocardial adenine nucleotides are cytosolic, most of the pyridine nucleotides are contained in the mitochondrial compartment [10]. Thus, a decline in myocardial NAD suggests a depletion of mitochondrial pyridine nucleotides. The degree to which depressed mitochondrial NAD would interfere with myocardial energy metabolism is currently open to speculation.

Summary

We have shown that ATP, total adenine nucleotides, and total pyridine nucleotides are depressed in the myocardium of individuals with dilated cardiomyopathy. This nucleotide depletion is correlated with diastolic dysfunction but the cause has not been established. Mitochondrial damage and subendocardial ischemia may play a role in some cases, but a variety of causal factors are undoubtedly responsible for the metabolic abnormalities seen in dilated cardiomyopathy.

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Differential cardioprotection with selective inhibitors of adenosine metabolism and transport: Role of purine release in ischemic and reperfusion injury

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Abstract

In a previous report, we have demonstrated that simultaneous inhibition of nucleoside transport and adenosine deaminase accumulates endogenous adenosine and protects the myocardium against stunning. The differential cardioprotective effects of erythro-9(2-hydroxy-3-nonyl)-adenine (EHNA), a potent inhibitor of adenosine deamination but not transport, and *p*-nitrobenzylthioinosine (NBMPR), a selective blocker of adenosine and inosine transport, are not known.

Thirty-seven anaesthetized adult dogs were instrumented to monitor left ventricular performance using sonomicrometery. Dogs were randomly assigned into four groups. The control group (n = 8) received only the vehicle solution. Treated groups received saline containing 100 μM EHNA (EHNA-group, n = 7), 25 μM NBMPR (NBMPR-group, n = 7), or a combination of 100 μM EHNA and 25 μM NBMPR (EHNA/NBMPR-group, n = 10). Hearts were subjected to 30 min of normothermic global ischaemia and 60 min of reperfusion while on bypass. Adenine nucleotides, nucleosides, oxypurines and NAD^+ were determined in extracts of transmural myocardial biopsies using HPLC. TTC staining revealed the absence of necrosis in this model.

Drug administration did not affect myocardial ATP metabolism and cardiac function in the normal myocardium. Ischemia caused about 50% ATP depletion and accumulation of nucleosides. The ratio between adenosine/inosine at the end of ischemia was 1:10, 1:1, 1:1 and 10:1 in the control, EHNA-, NBMPR- and EHNA/NBMPR-group, respectively. Upon reperfusion, both nucleosides washed out from the myocardium in the control and EHNA-group while retained in the myocardium in the NBMPR and EHNA/NBMPR groups. Ventricular dysfunction 'stunning' persisted in the control group (52%) and in the EHNA-treated group (32%) after 30 min of reperfusion. Significant improvement of function was observed in the EHNA group only after 60 min of reperfusion. LV function recovered in the NBMPR- and EHNA/NBMPR-treated groups during reperfusion. ATP recovery occurred only when animals were pretreated with the combination of EHNA/NBMPR and remained depressed in the control group and EHNA and NBMPR-treated groups. At post mortem, TTC staining revealed the absence of myocardial necrosis.

Superior myocardial protection was observed with inhibition of nucleoside transport by NBMPR alone or in combination with inhibition of adenosine deaminase by EHNA. Selective blockade of nucleoside transport by NBMPR is more cardioprotective than inhibition of adenosine deaminase alone in attenuating myocardial stunning. It is not known why EHNA partially inhibit adenosine deaminase, *in vivo*. (Mol Cell Biochem **180**: 179–191, 1998)

Key words: adenosine and inosine, adenine nucleotides and nucleosides, free radicals-mediated injury, ischemic and reperfusion injury, myocardial stunning, nucleoside transport, ventricular arrhythmias, ventricular function
Abbreviations: EHNA – erythro-9(2-hydroxy-3-nonyl)-adenine; NBMPR – *p*-nitrobenzylthioinosine

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Introduction

Purines play a major physiologic and pathophysiologic role in the heart (for review see [1–3]). Administration of exogenous adenosine proved to be cardioprotective against ventricular tachycardia, myocardial stunning and infarction [4–8]. Nevertheless, several side effects have been identified with administration of large and frequent doses of adenosine. For example adenosine induces vasodilation and hypotension, asthma and angina in certain patients, and causes renal constriction and elevation of urate in plasma [9–15]. One major problem with exogenous adenosine is that it is rapidly broken down to inosine, taken up by the endothelium and incorporated into S-adenosylhemocystein, thus reducing the steady state level of adenosine in circulation. As a result, multiple infusions or higher concentrations of adenosine may be required to achieve pharmacologic efficacy. On the other hand, site-specific modulation of endogenous adenosine, generated during ischemic stress, may provide an alternative approach for myocardial protection thus avoiding the peripheral side effects of exogenous adenosine [16–23].

Endogenous adenosine is transiently produced during ischemia and rapidly deaminated to inosine (Fig. 1). It is well established that inosine is the end products of ATP catabolism in isolated cardiomyocytes [24] and in ischemic dog myocardium [16–23, 25]. During reperfusion, both nucleosides are released via nucleoside transport protein located on the sarcolemmal membranes. In the interstitial space, as well as in the endothelial cells, adenosine and inosine are further catabolized to oxypurines (hypoxanthine and xanthine). In the presence of molecular oxygen in blood, superoxide radical and uric acid are formed. This reaction is catalyzed by endothelial xanthine oxidase [26, 27]. *In vitro* assays demonstrated that human and rabbit myocardial homogenate lack xanthine oxidase activity [28, 29]. Indeed, oxygen-derived free radicals have been implicated in the pathogenesis of postischemic reperfusion injury in animal models and in human [16–23, 31–34]. Site-specific blockade of nucleoside transport during ischemia or upon reperfusion has been shown to attenuate myocardial stunning in dogs [16–23] by limiting free radical production or by activating adenosine receptor. The differential effects of inhibition of adenosine deaminase or selective blockade of nucleoside transport on myocardial injury and protection is not known.

The aim of this study was to determine whether inhibition of adenosine deaminase or selective blockade of endogenous adenosine transport is more cardioprotective against global myocardial stunning. EHNA, a specific adenosine deaminase inhibitor and NBMPR, a selective nucleoside transport inhibitor, were used alone or in combination to selectively manipulate endogenous adenosine and inosine production and block their release in the ischemic myocardium without affecting other non-ischemic organs. Ventricular performance was correlated with myocardial adenine nucleotides, nucleosides and purines before and after reperfusion in the presence or absence of EHNA and/or NBMPR. Results from the present study demonstrate that superior myocardial protection was achieved with selective blockade of nucleoside transport blocker NBMPR, regardless of adenosine accumulation or ATP replenishment during reperfusion.

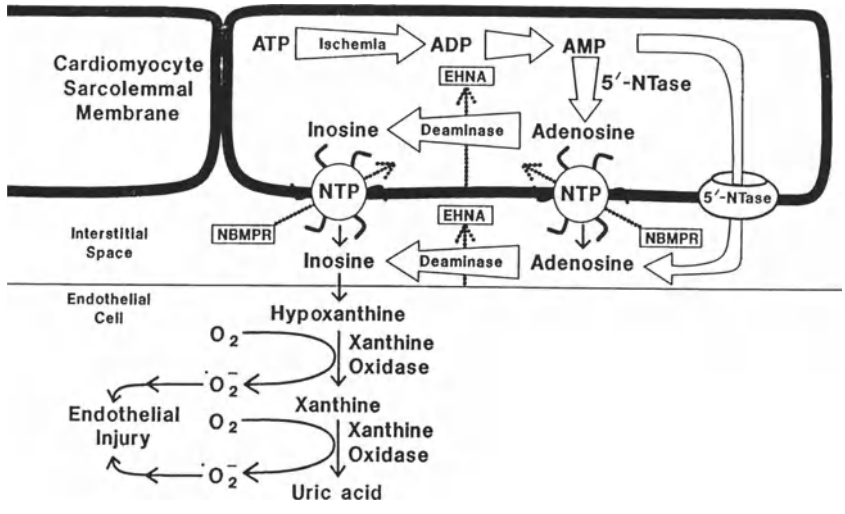

Fig. 1. Purine metablolism during ischemia and reperfusion.

Materials and methods

Materials

Biochemical reagents and EHNA were purchased from Sigma Chemical Company (St. Louis, MO, USA). NBMPR was obtained from Aldrich (Milwaukee, Wis, USA).

Animal model

The following studies conform to the guiding principles of the American Physiological Society. All animals were treated humanely in accordance with the United States Public Health Service Standards as outlined in 'Principles of Laboratory Animal Care' formulated by the National Society of Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institute of Health.

Thirty-seven microfilaria-free adult dogs of either sex weighing 17–25 kg were anaesthetized with sodium pentobarbital, 35 mg/kg, as an initial intravenous injection followed by 10 mg/kg when needed (Nembutal, Abbott Laboratories, Chicago, Ill, USA). Dogs were intubated and ventilated using a Bennett MA1 respirator (Puritan-Berkeley, CA, USA). The azygous vein was ligated, and the right phrenic nerve was transected to eliminate diaphragmatic contractions. The sinoatrial node was crushed, and the heart atrially paced at 150 beats per min using a Medtronic 5880A pacemaker (Minneapolis, MN, USA). Porcine based heparin was intravenously injected (400 U/kg) as an initial bolus followed by 200 U/kg/h (Elkins-Sinn Inc., Cherry Hill, NJ, USA). Cardiopulmonary bypass was established, employing subclavian artery cannulation and atrial venous cannulation using a two-stage cannula. A membrane oxygenator (Medtronics, Minneapolis, MN) was used and primed with non-cross-matched homologous blood. Mean arterial reperfusion pressure was maintained at 60 mmHg while on bypass. Arterial blood gases, pH and hematocrit were routinely determined and maintained at the following levels: PO_2 = 100–140 torr, pCO_2 = 30–40 torr, pH = 7.32–7.48, and hematocrit at about 30%.

Assessment of left ventricular performance

After weaning the dog from bypass, the left ventricular performance was assessed from the relationship between stroke work and end-diastolic dimension using a sensitive and load-independent index of contractility [35, 36]. Briefly, all pressure measurements were obtained utilizing intraventricular micromanometer-tipped catheters (Miller Instruments, Houston, TX, USA). Left ventricular dimension data were obtained using pulse transit sonomicrometry (Triton Technology, San Diego, CA, USA). One pair of LTZ-piezoelectric hemispheric crystal (Channel Industries, Santa Barbara, CA, USA) were sutured to the anterior and posterior of the epicardial surface of the left ventricle wall in the minor axis. The spacing between the minor axis crystals ranged from 4–6 cm. Analog data were digitized at 200 Hz and stored on magnetic disk using a microcomputer (DEC DPD 11/23, Digital Equipment Corp., Maynard, MA, USA). Subsequent analysis was performed using interactive software developed in our laboratory. Several parameters such as heart rate, systolic and diastolic left ventricular and arterial pressure, positive and negative derivatives of pressure and ventricular dimensions and work loops were simultaneously monitored on screens and recorded on magnetic disks. In order to create work loops, the venous line and left ventricle vent were clamped, and the left ventricle and systemic pressure were allowed to rise to 100–120 mmHg using the bypass roller pump followed by separating the animal from bypass. Left ventricular volume was gradually removed from the heart, thus generating a family of progressively diminishing pressure-dimension work loops during which all hemodynamic parameters were also changing. The slope of the integrated work loops plotted against enddiastolic length has been established to be a load-independent index of contractility and represents an accurate measurement of ventricular function [35, 36].

Assessment of adenine nucleotide pool metabolism

Transmural Serial Tru-Cut needle (Travenol Laboratories, Inc., Deerfield, IL, USA) biopsy specimens (5–10 mg) were obtained prior to ischemia, before and after drug administration, after 30 min of normothermic ischemia, and after 30 and 60 min of reperfusion. Biopsies were immediately frozen and stored in liquid nitrogen. Each biopsy specimen was extracted in 12% trichloroacetic acid (4°C) for 30 min with frequent homogenization. The soluble acid extract was separated from denaturated protein by centrifugation and neutralized with 2:1 (v/v) of tri-n-octylamine/freon mixture (1:3 v/v) while the protein in the pellet was determined as previously described [37]. The neutralized extracts were stored at –70°C until analysis. Myocardial adenine nucleotide pool intermediates were eluted and quantified using high performance liquid chromatography (HPLC) using external standards [38].

Protocol

Dogs were randomly assigned to one of four groups: (a) control (n = 8); (b) EHNA (n = 7); (c) NBMPR (n = 7); and

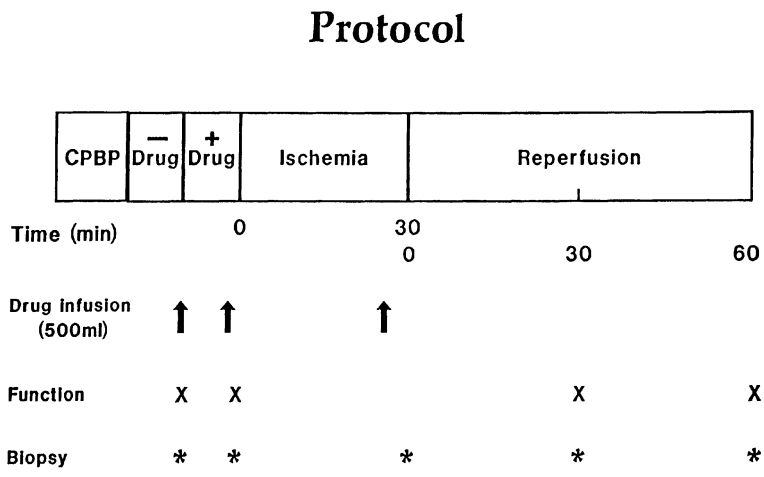

Fig. 2. Protocol: Dogs were randomly assigned to one of four groups: (1) control (n = 8); (2) EHNA (n = 7); (3) NBMPR (n - 7); and (4) EHNA/NBMPR (n = 10). The protocol followed is illustrated in the Figure. Three boluses (500 ml each) of saline with or without inhibitors (25 μM NBMPR and 100 μM EHNA) were administered into the cardiopulmonary reservoir. The first bolus was infused prior to ischemia to assess the effect of inhibitors on the functional and metabolic status of the normal heart before ischemia. The second bolus was infused immediately before cross-clamping and the third bolus upon reperfusion. The half-life of these inhibitors is more than 60 min and the respective K_i values within the nanomolar ranges *in vitro*.

(d) EHNA/NBMPR (n = 10). The protocol followed in this study is illustrated in Fig. 2. Three boluses (500 ml each) of the vehicle solution (saline containing 0.05% dimethysulfoxide, DMSO) with or without inhibitors (25 μM NBMPR and 100 μM EHNA) were administered into the cardiopulmonary reservoir. The first bolus was infused prior to ischemia to assess the effect of inhibitors on ventricular contractility of normal canine myocardium. Myocardial biopsy was obtained to determine the adenine nucleotides and nucleosides of the normal heart before ischemia. The second bolus was infused immediately before cross-clamping to ensure effective concentrations in the myocardium during ischemia, and the third bolus upon reperfusion to ensure an adequate amount of inhibitor concentration during reperfusion. The half-life of EHNA is 1–2 h, *in vivo* in mice (Dr. Donald Nelson, Burroughs Wellcome, Research Triangle Park, NC, USA). Lamb and Nelson [39] have demonstrated that a single oral dose (50 mg/Kg) totally inhibited adenosine deaminase activity for 4 h. The half-life of NBMPR is about 120 in rats (Dr. A. Paterson, Department of Pharmacology, University of Alberta, Canada). The respective K_i values for these drugs are within the nanomolar ranges *in vitro*.

Statistical analysis

Data are presented as mean ± S.E.M. Sequential measurements were compared using repeated measures analysis of variance (ANOVA) using SAS (Statistical Analysis System Institute, Cary, NC, USA). Differences were considered significant if the probability value for comparison of least squares means was less than 0.05.

Results

Myocardial adenine nucleotide pool metabolism

Myocardial adenine nucleotide levels before, during and after ischemia are depicted in Fig 3. Infusion of the vehicle solution with or without EHNA, NBMPR or a combination of EHNA and NBMPR did not effect ATP levels in the normal myocardium before ischemia. Myocardial ATP levels fell to about 50% in all groups at the end of 30 min of normothermic global ischemia ($p < 0.001$ vs. preischemic levels). There were no significant differences between groups in myocardial ATP levels at the end of the ischemic period. Myocardial ATP levels recovered to 26.0 ± 0.9 nmol/mg protein in the EHNA/NBMPR-treated group at the end of 60 min of reperfusion (p = NS vs. preischemia 28.5 ± 1.4 nmol/mg protein). Myocardial ATP levels remained depressed by 50% of normal in the control, EHNA- and NBMPR-treated groups (Fig. 3A).

The levels of ADP and AMP in the myocardium were not significantly affected by inhibitor administration prior to ischemia. ATP depletion during normothermic ischemia was associated with a transient accumulation of ADP and AMP (Fig. 3B and 3C). This was followed by a rapid depletion of ADP levels to levels below the preischemic level. Two-way

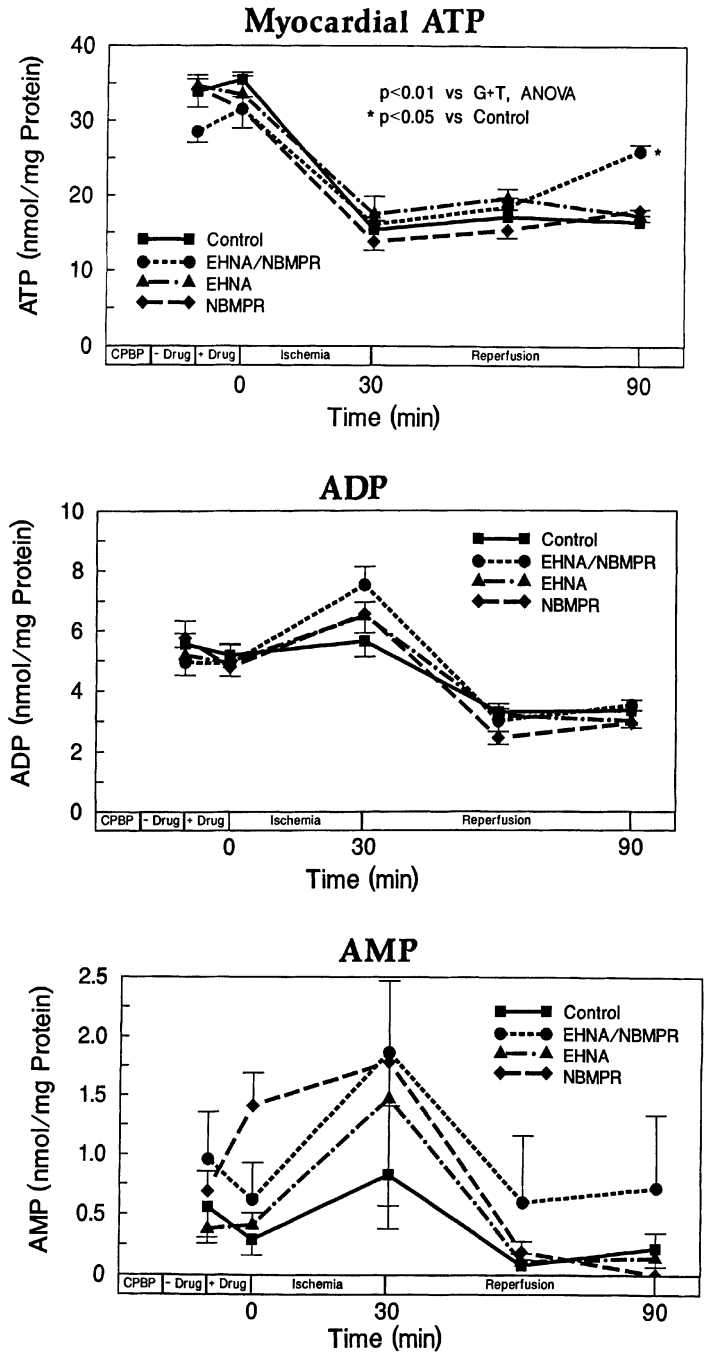

Fig. 3. Effect of EHNA and NBMPR on myocardial adenine nucleotide levels during global ischemia and reperfusion: Adenine nucleotides were determined in myocardial biopsies obtained before and after saline (control) or drug administration and at the end of 30 min of ischemia and reperfusion. Represented are effects of myocardial (A) adenosine-5'-triphosphate (ATP); (B) adenosine-5'-diphosphate (ADP) and (C) adenosine-S'-monophosphate (AMP). There are significant differences between groups ($p < 0.05$, ANOVA). Asterisks represent significant differences between groups at certain points.

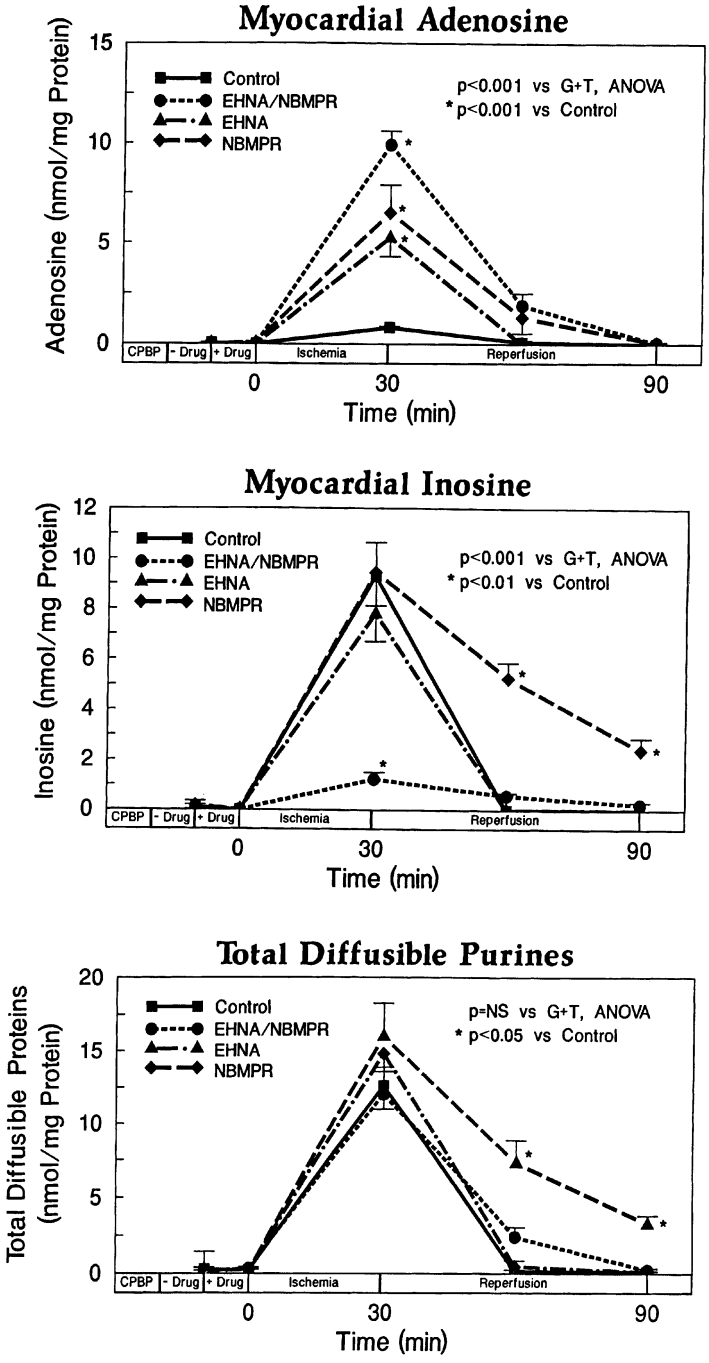

Fig. 4. Effects of EHNA and NBMPR on myocardial adenine nucleoside levels during global ischemia and reperfusion: Adenine nucleosides were determined in myocardial biopsies before and after saline (control) or drug administration and at the end of ischemia and during reperfusion. Represented are effects on myocardial adenosine (A) and inosine (B). (C) illustrates the effect of ischemia and reperfusion on total difusible purines (adenosine+inosine+ hypoxanthine+xanthine). Statistically significant differences were found between groups ($p < 0.05$, ANOVA). Asterisks represent significant differences at certain points.

analysis of variance revealed no significant differences between the groups and the time of experiment with respect to myocardial ADP and AMP. Total adenine nucleotides were decreased by 50% at the end of 30 min of ischemia in all groups ($p < 0.05$ vs. preischemia). During reperfusion, myocardial total adenine nucleotide levels recovered only in EHNA/NBMPR-treated groups and remained depressed in all other groups (Fig. 3D).

Myocardial adenide nucleosides

Adenine nucleosides (adenosine and inosine) were not detectable in the normal myocardium before or after drug administration. In the control group, the level of adenosine varied from non-detectable at preischemia to 0.8 ± 0.1 nmol/mg protein at the end of ischemia. Adenosine disappeared from the myocardium during reperfusion (Fig. 4A). Inosine was the major (>90%) nucleoside accumulated during ischemia in the control group. However, prophylactic treatment with EHNA or NBMPR before ischemia, resulted in a marked increase in the levels of myocardial adenosine at the end of the ischemic period (Fig. 4A). Adenosine accumulated in both EHNA (5.6 ± 0.9 nmol/mg protein) and NBMPR (6.5 ± 1.4 nmol/mg protein) groups ($p < 0.05$ vs. control group). The ratio of adenosine/inosine when each drug is used alone was about 1:1. When a combination of EHNA/NBMPR was infused before ischemia, a 10 fold increase in myocardial adenosine levels (9.9 ± 0.7 nmol/mg protein) was observed at the end of the ischemic period ($p < 0.05$ vs. other groups) (Fig. 4A). The ratio between adenosine and inosine in the EHNA/NBMPR-treated group was 10:1. It is interesting to note that myocardial adenosine was washed out within 30 min of reperfusion in the control- and EHNA-treated groups only, while a significant amount of adenosine and inosine were detectable in NBMPR- and EHNA/NBMPR-treated groups during reperfusion ($p > 0.05$ vs. pre-ischemia, control, and EHNA-treated groups). Inosine was the least accumulated nucleoside only in the EHNA/NBMPR-treated group (Fig. 4B). Inosine levels were significantly higher at the end of the ischemic period in the control > EHNA- > NBMPR-treated groups. During reperfusion, inosine was rapidly lost from the myocardium during reperfusion in the control and EHNA-treated groups (Fig. 4B). Ischemia-generated inosine did not totally disappear from the myocardium during reperfusion in NBMPR-treated groups. Despite low levels of inosine in the EHNA/NBMPR-treated group, it was still detectable during reperfusion (Fig. 4B).

Total nucleoside levels were not detectable at preischemia and were not elevated following the administration of EHNA, NBMPR or both. The levels of total adenine nucleosides were similar in all groups (p = NS). Total adenine nucleosides washed out within 30 min of reperfusion in the control and EHNA-treated groups, but remained detectable in NBMPR and EHNA/NBMPR-treated groups. Despite significant accumulation of adenosine during ischemia, myocardial ATP levels continued to decline until the end of the ischemic period (Figs 3A and 4A).

Myocardial oxypurines and NAD^+

Myocardial oxypurines (hypoxanthine and xanthine) levels were very low at the end of the ischemic period compared to adenine nucleoside levels. However, hypoxanthine was significantly lower in the EHNA/NBMPR-treated groups compared to others groups ($p < 0.05$). The highest concentration of hypoxanthine was found in the control group at the end of ischemia. Myocardial xanthine was much lower in the myocardium throughout the study, ranging from non-detectable to 0.2 ± 0.05 nmol/mg protein at the end of the ischemic period. The total oxypurine levels represented a minimal fraction of adenine nucleotide and nucleoside pools during the ischemic period and washed out during reperfusion in all groups (p = NS between groups). It is well established that inosine is the end product of ATP catabolism at the end of ischemia while hypoxanthine and xanthine are mainly produced during reperfusion. The effect of ischemia and reperfusion on the levels of total diffusible purines (adenosine+inosine+hypoxanthine+xanthine) in the presence or absence of EHNA/NBMPR is depicted in Fig. 4C.

Normothermic ischemia and reperfusion did not significantly affect myocardial NAD^+ in the presence or absence of EHNA and NBMPR (Fig. 5). Myocardial NAD^+ levels were not altered by drug administration before or after ischemia. Two-way analysis of variance demonstrated no significant differences between groups with respect to the time of the experiment. Myocardial levels of NAD^+ suggest that 30 min of ischemia caused reversible injury.

Left ventricular performance

After weaning the dog from bypass, the left ventricular performance was assessed from the slope of the linear relationship between stroke-work and end-diastolic length before ischemia and at 30 and 60 min of reperfusion. During LV emptying maneuver, all hemodynamic parameters, pressures and first derivatives changed as work loops diminished.

Administration of the vehicle solution or drugs did not significantly affect left ventricular contractility before ischemia. However, severe ventricular dysfunction 'stunning' was observed, following 30 min of normothermic global ischemia, in the control group. The heart function recovered to only 42.94 ± 9.8 dyn/cm^2 × 10^3 and 52.29 ±

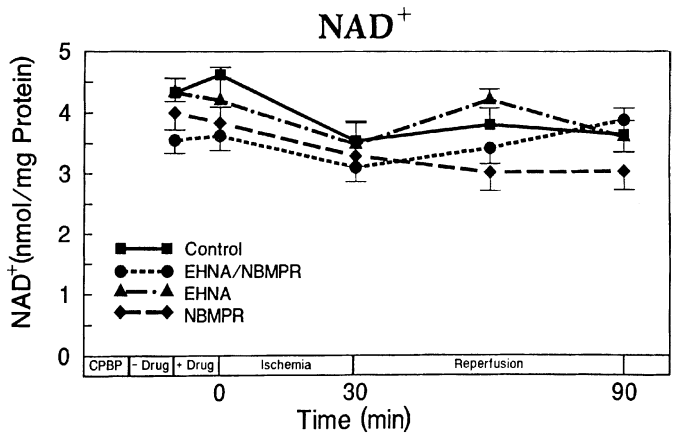

Fig. 5. Effect of ischemia and reperfusion on myocardial NAD^+ levels in the presence or absence of EHNA/NBMPR. There were no statistical differences between groups with respect of myocardial NAD^+.

8.5 dyn/cm^2 × 10^3 after 30 and 60 min of reperfusion, respectively ($p < 0.05$ vs. preischemic function after vehicle administration, slope = 76.78 ± 7.6 dyn/cm^2 × 10^3) (Fig. 6). Left ventricular dysfunction was greatly depressed in the EHNA-treated group (slope = 35.06 ± 3.84 dyn/cm^2 × 10^3) after 30 min of reperfusion but significantly improved to 71.67 ± 7.1 dyn/cm^2 × 10^3 (89.2% of preischemic function, slope = 75.28 ± 6.4 dyn/cm^2 × 10^3) after 60 min of reperfusion (p = NS vs. preischemia). Pretreatment with NBMPR resulted in significantly greater functional recovery 65.51 ± 12.9 and 86.47 ± 8.9 dyn/cm^2 × 10^3 compared to preischemic function (87.51 ± 6.1 dyn/cm^2 × 10^3) after 30 and 60 min of reperfusion, respectively (p = NS vs. preischemia). In the EHNA/NBMPR-treated group, left ventricular function returned to 71.94 ± 8.9 and 89.63 ± 12.6 dyn/cm^2 × 10^3 (83 and 104% of preischemic function 72.36 ± 7.1 dyn/cm^2 × 10^3) after 30 and 60 min of reperfusion, respectively (p = NS vs. preischemia). It is interesting to observe that the intercept of linear relationship between ventricular pressure and dimensions did not change significantly with drug treatment or during postischemic reperfusion.

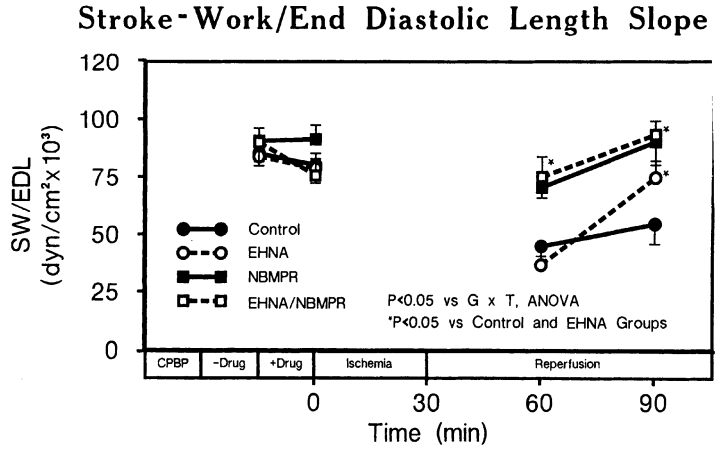

Fig. 6. Effects of EHNA and NBMPR on left ventricular performance before and after global ischemic injury: The slope of the relationship between the stroke-work/end diastolic dimension of the left ventricle at each time, except during ischemic arrest, is plotted. Statistically significant differences were found between groups ($p < 0.05$, ANOVA). Significant differences between groups at certain points are represented by an asterisk. It is important to mention that there have been no significant changes in the intercept or the linear relationship between left ventricular stroke work/end diastolic dimensions before or after ischemia in the presence or absence of EHNA/NBMPR.

Discussion

The present study was designed to determine the differential role of adenosine catabolism and nucleoside (adenosine and inosine) transport in myocardial injury and protection in a canine model of global ischemia and reperfusion. EHNA is known to specifically inhibit adenosine deaminase activity during ischemia without affecting its release mechanism via the nucleoside transport protein. NBMPR was used to selectively block the release of endogenous nucleosides during ischemia and reperfusion. A combination of EHNA and NBMPR was employed to selectively entrap endogenous adenosine at the side of production during ischemia. Continuous entrapment of adenosine and inosine prevents free radical formation during reperfusion subsequent endothelial cell injury and meanwhile allows adenosine phosphorylation and salvage for ATP repletion [16]. In this study, we assessed the differential cardioprotective actions of EHNA and NBMPR when used separately or in combination. Four scenarios were possible: (a) release of nucleosides (inosine >> adenosine) in the control group; (b) release of nucleosides (adenosine > inosine in the EHNA group); (c) entrapping nucleosides (inosine > adenosine in the NBMPR group; and (d) entrapping mainly adenosine in the EHNA/NBMPR group.

Results from the present study demonstrate that administration of EHNA or NBMPR alone, or in combination, did not have inotropic effects and did not alter adenine nucleotide or nucleoside metabolism in normal myocardium before ischemia in dogs. These drugs also did not prevent ischemia-induced ATP depletion nor stoichiometric accumulation of endogenous adenine nucleosides during ischemia but significantly changed the ratio between adenosine/inosine. Pretreatment with a combination of EHNA/NBMPR induced site-specific modulation of endogenous adenosine metabolism and transport during ischemia and during reperfusion. Therefore, ATP repletion during reperfusion was mainly related to significant entrapment of endogenous adenosine in the EHNA/NBMPR-treated group during ischemia. Despite significant and progressive accumulation of adenosine during ischemia, myocardial ATP levels continued to decline until the end of the ischemic period. Other previous reports have suggested that pretreatment with exogenous adenosine resulted in slowing down the rate of ATP depletion during ischemic canine myocardium [40]. It is possible that exogenous adenosine may have a different mechanism of protection than intracellular adenosine in EHNA/NBMPR-treated group. In the latter group, ATP recovered to almost preischemic level. It is most likely that adenosine was entrapped in a compartment where its adenosine kinase is present and subsequent salvage to ATP is taken place.

It is interesting to note that ATP repletion occurred only when the ration between adenosine and inosine is about 10:1 in the EHNA/NBMPR treated group. However, lack of ATP recovery was observed when the ration of adenosine to inosine at the end of ischemia was about 1:1 when nucleosides were released in the EHNA group or entrapped in the NBMPR group. The mechanism(s) by which the steady state levels of adenosine regulates ATP repletion is unknown from the present study. Inosine is not directly a salvageable precursor or ATP synthesis. Therefore, it remains detectable in the myocardium during reperfusion only when NBMPR was present.

Despite numerous investigations, the sites and mechanisms and intra and extracellular pools of adenosine and its subcellular compartmentalization have not been fully delineated. In the present study, the inhibitor concentration was enough to inhibit adenosine deaminase activity and effectively block nucleoside transport. The inhibition constants (K_i's) of these inhibitors are within nanomolar concentrations and drugs concentrations were in micromollar ranges. Also, drugs were infused in three boluses throughout the study to ensure pharmacologic efficacy during the study. Accumulation of nucleosides during ischemia and entrapment during reperfusion is an evidence for adequate distribution of drugs in the myocardium. However, variable levels of adenosine and inosine were observed when EHNA or NBMPR were infused separately. This may be related to subcellular distribution of adenosine deaminase that regulating adenosine catabolism. In the presence of both EHNA and NBMPR these variations in the adenosine/inosine ratios were abolished and a maximal production of adenosine during ischemia was achieved. The question remains to whether these drugs act on different compartments or how they act in a synergistic fashion to maximize adenosine levels requires further investigation. We believe that EHNA inhibits adenosine deaminase activity whether the enzyme is located inside the cardiomyocyte, endothelial cells, erythrocytes, in blood circulation or the interstitial space. Isolated endothelial cells are known to be relatively more tolerant to *in vitro* ischemia and hypoxia than cardiomyocytes. It has been shown that about 98% of ATP depleted in the myocardium originates from cardiomyocytes and only 2% from endothelial origins [24]. Isolated cell models are useful in providing important information to differentiate between cellular and subcellular compartments. However, these models lack the contribution of each type of cells in adenosine production and catabolism. Recent *in vivo* work from our laboratory indicated that even new and more potent inhibitors of adenosine deaminase than EHNA such as PVP-III-27 (9′-Hydroxy-(+)-EHNA), PVP-III-30 (8′-Hydroxy-(+)-EHNA), VMV-I-64 (8′,9′-Dihydroxy-(+)-EHNA) and (+) EHNA, failed to completely inhibit adenosine deaminase in ischemic rat myocardium, *in vivo* [41]. These inhibitors proved to very potent than (±) EHNA as inhibitor of adenosine deaminase, *in vitro* (K_i (nM) = 3.8 ± 0.4, 6.4 ± 0.8, 15.8 ± 0.4 and 0.82 ± 0.2, respectively [42].

Therefore, we believe that concentration of EHNA and its half life are appropriate to effectively inhibit adenosine deaminase for the duration of our study. Our results may also suggest that differential effects of EHNA and NBMPR may be related to enzyme compartmentalization rather than lack of efficacy. The reason for partial inhibition of adenosine deaminase *in vivo* is not known and warrant further investigation. It is difficult to determine accurately adenosine compartmentalization in cardiac, endothelial and smooth muscle cell and in the interstitium. Microdialysis techniques have been useful measurement of 'interstitial' adenosine formation during ischemia [43]. EHNA and dipyridamole increased the interstitial adenosine in dogs [44]. These measurements are useful but do not precisely differentiate between cellular and subcellular compartments of adenosine production and metabolism in the intact heart. Adenosine is also produced from other sources. ATP co-released with catecholamines from nerve endings and subsequently broken down to adenosine and inosine. Endothelial cells are also a source of ADP and adenosine. Therefore, interstitial adenosine may be derived from extracellular ATP and not solely from cardiomyocytes, *per se*. Although determination of adenine nucleotide pool intermediates in tissue biopsy samples does not provide insight into enzyme distribution and metabolise compartmentalization, it does provide unique and valuable information on the steady state transmural ATP derangement and accumulation of nucleosides in the myocardium during ischemia and reperfusion. Based on our results, EHNA/NBMPR entrapped intracellular adenosine which is rapidly phosphorylated to AMP, ADP and ATP. In addition, EHNA/NBMPR also block the uptake of extracellular adenosine by endothelial cells as well as cardiomyocytes.

Several unrelated cardioprotective drugs have been known to be potent vasodilators. Mechanisms of vasodilation of dipyridamole [45], several calcium antagonists [46], local anesthetics such as lidocaine [47] and lidoflazine [48, 49] or 5-amino-4-imidazole carboxamide riboside (AICAR) [50] have been related, in part, to modulation of adenosine production, metabolism and/or transport. Lidoflazine, among derivatives of N-diaryldialkyl-piperazine such as mioflazine, cinnarizine, flunarizine and R75231, found to have some actions like dipyridamole in addition to their calcium antagonistic properties [51]. These compounds are very hydrophobic with high affinity to membrane phospholipid [52, 53]. These derivatives accumulate adenosine during ischemia but they do not allow ATP resynthesis [54] or they cardioprotective when administered after ischemia. Unlike these dipyridamole and piperazine derivatives, NBMPR is a nucleoside known to be a selective inhibitor of the nucleoside transport [55] and is also cardioprotective when administered only at postischemic reperfusion in the absence of accumulated adenosine [18, 19].

The mechanism(s) of ventricular dysfunction observed during the first early phase of reperfusion (30 min) in the EHNA-treated group is not known. In this group, adenosine and inosine were equally accumulated during ischemia but released during reperfusion. Impairment of ventricular function in the EHNA-treated group could be mediated by the formation of oxypurines and the production of free-radicals causing temporary 'myocardial global stunning.' However, marked improvement in ventricular function in the same group occurring during the late phase of reperfusion (after 60 min) could be related to cardioprotective actions of 'circulating adenosine.' Pretreatment with exogenous adenosine is cardioprotective in attenuating myocardial global stunning, limiting myocardial infarction, or inhibiting neutrophil activation during reperfusion. In a canine model of LAD occlusion and reperfusion, pretreatment with EHNA/NBMPR attenuated myocardial stunning induced by 15 min ischemia and 4 h reperfusion [56]. Complete functional recovery was achieved at the end of reperfusion in groups treated with nucleoside transport blocker in the presence or absence of adenosine deaminase inhibitors. Myocardial ATP recovered to near normal levels in the EHNA/NBMPR-treated groups when adenosine was the major nucleoside entrapped during ischemia and reperfusion but remained depressed in the control, EHNA, and NBMPR-treated groups. Regardless of ATP recovery, ventricular function returned to normal after 60 min in all drug-treated groups.

Myocardial ATP levels have long been considered an important index of functional recovery following ischemic injury [57]. However, lack of correlation between myocardial ATP and ventricular function has been recently reported [58, 59]. These discrepancies may be attributed to the fact that the normal myocardium contains more ATP (2–5 mM) than it may need to maintain basic cardiac function and metabolism (50–100 μM); and lack of separation between ischemic and reperfusion injury. Purine release has been used as an index of ischemia [60, 61]. Selective blockade of purines allowed separation between components of ischemic and reperfusion injury has been demonstrated [20].

Cardioprotective actions of exagenous adenosine have been demonstrated to be mediated by A_1-receptors [62, 63]. The phenomenon of ischemic preconditioning has been related to activation of A_1-receptors in rabbits [64] and could be abolished by A_1-receptor antagonists (8-sulfonylphenytheophylline) but not DPCPX (James Downey, personal communication). Other studies have demonstrated failure of exogenous adenosine to mimic ischemic preconditioning in limiting ventricular dysfunction assessed by recovery of developed pressure described in isolated rat [65] and rabbit [66] models of global ischemia and reperfusion. These discrepancies may be related to species- and model-related differences [67]. Administration of EHNA/NBMPR before ischemia resulted in 10 fold greater accumulation of endo-

genous adenosine than the control group at the end of ischemia. It is possible that the remarkable myocardial protection with EHNA/NBMPR could be mediated, in part, by Al-receptor stimulation. The question whether or not the myocardial protection provided by EHNA/NBMPR was mediated by A_1-receptors was not addressed in the present study. However, the role of A_1-receptor in EHNA/NBMPR cardioprotective action has been demonstrated [68]. Briefly, selective blockade of A_1-receptor by a selective antagonist (8-cyclopentyl-1,3-dipropyl-xanthine, DPCPX) during ischemia, in the presence of absence of EHNA/NBMPR revealed that adenosine receptor activation by endogenous adenosine is about 20% of overall protection mediated by EHNA/NBMPR. Therefore, cardioprotection induced by site specific entrapment of adenosine may involve other mechanisms of protection in addition to that mediated by A_1-receptor.

Despite the fact that endogenous adenosine was also transiently accumulated in the ischemic myocardium but rapidly deaminated to inosine in the untreated control group, it failed to protect the myocardium against stunning. Therefore, it seem important that adenosine level has to reach certain level before it becomes cardioprotective. Work from our laboratory demonstrated that ventricular function completely recovered when EHNA/NBMPR was administered only during reperfusion in the absence of endogenous adenosine [18, 19]. Furthermore, intracoronary infusion of exogenous hypoxanthine and xanthine, in a group of dogs pretreated with EHNA/NBMPR before ischemia and accumulated endogenous adenosine at the end of ischemia, resulted in ventricular dysfunction similar to that of the control group [16]. The question remains to whether transient accumulation of low levels of adenosine in untreated animals is adequate enough to stimulate adenosine receptors and augment ventricular functional recovery.

The most important findings of this study could be summarized as follows: (a) selective blockade of adenosine nucleoside transport was an effective pharmacologic intervention to prevent ventricular dysfunction associated with reperfusion following reversible ischemic injury 'stunning' in absence of necrosis; (b) the heart can function adequately with about 50% of its normal ATP levels if purine release was blocked by EHNAINBMPR; (c) restoration of myocardial ATP after ischemia is dependent on the availability of entrapped intracellular adenosine, but not inosine or exogenous adenosine. We concluded that nucleoside transport plays an important role in myocardial metabolic derangement during ischemia and in reperfusion-mediated myocardial stunning. Myocardial protection with EHNA/NBMPR may be clinically feasible to prevent reperfusion injury triggered by purine release associated with coronary artery revascularization, balloon angioplasty or thrombolytic therapy.

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Effects of nucleoside transport inhibitors and adenine/ribose supply on ATP concentration and adenosine production in cardiac myocytes

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Abstract

Adenosine plays an important role in protection of the heart before, during and after ischemia. Nucleoside transport inhibitors (NTI) increase adenosine concentration without inducing ischemia by preventing its uptake and metabolism in cardiac cells. However, prolonged effects of nucleoside transport inhibitors on adenosine and nucleotide metabolism and its combined effect with nucleotide precursors has not been established in cardiomyocytes. The aim of this study was to investigate the effect of two nucleoside transport inhibitors, dipyridamole (DIPY) and nitrobenzylthioinosine (NBTI) alone or combined with adenine and ribose on adenosine production and ATP content in cardiomyocytes.

Rat cardiomyocytes were isolated using collagenase perfusion technique. Isolated cell suspensions were incubated for up to 480 min with different substrates and inhibitors as follows: (1) control; (2) 100 μM adenine and 2.5 mM ribose; (3) 10 μM DIPY; (4) 1 μM NBTI; (5) DIPY, adenine and ribose and (6) NBTI, adenine and ribose. Five μM EHNA (erythro-9(2-hydroxy-3-nonyl)adenine, an inhibitor of adenosine deaminase) was added to all incubations. After incubation, extracts of myocyte suspension were analysed by HPLC for adenine nucleotides and metabolite concentrations.

ATP content decreased in cardiomyocytes after 8 h of incubation with DIPY, while no change was observed with NBTI or without inhibitors. Adenosine concentration increased with both DIPY and NBTI. In the presence of adenine and ribose an elevation in ATP concentration was observed, but no significant change in adenosine content. In the presence of DIPY or NBTI together with adenine and ribose, an enhancement in cardiomyocyte ATP concentration was observed together with an increase in adenosine content. This increase in adenosine production was especially prominent with DIPY.

In conclusion, dipyridamole causes a decrease in ATP concentration in isolated cardiomyocytes by mechanisms other than nucleoside transport inhibition. Addition of adenine/ribose with dipyridamole prevents the depletion of ATP. Combination of adenine/ribose with nucleoside transport inhibitors may also further enhance adenosine concentration and thus, could be more effective as pharmacological agents for treatment. (Mol Cell Biochem **180**: 193–199, 1998)

Key words: adenosine, nucleoside transport inhibitors, heart metabolism, adenine nucleotides

Introduction

Adenosine is an important catabolite of adenine nucleotides with potent coronary vasodilatory, antiplatelet, antileukocyte and antiarrhythmic properties [1–4]. Release of adenosine is increased predominantly as the consequence of an imbalance between oxygen supply and demand, and the main sites of adenosine formation and breakdown in the heart are the cardiac vascular endothelium and the cardiomyocytes. The clinical relevance of these actions of adenosine make it an important therapeutic and diagnostic tool.

Infusion of adenosine has been demonstrated to be effective in providing protection against damage during myocardial ischemia [5, 6]. However, therapeutic use of adenosine has some drawbacks, since adenosine has a very short half life and may trigger receptors throughout the body with unwanted effects. To harness the beneficial effects of adenosine in a localised manner, current investigations include studies on increasing endogenous adenosine concentration by preventing its re-uptake or by inhibiting the

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enzymes responsible for its degradation, especially by the endothelial cells. Nucleoside transport inhibitors increase adenosine concentration by preventing its re-uptake mainly by the endothelial cells but also by cardiomyocytes [7]. There are a considerable number of compounds with nucleoside transport inhibition properties. These include dipyridamole which is used clinically, dilazep, nitrobenzylthioinosine (NBTI), and the lidoflazine group of inhibitors – draflazine, R75231 and mioflazine. Nucleoside transport inhibitors have been proposed as cardioprotective agents during heart transplantation [8], for the reduction of infarct size [9, 10] and for the improvement in myocardial function. Cardioprotective effects have been demonstrated with dipyridamole [11], mioflazine [12], NBTI [13] and dilazep [14]. Dipyridamole has been used therapeutically during coronary artery bypass surgery and angioplasty in humans, and proved to be very effective in reducing postoperative bleeding, platelet deposition and perioperative ischemic injury [15, 16]. Studies in humans with dipyridamole were promising but the pharmacokinetics have proved to be more complex and the bioavailability is variable, making it difficult to show its benefits.

Little attention has been given to the possible deleterious consequences of increased adenosine efflux from the cell and the possible depletion of the nucleotide pool. The effect of the combined application of these compounds with precursors of nucleotides such as adenine and ribose are also not known. The aim of the present study was therefore to evaluate the consequences of nucleoside transport inhibition on ATP concentration and adenosine production in cardiomyocytes and to study metabolic effects of the combined application with adenine and ribose.

Materials and methods

Rat cardiac myocyte isolation

All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health (NIH publication no. 85-23, revised 1985), and the European Convention on Animal Care. Cardiomyocytes were isolated using collagenase perfusion technique from the hearts of Sprague-Dawley rats weighing 250–300 g as described previously [17]. The final preparation contained 70–85 % of rod shaped cells. After isolation and purification cells were finally suspended in buffer containing 120 mM NaCl, 2.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 11 mM glucose, 2 mM pyruvate and 2% bovine serum albumin. Five minutes prior to start of experiment, erythro-9(2-hydroxy-3-nonyl) adenine (EHNA) was added at 5 μM concentration. Subsequently, the cell suspension was transferred in 0.5 ml aliquots to round bottomed sealed plastic test tubes and substrates and inhibitors were added.

Experimental protocol

Dipyridamole (DIPY) and nitrobenzylthioinosine (NBTI) were dissolved in dimethyl sulfoxide (DMSO), the volume of the inhibitors added to the tubes were less than 1% of the incubation volume, at a final concentration of 10 μM of DIPY and 1 μM of NBTI with and without the addition of 100 μM adenine and 2.5 μM ribose. The incubation tubes were set up as follows: (A) EHNA alone (control); (B) with EHNA plus adenine and ribose; (C) with EHNA and DIPY; (D) with EHNA and NBTI; (E) with EHNA, DIPY plus adenine and ribose; (F) with EHNA, NBTI plus adenine and ribose.

Incubation was carried out at 37°C in a shaking water bath for 15, 180 or 480 min and initial samples were taken at the start. Incubation was terminated by the addition of 25 μl of 6 M $HClO_4$. Tubes were then centrifuged (4°C, 3700 g, 30 min) and 0.4 ml of the supernatant taken for neutralisation with 30 μl of 3 M K_3PO_4. After removal of the potassium perchlorate precipitate by centrifugation, samples were analysed by HPLC as described below. To evaluate total cellular protein content, 0.5 ml of the cell suspension was carefully placed on top of 0.6 ml of 1-bromododecane, followed by centrifugation to separate the myocytes from the medium [18]. The myocyte pellet was then solubilized in 0.5 M NaOH. Protein concentration was evaluated using the method of Bradford [19].

Determination of ATP, adenosine and other metabolises by HPLC

All determinations of metabolise concentrations were performed using high performance liquid chromatography (HPLC). The equipment used was Merck-Hitachi chromatograph attached to a Carnegie-Medicin auto sampler and Turbochrom (PE -Nelson) workstation for data processing. The reversed-phase method used for determination of ATP, adenosine and NAD concentration in the myocytes, has been described in detail previously [20, 21].

Statistics

All results are presented as means (S.E.M.) of 9–16 different myocyte preparations. Values after 15, 180 and 480 min of incubation were compared with initial values using paired Student's *t*-test. For comparison between groups at the same time point, one way analysis of variance was used followed by Student-Newman-Keuls test. The difference between means was considered to be significant when $p < 0.05$.

Results

Changes in ATP content in cardiomyocytes in the presence of nucleoside transport inhibitors, EHNA and nucleotide precursors

The initial absolute contents of nucleotides and nucleosides in the myocyte suspension including both the intracellular and extracellular metabolites are shown in Table 1. Table 2 and 3 shows the amounts after 180 and 480 minutes of ATP, ADP, AMP and NAD with and without the combination of the different treatments. There was a significant increase in ATP content in the presence of NBTI + A/R and this is maintained even after 480 min (Table 3). Dipyridamole alone demonstrated a trend towards a decline of ATP concentration but in the presence of dipyridamole + adenine/ribose the ATP content was maintained and is slightly enhanced after 480 min (Table 3). Adenosine content was increased in the dipyridamole + adenine/ribose group after 180 min (Table 2) and after 480 min, while the increase of adenosine in the NBTI + adenine/ribose group was only apparent after 480 min (Table 3).

ATP levels expressed as the change from time zero in the presence of EHNA alone (control) demonstrated an initial decrease but no further decrease was seen over the 8 h of incubation (Fig. 1). In the presence of dipyridamole alone the change in ATP content showed a steady decrease over time, however, NBTI did not affect ATP levels when compared to control myocytes. Adenine and ribose caused an enhancement in ATP concentration. A 10% increase in ATP content was demonstrated after 8 h when dipyridamole was incubated with adenine and ribose, while with NBTI plus adenine and ribose showed a 25% increase in ATP content, which was similar to changes with adenine and ribose.

Changes in adenosine content in cardiomyocytes in the presence of nucleoside transport inhibitors, EHNA and nucleotide precursors

Adenosine formation in the presence of EHNA alone or in the adenine and ribose group alone was not stimulated (Fig. 2). Dipyridamole and NBTI showed a slow stimulation of endogenous adenosine content after 3 h of incubation. The greatest change seen was in the groups where adenine and ribose were present in addition to dipyridamole and NBTI. This increase became evident after just 15 min in the dipyridamole plus adenine/ribose group and a further in-

Table 1. Initial concentrations of ATP, ADP, AMP, adenosine and NAD in rat myocytes.

ATP	ADP	AMP	Adenosine	NAD
		nmol/mg cellular protein		
17.18 ± 1.34	2.77± 0.21	0.15 ± 0.05	0.15± 0.04	4.52 ± 0.71

Values are means ± S.E.M. of 9–16 myocyte preparations.

Table 2. Concentrations of ATP, ADP, AMP, adenosine and NAD in rat myocytes at time 180 min.

Treatment	ATP	ADP	AMP	Adenosine	NAD
			nmol/mg cellular protein		
Control	15.67 ± 1.61	2.69 ± 0.17	0.11 ± 0.07	0.42 ± 0.08	3.90 ± 0.79
A/R	20.38 ± 1.70	2.47 ± 0.17	0.25 ± 0.02	0.25 ± 0.03	2.21 ± 0.29
DIPY	15.02 ± 0.95	2.67 ± 0.15	0.13 ± 0.05	0.38 ± 0.06	3.17 ± 0.27
NBTI	16.81 ± 1.20	2.48 ± 0.16	0.13 ± 0.04	0.37 ± 0.08	3.88 ± 0.08
DIPY + A/R	17.81 ± 1.29	2.69 ± 0.13	0.24 ± 0.08	1.05 ± 0.23#	2.86 ± 0.32
NBTI + A/R	21.46 ± 1.76*	2.62 ± 0.16	0.14 ± 0.03	0.38 ± 0.09	2.56 ± 0.30

A/R – Adenine/Ribose; DIPY – Dipyridamole; NBTI – Nitrobenzylthioinosine. * $p < 0.05$ vs. dipyridamole. #$p < 0.05$ vs. control, A/R, DIPY, NBTI and NBTI + A/R. Values are means ± S.E.M. of 9–16 myocyte preparations.

Table 3. Concentrations of ATP, ADP, AMP, adenosine and NAD in rat myocytes at time 480 min.

Treatment	ATP	ADP	AMP	Adenosine	NAD
nmol/mg cellular protein					
Control	16.37 ± 1.42	2.54 ± 0.14	0.07 ± 0.04	0.25 ± 0.06	3.94 ± 0.92
A/R	17.98 ± 1.42	2.17 ± 0.21	0.24 ± 0.05	0.26 ± 0.08	1.78 ± 0.21
DIPY	14.47 ± 0.95	2.48 ± 0.17	0.19 ± 0.04	0.43 ± 0.05	3.18 ± 0.46
NBTI	16.71 ± 1.23	2.49 ± 0.16	0.17 ± 0.07	0.41 ± 0.07	3.72 ± 0.90
DIPY + A/R	18.39 ± 1.30	2.50 ± 0.14	0.18 ± 0.03	1.25 ± 0.25#	2.48 ± 0.32
NBTI + A/R	21.50 ± 1.93*	2.88 ± 0.26	0.09 ± 0.09	0.99 ± 0.18#	1.98 ± 0.19

A/R – Adenine/Ribose; DIPY – Dipyridamole; NBTI = Nitrobenzylthioinosine. *$p < 0.05$ vs. dipyridamole. #$p < 0.05$ vs. control, A/R, NBTI and DIPY. Values are means ± S.E.M. of 9–16 myocyte preparations.

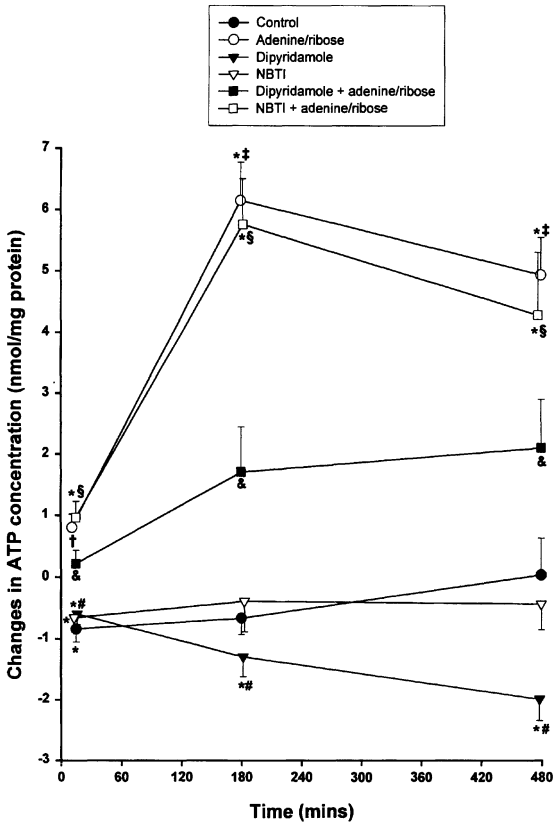

Fig. 1. Changes in ATP content in isolated rat cardiomyocytes incubated for 480 min in HEPES-buffered physiological medium containing EHNA with additional presence, where indicated, of adenine /ribose, or DIPY, or NBTI, or DIPY and adenine/ribose, or NBTI and adenine/ribose. Values represent the means ± S.E.M. n = 9–16. *p < 0.05 vs. initial. #p < 0.05 vs. adenine/ribose, NBTI + adenine/ribose and DIPY + adenine/ribose. §p < 0.05 vs. control, NBTI and DIPY + adenine/ribose. †p < 0.05 vs. control and NBTI. ‡p < 0.05 vs. control, NBTI and DIPY + adenine/ribose.

crease was observed after 3 h and 8 h. In the NBTI plus adenine and ribose group the increase in adenosine was only evident after 3 h of incubation.

Changes in NAD content in cardiomyocytes in the presence of nucleoside transport inhibitors and nucleotide precursors

NAD concentration (Fig. 3) showed a steady decline in all of the groups, including the control. However, after 3 h the NAD changes reached a plateau in the control group. There were no differences between the groups.

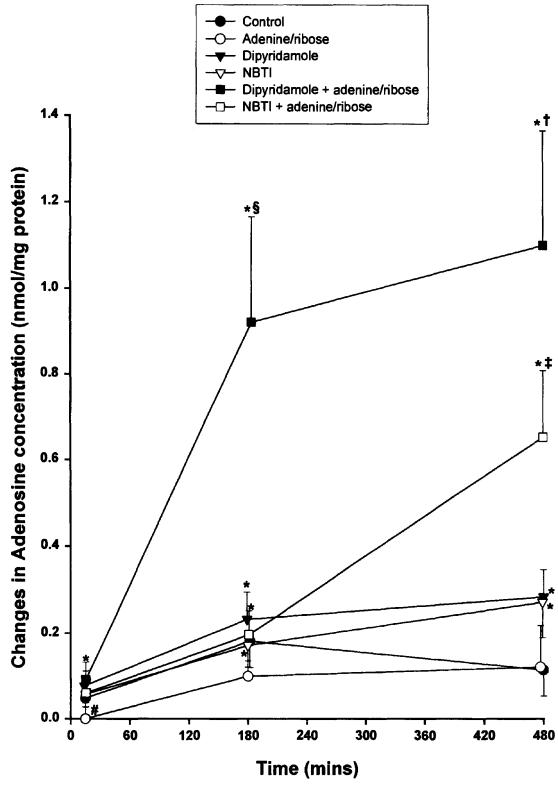

Fig. 2. Changes in adenosine content in isolated rat cardiomyocytes incubated for 480 min in HEPES-buffered physiological medium containing EHNA with additional presence of adenine/ribose, or DIPY, or NBTI, or DIPY and adenine/ribose, or NBTI and adenine/ribose. Values represent the means ± S.E.M. n = 9–16. *p < 0.05 vs. initial. #p < 0.05 vs all other groups. §p < 0.05 vs. control and adenine/ribose. †p < 0.05 vs. control.

Discussion

This study has demonstrated that the combined application of nucleoside transport inhibitors (NTI) with adenine/ribose markedly increases adenosine concentration in isolated cardiomyocytes. Furthermore, adenine/ribose supply prevented the depletion of ATP caused by the most frequently used drug in this class – dipyridamole (DIPY).

Effect of nucleoside transport inhibitors on adenosine concentration

Increase in adenosine concentration is a crucial mechanism of the pharmacological effects of nucleoside transport inhibitors (NTI) resulting in its vasoactive and antiplatelet activities. Adenosine produced intracellularly can be

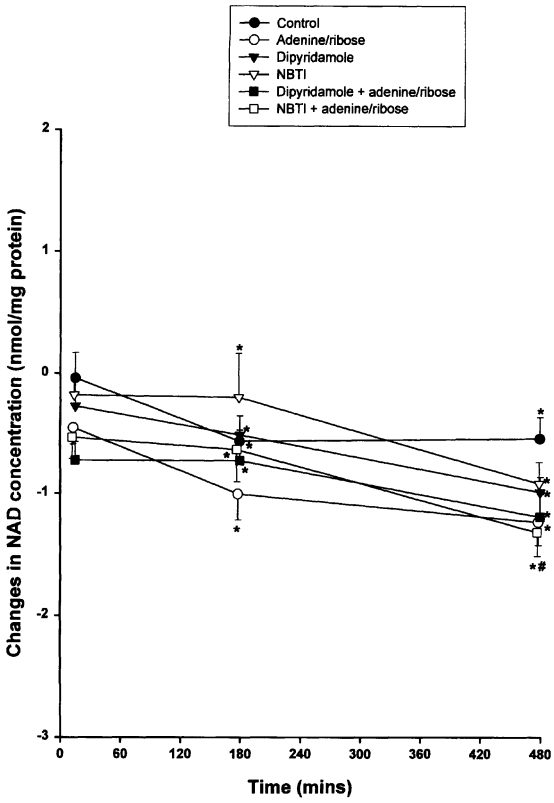

Fig.3. Changes in NAD content in isolated rat cardiomyocytes incubated for 480 min in HEPES-buffered physiological medium containing EHNA with additional presence of adenine/ribose, or DIPY, or NBTI, or DIPY and adenine/ribose, or NBTI and adenine/ribose. Values represent the means ± S.E.M. n = 9–16. *p < 0.05 vs. initial. #p < 0.05 vs. control.

released from cells by facilitated or passive diffusion and the rate is controlled by the intracellular concentration [22, 23]. Myocytes are the most important source of adenosine [24], which is then taken up by the endothelial cells under physiological conditions. However, if this is prevented by NTI, adenosine concentration increases [11]. Autoradiographic studies with [^{3}H]-NBTI [25], demonstrated that there are more NTI binding sites in endothelial cells associated with coronary vessels than with myocytes. The endothelium therefore acts as a barrier scavenging the adenosine produced by myocytes during hypoxia or ischemia [26]. It has also been suggested that NTI could further increase adenosine release by inhibiting the uptake of adenosine to a larger extent than its release [27, 28]. This in turn would lead to a deprivation in the cells of the nucleotide precursors. It is therefore important to establish what are the effects of long term exposure to NTI in single cells on both ATP levels and adenosine concentration. We have shown that in the presence

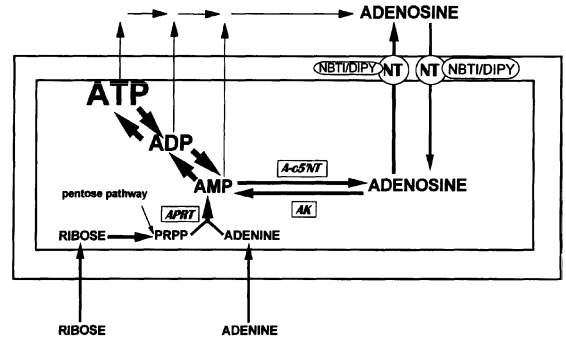

Fig. 4. Adenine nucleotide synthesis and effects of NTI in cardiomyocytes. Adenine is rapidly incorporated, providing there is a supply of ribose for PRPP synthesis. Abbreviations are: (A-c5'NT) AMP specific cytosolic 5'-nucleotidase, (AK) adenosine kinase, (APRT) adenine phosphoribosyltransferase, (NT) nucleoside transporter.

of NTI, adenosine concentration increased in myocyte suspension during long term incubation while changes in control incubations were not significant. This is in line with the suggestion that adenosine uptake is affected to a larger extent than its release. An alternative explanation could be the release of nucleotides which are then degraded by extracellular ectoenzymes with adenosine as the end product (in adenosine deaminase inhibited cells). These mechanisms may eventually lead to a starvation of the cells from nucleotide precursors and a fall in ATP content.

Effects of nucleoside transport inhibitors on ATP concentration

We have shown that the nucleotide pool decreased significantly in the cells treated with DIPY after 8 h exposure. However, this does not seem to be the general effect of NTI since it was not observed with NBTI. DIPY exerts many other non-specific effects not related to nucleoside transport inhibition, such as inhibition of phosphodiesterases, increase in prostacyclin formation, interference with the transport of glucose, choline and phosphate [29] and these mechanisms could lead to further detrimental effects. The evidence for the inhibition and/or the oxidation of glucose was demonstrated by dipyridamole in isolated rat myocytes where the oxidation of palmitate was stimulated [30] therefore resulting in a switch away from glucose metabolism and possibly decreased ATP turnover. Decrease in ATP levels was not observed after short term heart perfusion experiments with DIPY, showing that ATP was well maintained [31], while prolonged effects of DIPY or other NTI on the nucleotide pool were not studied. However, in contrast to this previous study even short term application of DIPY may lead to

functional and metabolic deterioration during postischemic reperfusion [32]. The preparation of isolated adult cardiomyocytes involves a rigorous regime of collagenase digestion and aerobic perfusion with glucose that may confer a degree of preconditioning or loss of metabolites. We cannot rule out that preconditioning of the myocytes during the isolation procedure may have affected the results to some extent but this would rather protect against ATP depletion caused by DIPY than induce the changes that we are seeing. The procedure of isolation in this protocol resulted in 70–85% rod shaped cells and this has been shown not to have an effect on the total adenine nucleotide pool at any stage of the washing steps [18] when compared to intact heart tissue. However, the fact that the adenylate energy charge (2 ATP + ADP)/(2TAN), was maintained at 0.91 with DIPY, suggest that DIPY may interfere with nucleotide metabolism without affecting energy turnover. The gradual decrease in NAD concentration observed in all of the treatment groups may indicate some metabolic deterioration of the cells but alternatively this may be explained by a shift in the redox state of NAD to its reduced form of NADH which was not quantified in this study.

Effects of combined application of adenine/ribose and NTI

Cardiac myocytes possess a significant potential to salvage adenosine to regenerate adenine nucleotides, however once adenosine has been deaminated to inosine the myocytes are unable to reutilize inosine in the salvage pathway [23]. The combined inhibition of adenosine deaminase activity and membrane nucleoside transport is considered as a possible strategy to prevent metabolism of adenosine and to enhance its pharmacological effects. EHNA which competitively inhibits adenosine hydrolysis to inosine, has been shown to reduce myocardial stunning [33, 34] and to improve myocardial function after ischemia [35]. Nucleoside transport inhibition prevents re-entry of adenosine into the cell after its formation and in combination with EHNA possible extracellular deamination of adenosine is also prevented [12].

Supply of precursors alternative to the pathway of adenosine rephosphorylation of adenosine – ribose and adenine, which are involved in the pathway of adenine nucleotide synthesis, may provide several benefits in cells treated with NTI and adenosine deaminase inhibitors (Fig. 4), [36]. These include possible prevention of nucleotide pool alterations and further enhancement of adenosine production due to the increased nucleotide precursor pool. We have shown that the presence of adenine and ribose markedly increases ATP pool in the cardiac cells but no increase in adenosine concentration was observed. However, combined application of adenine/ribose with NTI increased not only ATP level but also adenosine concentration. This increase in adenosine concentration was markedly greater in cells treated with DIPY while ATP increase was smaller than with NBTI. This could be again related to a number of known unspecific effects of DIPY. Inhibition of ATP utilization could be an alternative to nucleotide resynthesis mechanism of ATP preservation/enhancement caused by adenine/ribose. However, it seems to be unlikely because adenine/ribose does not affect any aspect of cardiac function during short term cardiac perfusion [37].

Summary

Nucleoside transport inhibition offers major promise in the regulation of localised adenosine production. We have demonstrated that the metabolic consequences of the application of some of the drugs in this class, may induce deleterious effects in cardiac myocytes, such as a decrease in ATP concentration, but it is possible to reverse this by providing the precursors of nucleotides – adenine and ribose. Further work is needed to quantify these changes in the *in vivo* situation and to explore the potential beneficial effects after combined application with adenine/ribose on functional parameters.

Acknowledgements

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