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Triglyceride Turnover and Contribution to Energy Substrate Use in the Heart

BY

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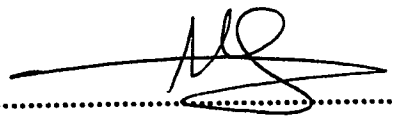

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**Triglyceride turnover and contribution to energy substrate use in the heart**" submitted by Maruf Saddik in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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To my parents

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ABSTRACT

Myocardial triglyceride turnover was determined in control, reperfused/ischemic, and in diabetic rat hearts. Hearts were initially perfused under aerobic conditions for a 60 minute "pulse" perfusion with 1.2 mM [1-^{14}C]palmitate to label the endogenous lipid pools, followed by 10 minutes of Langendorff perfusion (in control and diabetic rat hearts) or 30 minutes of no-flow ischemia. Hearts were then perfused for a further 60 minute period of aerobic "chase" in the presence or absence of 1.2 mM [9,10-^{3}H]palmitate. Endogenous triglyceride lipolysis and synthesis were measured during the "chase" perfusion, while rates of exogenous palmitate oxidation were measured both during the "pulse" and during the "chase" perfusion. A second series of perfusions was performed to measure glycolysis and glucose oxidation. In this series, hearts were perfused under similar conditions, except that unlabeled palmitate was used during the "pulse" and that 11 mM [2 or 5-^{3}H/U-^{14}C]glucose and unlabeled palmitate was present during the "chase". During the "chase", both glycolysis and glucose oxidation rates were measured.

In control hearts, myocardial triglycerides proved to be a significant contributor of fatty acids for myocardial oxidative metabolism; this contribution was more dramatic in hearts perfused without exogenous fatty acids where myocardial triglycerides contributed to almost 60% of myocardial ATP requirements. In diabetes mellitus, myocardial triglyceride contribution to overall myocardial ATP requirements was not different from control in the presence of a high concentration of fatty acid. However, an increased contribution was seen in diabetic rat hearts when hearts were perfused in the

absence of fatty acids. During reperfusion of ischemic hearts, a 20% increase in exogenous fatty acid oxidation rate was seen compared to the pre-ischemic rate. Steady state endogenous triglyceride lipolysis did not change. However, a significant increase in triglyceride synthesis was observed.

This study highlights the importance of myocardial triglycerides as an important energy source for the heart especially under perfusion conditions that were and are still used where hearts were perfused in the absence of any fatty acid. It also provides a direct evidence for the importance of cardiac acetyl-CoA carboxylase in regulating myoardial fatty acid oxidation and improves our understanding of the relation between myocardial fatty acid and glucose use.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CoA	coenzyme A
cAMP	3,5 cyclic adenosine monophosphate
CPT I	carnitine palmitoyltransferase I
CPT II	carnitine palmitoyltransferase II
DGAT	diacylglycerol acyltransferase
EDTA	ethylene diamine tetraacetic acid
FFA	free fatty acid
GPAT	glycerol 3-phosphate acyltransferase
HR	heart rate
NEFA	non-esterified fatty acid
NMR	nuclear magnetic resonance
PAP	phosphatidate phosphohydrolase
PDC	pyruvate dehydrogenase complex
PET	positron emission tomography
PFK	phosphofructokinase
PL	phospholipid
PSP	peak systolic pressure
TCA	tricarboxylic acid
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

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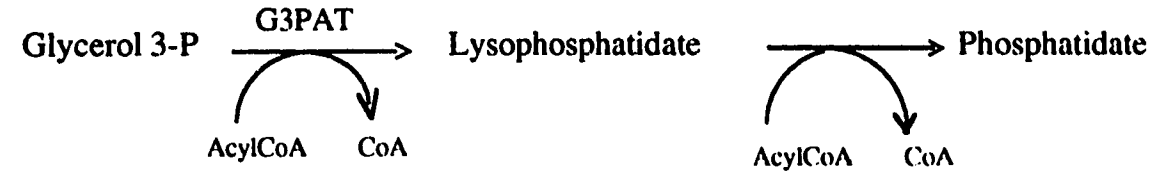
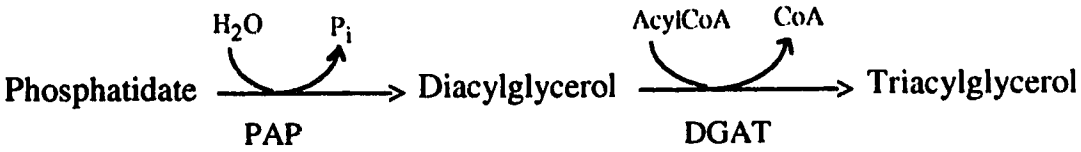
INTRODUCTION

Triglycerides represent an efficient means of storing energy in mammalian tissues. The main portion of triglycerides in our body is stored in adipose tissue from which fatty acids can be mobilized and released into the circulation. The heart uses these fatty acids to meet its ATP requirement. Fatty acids utilized by the heart can either be derived directly from the circulation (as free fatty acids) or indirectly through hydrolysis of triglyceride rich lipoproteins.

The heart, like adipose tissue, also has a mobilizable endogenous triglyceride pool. This triglyceride pool exists in both the subepicardial cardioadipocytes and in the cardiomyocytes. Triglycerides are neutral lipids and are simply a triacylated glycerol where one acyl group is attached to each one of the three glycerol oxygen atoms. Triglycerides can differ from each other depending on the acyl groups attached to these three glycerol carbon atoms.

There are three important enzymes in the triglyceride synthetic pathway: glycerol 3-phosphate acyltransferase (GPAT; the rate limiting enzyme), phosphatidic acid phosphohydrolase (PAP; probably rate limiting as well), and diacylglycerol acyltransferase (DGAT) as shown in the diagram below. Subcellular fractionation studies revealed that most of the lipogenic enzymes are recovered in the microsomal fraction. Therefore esterification and synthesis of triglyceride is believed to take place in the outer membrane of the endoplasmic reticulum (1).





Regulation of the myocardial triglyceride synthetic pathway is not as well understood as in the liver or adipose tissue. However, it has been demonstrated that it can be regulated by the concentration of exogenous fatty acids as well as by other factors including myocardial CoA levels. High levels of exogenous fatty acids are well known to enhance triglyceride synthesis but the exact mechanism for such an enhancement is not clear. However, it has been shown in the liver, that fatty acids can enhance triglyceride synthesis through enhancing translocation of PAP from the cytosol to the endoplasmic reticulum (2). It is possible that a similar mechanism may exist in the heart. In addition to being important for activating fatty acids prior to their metabolism, increases in myocardial CoA levels will shunt fatty acids towards triglyceride synthesis (3).

Myocardial triglyceride stores are also subject to lipolysis. In the heart there are two important lipases: tissue triglyceride lipase (hormone sensitive lipase) and lipoprotein lipase (transported to the capillary endothelium after being synthesized in the myocyte). Although lipoprotein lipase is known to be

released from the heart (such as following administration of heparin) (4), its major action is to catalyze the breakdown of triglycerides in triglyceride rich lipoproteins which are an important energy source to the heart. Synthesis and release of lipoprotein lipase were reported to be enhanced with catecholamines or with cAMP analogues (5,6). The two tissue triglyceride lipases can be further separated into an acid lipase (pH optimum 4.5-5.0) (7) and a neutral pH lipase (8). Myocardial triglyceride lipolysis is known to be regulated by different factors. Hormones such as catecholamines and glucagon are known to stimulate triglyceride lipolysis in the heart (see 9 for review) possibly through increasing cAMP levels (5,10). Myocardial triglyceride lipolysis is also regulated by the supply of fatty acids. Actually the presence of fatty acids has been reported to inactivate neutral triglyceride lipase (11).

Although myocardial triglyceride lipolysis and synthesis are two different and opposite processes, the two processes are inter-related. For instance, when there is an enhancement of lipolysis, the concentration of free fatty acids in the cytosol increases and this in turn activates the triglyceride synthetic pathway.

Myocardial triglycerides are known to contribute fatty acids to the heart for oxidative metabolism. Although the actual extent to which they contribute to oxidative metabolism is not clear, their relative contribution has been shown to be smaller in the presence of high concentrations of exogenous fatty acids or glucose. In contrast, triglyceride lipolysis and its contribution to myocardial energy requirement increase during exercise (or in any situation leading to increase in heart work), in the absence of exogenous fatty acids or other substrates, and with the addition of ß-adrenergic agonists (e.g., isoproterenol) (9).

In order for fatty acids to be metabolised, they first need to be activated through binding to CoA, forming acyl-CoA. However, the inner mitochondrial membrane is impermeable to acyl-CoA. Therefore, to be able to enter the inner mitochondrial membrane, acyl-CoA substitutes carnitine for its CoA moiety, a step mediated by carnitine palmitoyl transferase 1 (CPT 1). The acylcarnitine thus formed can then enter the mitochondria (via carnitine acyltranslocase) and through the action of carnitine palmitoyl transferase II (CPT II) exchange carnitine for CoA to reform acyl-CoA. Acyl-CoA is now available to enter the ß- oxidation spiral. It has been suggested that the cytosolic carnitine/CoA ratio is important in determining the fate of fatty acid taken up by the heart (12-14). The higher this ratio, more fatty acids are oxidized; the lower this ratio, more fatty acids are shunted to neutral lipid synthesis (mostly triglycerides).

Models used to study myocardial triglyceride turnover and energy substrate use:

1. Isolated cardiomyocytes:

Many studies have used isolated myocytes to study energy substrate use in these cells under both aerobic and hypoxic conditions. The major problem with these studies, however, is that these cells though contracting, are subjected to less work load compared to the working heart. As a result, the ATP requirement of these cells is lower than their *in vivo* counterparts. Therefore, oxidative rates obtained in these studies may be quite different from those seen in the functioning organ. Furthermore, using radiolabeled carbon substrates in these cells to measure energy substrate metabolism requires longer periods of

incubation to reach steady state rates due to these lower metabolic rates. In addition, these cell preparations are not ideal to study metabolic changes during or following ischemia because hypoxia and ischemia are not synonymous. While hypoxia is one part of ischemia, other changes during ischemia (such as defective washout of metabolic intermediates) is lacking in hypoxia.

2. In vivo studies:

Studies *in vivo* usually rely on measuring arterio-venous differences of various substrates and/or oxygen concentration. These differences are used to calculate substrate use rate. Myocardial triglyceride turnover in these studies can be determined by measuring myocardial triglyceride content in biopsies obtained from the heart. The major problem with these studies is the assumption that the arterio-venous differences in substrate concentration represents the rate of utilization of that substrate. This assumption has been shown to be incorrect since it overlooks the fact that some substrates are taken up by the heart but are not necessarily oxidized (e.g., glucose). In addition some endogenous substrates can be released by the heart and appear in the coronary sinus (e.g., fatty acids). This can lead to erroneous calculations of oxidation and uptake rates. In some of these studies more elegant techniques are used e.g., the use of [^{14}C]labeled substrates and measuring $^{14}CO_2$ production through sampling of blood from the coronary sinus. This methodology, however, is limited by the complexity of the procedures, number of subjects available, as well as by safety requirements and costs.

3. Positron emission tomographic techniques (PET) and Nuclear magnetic resonance (NMR) techniques:

PET is a new technique which has been recently applied in many studies to investigate myocardial metabolism under different conditions, including myocardial ischemia. NMR, on the other hand, can be useful to determine changes in cellular high energy nucleotides (usually through using ^{31}P NMR spectra) and calcium levels (using an NMR-detectable Ca^{2+} indicator, e.g., 5F-BAPTA) during certain events like ischemia. Besides being expensive, caution is required in interpreting results obtained using these techniques. Limitations of PET will be discussed in the following chapters. In addition, the NMR technique lacks the ability of easily providing quantitative data. However, they can be useful.

4. Isolated heart preparations:

Isolated intact hearts are widely accepted as efficient models to study myocardial metabolism. Two main models are in use, the Langendorff perfused heart and the isolated working heart. The Langendorff heart was introduced a century ago and involves retrograde perfusion of the aorta. The working heart model, first described by Morgan *et al.* (15), involves a left atrial cannulation and allows the left ventricle to pump against an afterload. The working heart has the advantage of providing an orthograde perfusion to the heart chambers as well as an ejecting left ventricle.

The isolated working heart preparation is suitable to study cardiac metabolism since oxidative rates can be measured directly under conditions of controlled workload. One disadvantage of the isolated heart model is the absence of neurohumoral regulation. However, this can be turned into an

advantage in studies in which neurohumoral effects on metabolism are determined, since hormones can be introduced into the system in a controlled manner. In addition, simultaneous measurements of metabolism and mechanical function in response to neurohumoral factors can be determined since different factors can be adjusted (e.g., preload, afterload, etc.) and monitored (e.g., heart rate, contractility, etc.) at the same time.

In the following sections myocardial triglyceride turnover and energy substrate use under different conditions will be discussed. Myocardial metabolism of unsaturated fatty acids will be discussed as well.

I. *Myocardial triglyceride turnover in aerobic hearts:*

It is well known that circulating fatty acids are an important myocardial energy source that provides 60-70% of the energy requirement of the heart (16). The majority of these fatty acids are derived from circulating lipoproteins or free fatty acids bound to albumin. Although previously regarded by some as a pure *in vitro* phenomenon (17), myocardial triglycerides can also contribute fatty acids for oxidative metabolism (18,19). The concentration of exogenous fatty acids plays an important role in the regulating triglyceride lipolysis (18). Increasing the concentration of exogenous fatty acids inhibits endogenous triglyceride lipolysis in isolated perfused hearts. High concentrations of fatty acids also stimulate triglyceride synthesis, possibly through stimulating the enzyme PAP (20). Although several studies have addressed the topic of triglyceride lipolysis in myocardial tissue (21-28), measurements of triglyceride turnover have only been indirect, i.e. by measuring glycerol release by the myocardium and/or myocardial triglyceride content. Myocardial triglyceride

content is not only variable among hearts, but measuring triglyceride content alone overlooks the fact that substantial triglyceride lipolysis may take place without measured changes in myocardial triglyceride content. This can arise due the concurrence of triglyceride synthesis during lipolysis. Glycerol production *per se* has never been documented as a good measure of triglyceride lipolysis and lipolysis of triglycerides into mono- or di-acylglycerols with subsequent triglyceride synthesis can result in an underestimation of triglyceride lipolysis when using glycerol as a measure of this lipolysis. In addition, phospholipids can also be a significant source of glycerol, especially following ischemia (28).

In addition to fatty acids, glucose is another major energy substrate for the heart. It has been shown that high levels of fatty acids interfere with glucose utilization; both glycolytic flux and glucose oxidation are inhibited by fatty acids (16,29-31) primarily through inhibition of both PFK, the rate limiting enzyme in the glycolytic pathway (31), and pyruvate dehydrogenase (PDC), the rate limiting enzyme in glucose oxidation, respectively (32). Several studies suggested that glucose contributes from 15% to 55% of oxidative metabolism of human hearts under fasting conditions (19,33-36) and that glucose extraction significantly increases during acute hyperglycemia where it becomes the major substrate for oxidative metabolism in the heart (37-39). However, in all these studies no direct measurement of glucose oxidation was undertaken and results were built on the assumption that all the glucose taken up by the heart is eventually oxidized. This assumption is inaccurate since Wisneski *et al.* (40) recently demonstrated in humans that despite an increase in glucose uptake during hyperglycemia, only 32% of the glucose extracted was oxidized,

suggesting that primary fate of the extracted glucose is not oxidative metabolism and the majority of it is probably stored as glycogen.

In addition to using fatty acids and glucose as carbon substrates for oxidative metabolism, the heart is also able to use lactate, acetate, pyruvate and under certain circumstances (especially starvation and diabetic ketosis) ketone bodies. Aminoacids are known to be minor energy substrates for the heart except when their levels increase.

Despite the importance of fatty acids and glucose to oxidative metabolism, the relationship between myocardial fatty acid oxidation (both exogenous and endogenous) and glucose use in the heart is still incompletely understood. This relationship can change dramatically in the presence of different concentrations of exogenous fatty acids, a situation that can readily occur under both physiologic and pathologic conditions. It seems fruitful therefore, to study systematically this relationship.

II. *Myocardial triglyceride turnover in reperfused/ischemic hearts:*

High levels of fatty acids are detrimental to the ischemic myocardium. Fatty acids and/or their intermediates have been shown to be arrhythmogenic (41-43), to interfere with suborganelle performance and with membrane integrity (44-47), and to decrease mechanical function during reperfusion (29,48-51). However, the mechanism behind this detrimental effect of fatty acids in ischemic injury is not yet clear. While it has been suggested by some that this detrimental effect is related to the build up of potentially harmful intermediates of fatty acid metabolism, namely long chain acyl-CoA and acylcarnitine (44-46), results from

our laboratory (29,30) and others (16) have indicated that fatty acids may exert part of their detrimental effect by interfering with myocardial glucose utilization during reperfusion following ischemia. For instance, a previous study in our lab (29) has shown that etomoxir, an agent which significantly improves recovery of mechanical function of ischemic hearts during reperfusion, also significantly increased glucose oxidation rates during reperfusion. This can occur at concentrations of etomoxir that do not affect levels of fatty acid intermediates.

A review of the literature demonstrates two major shortcomings of many of the previous studies which have indicated accumulation of fatty acid intermediates as a contributing mechanism to ischemic injury: First, in many of these studies isolated hearts perfused in the absence of fatty acids were used, a condition which itself markedly alters myocardial levels of fatty acid intermediates. Second, in some of these studies the detrimental effect of fatty acid intermediates was observed in cell free mixtures using relatively high concentrations of these intermediates. As a result, these studies may not reflect true cellular events, since there are several regulatory steps inside the cell which can maintain levels of these intermediates, namely the fatty acid binding proteins and the incorporation of these intermediates into the myocardial triglyceride pool.

Myocardial triglycerides are potentially important source of fatty acids in the ischemic and reperfused heart. The role of myocardial triglycerides in ischemic injury is still not clear. In intact animals, accumulation of triglycerides occurs at the periphery of the infarct zone (47), and an increase in myocardial triglyceride content can be seen in subepicardial (52) and subendocardial regions (53) of the heart. In contrast, no change in myocardial triglyceride content has also been reported (54). As a result, whether myocardial

triglyceride turnover changes during and following myocardial ischemia is still controversial. While Crass *et al.* (18,55) reported that triglyceride turnover is inhibited during ischemia and hypoxia, van Bilsen *et al.* (25), using glycerol accumulation as a measure of triglyceride lipolysis, have suggested that triglyceride turnover is accelerated during ischemia and ends immediately after restoration of flow. Van Bilsen *et al.* have also suggested that such an acceleration during ischemia can be harmful to the ischemic myocardium, since this turnover is expensive energetically (i.e. ATP is required for the esterification of fatty acids). Therefore, acceleration of triglyceride turnover could potentially deplete intracellular ATP levels which are already reduced in the ischemic myocardium (25). These authors have referred to this accelerated turnover of myocardial triglycerides during ischemia as the "futile cycle". In contrast, Schoonderwoerd *et al.* (20) suggested that triglyceride turnover during ischemia is beneficial by scavenging free fatty acids from the cytosol and thus inhibiting the accumulation of fatty acids and their intermediates to toxic levels. Again, however, all of the above studies have used an indirect approach to measure myocardial triglyceride turnover. Therefore, a direct method to measure triglyceride turnover during reperfusion of ischemic hearts would be desirable in order to understand the bioenergetics of the reperfused/ischemic myocardium. Such an understanding may be important in the sense that improving these energetics could have an impact on reperfusion recovery of mechanical function.

III. *Myocardial triglyceride turnover in diabetes mellitus:*

As mentioned previously, endogenous myocardial triglycerides are a potentially important source of fatty acids for ß-oxidation (19,22,55). The rate of lipolysis of myocardial triglyceride, and its contribution to overall myocardial ATP production, appears to be inversely related to the concentration of exogenous fatty acids (18). Levels of exogenous fatty acids are also a key determinant of glucose utilization by the heart (16,29). In the presence of high concentrations of fatty acids both glycolysis and glucose oxidation rates decrease (16,29,30). An example of this is diabetes, in which plasma levels of both free fatty acids and triglyceride-rich lipoproteins increase. This can lead the heart to become almost entirely dependent on fatty acid oxidation to meet its energy requirements (56-58).

Myocardial triglyceride content can be markedly increased in diabetes (59-62). However, the relative contribution of this expanded pool to overall myocardial energy requirements, and the relationship between endogenous and exogenous fatty acid oxidation and glycolysis and glucose oxidation in hearts of diabetic animals has not been well characterized. In addition, many previous studies have been performed on isolated hearts perfused in the absence of exogenous fatty acids, despite the fact that levels of plasma free fatty acids and triglyceride rich lipoproteins increase in diabetes.

An element of cardiac dysfunction is known to exist in diabetes and has been attributed to changes in myocardial energy substrate use (63-66). Agents which optimize glucose metabolism have been shown to improve myocardial performance of isolated diabetic hearts when added to the perfusing medium (67). It is important therefore to have a better understanding of myocardial

triglyceride turnover in diabetes mellitus since it may serve as a source of fatty acids which inhibit myocardial glucose use.

IV. Acetyl-CoA carboxylase (ACC) regulation of fatty acid oxidation in the heart:

ACC is known to be a key regulatory enzyme in fatty acid synthesis. It catalyses carboxylation of acetyl-CoA into malonyl-CoA (78). Since malonyl-CoA regulates fatty acid oxidation through inhibiting carnitine palmitoyltransferase I (CPT I), ACC probably plays a pivotal role in regulating fatty acid oxidation as well. It is widely distributed in different tissues including those where fatty acid synthesis is not prominent, e.g., the heart and skeletal muscle (79-82). Two isoforms (265- and 280-kDa) are known to exist so far (79,80) and more isoforms are thought to exist as well (83,84). Much attention has been given to study ACC-265 (predominant in highly lipogenic tissues, e.g., liver and white adipose tissue) which has been cloned recently while little is known about ACC-280 which is predominant in low lipogenic tissues (79,80), e.g., heart and skeletal muscle. Whether these two isoforms are differentially regulated or serve different functions is not yet known. However, Bianchi *et al.* (80) have suggested that this isozyme could be important in regulating fatty acid oxidation in heart and skeletal muscle through forming malonyl-CoA. Unfortunately, fatty acid oxidation rates were not measured in their study. Studies designed to correlate fatty acid oxidation to ACC activity in the heart are therefore required.

ACC was shown to be regulated over two different time frames. A rapid regulation (minutes) involves changes in covalent phosphorylation, allosteric regulation and polymerization (85). A long term (hours-days) regulation

involves changes in enzyme content (86) together with changes in enzyme activity caused by the aforementioned rapid regulation (87,88). While ACC was reported to be dependent on citrate for activation (89,90), Thampy *et al.* (88) found that it is the phosphorylated form of the enzyme that is citrate dependent; quickly frozen livers yielded a more active, citrate-independent ACC with lower phosphate content. Davies *et al.* (91) recently reported that the higher phosphorylated state of ACC was seen when freeze-clamping was not used and that this higher phosphorylation correlated with a large increase in AMP and decrease in ATP (presumably caused by hypoxia during removal of the liver) and with increased activity of AMP-activated protein kinase.

V. Purpose of this study:

The necessity for a new model to measure myocardial triglyceride turnover is thus apparent. Therefore, a new technique is developed to measure directly triglyceride turnover in the heart using radiolabeled substrates. In short, hearts were perfused first in the working mode with Krebs-Henseleit buffer containing 11 mM glucose + 1.2 mM [1-^{14}C]palmitate to label the endogenous lipid pools (pulse). $^{14}CO_2$ was collected during this "pulse" perfusion and was used as a measure of exogenous [^{14}C]palmitate oxidation (see Figure 1). After a washout retrograde perfusion (or ischemia) the hearts were perfused again in the working mode with Krebs-Henseleit buffer containing 11 mM glucose in the presence or absence of [9,10-^{3}H]palmitate ("chase" perfusion). $^{14}CO_2$ production during the "chase" was a measure of endogenous [^{14}C]palmitate oxidation while $^{3}H_2O$ production was a measure of exogenous [^{3}H]palmitate oxidation. Another series of hearts was perfused using the same protocol

except that 1.2 mM unlabeled palmitate was used during the "pulse" and [5-^{3}H/U-^{14}C]glucose in the presence or absence of unlabeled palmitate was used during the "chase" (see Figure 2). In this series of hearts $^{14}CO_2$ production was used as a measure of glucose oxidation while $^{3}H_2O$ was a measure of glycolysis. Heart mechanical function was monitored throughout the perfusion protocols. After each perfusion protocol, hearts were frozen to the temperature of liquid nitrogen and were used later to determine levels of different metabolites. By measuring rates of exogenous and endogenous fatty acid oxidation together with rates of glycolysis and glucose oxidation, it was possible to calculate the relative contribution of myocardial triglyceride to overall ATP production in the heart.

Finally, many studies were conducted to study the enzyme acetyl-CoA carboxylase. Most of these studies were conducted on highly lipogenic tissues like liver and white adipose tissue. Recently a second isozyme of this enzyme has been found to predominate in low lipogenic tissues like the heart and has been suggested to regulate fatty acid oxidation in these tissues. However, no study has been designed previously to directly correlate cardiac enzyme activity with fatty acid oxidation in the heart. A new study to characterize this enzyme in the heart in terms of its regulation is therefore required. In addition, measurement of this enzyme activity in the heart should be correlated with myocardial fatty acid oxidative rates since as mentioned above, this enzyme may play an important role in regulating fatty acid oxidation. As a result, a set of experiments were performed where both enzyme activity and fatty acid oxidation were measured in the heart.

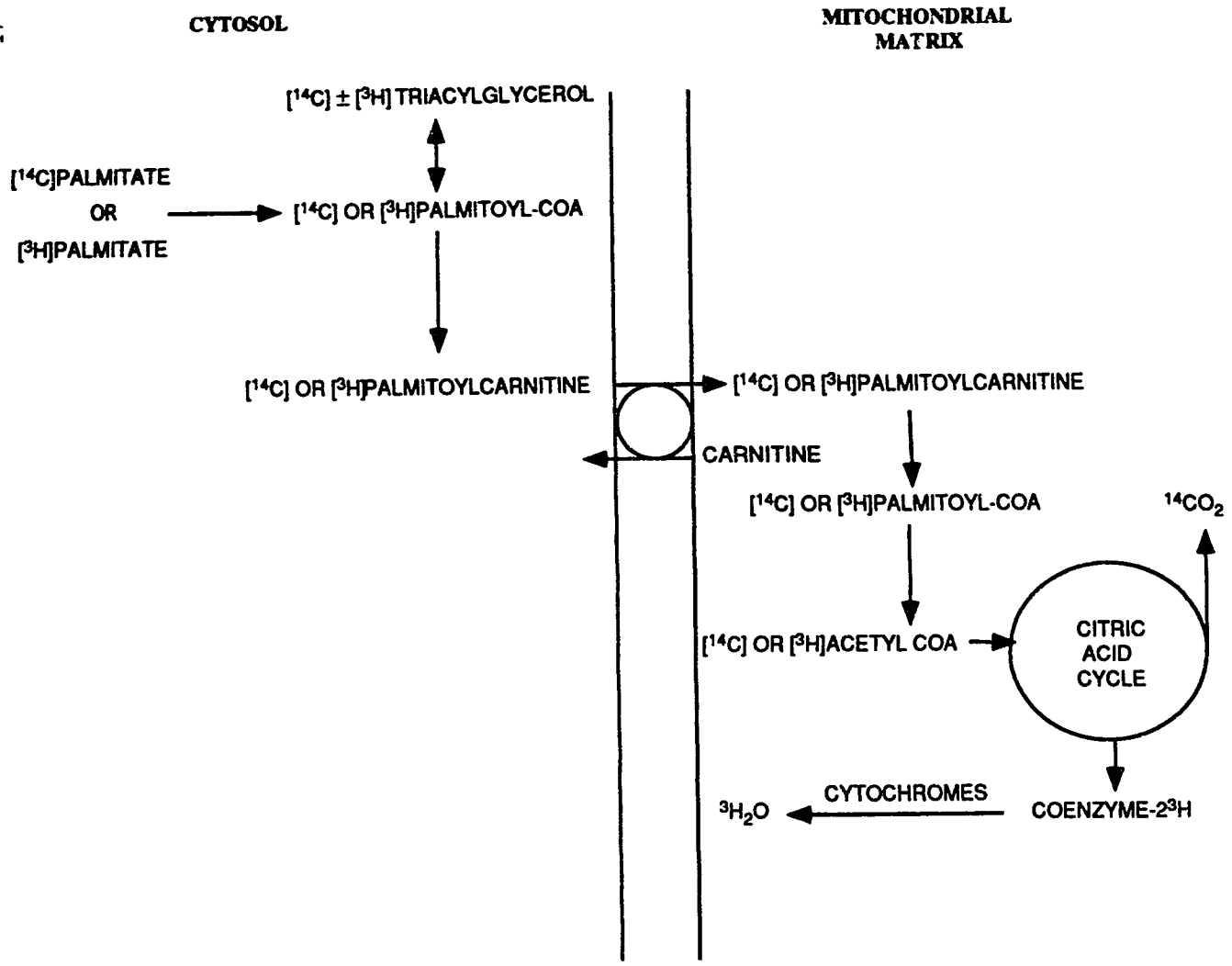


Figure 1. Fate of radio-labeled palmitate in the heart

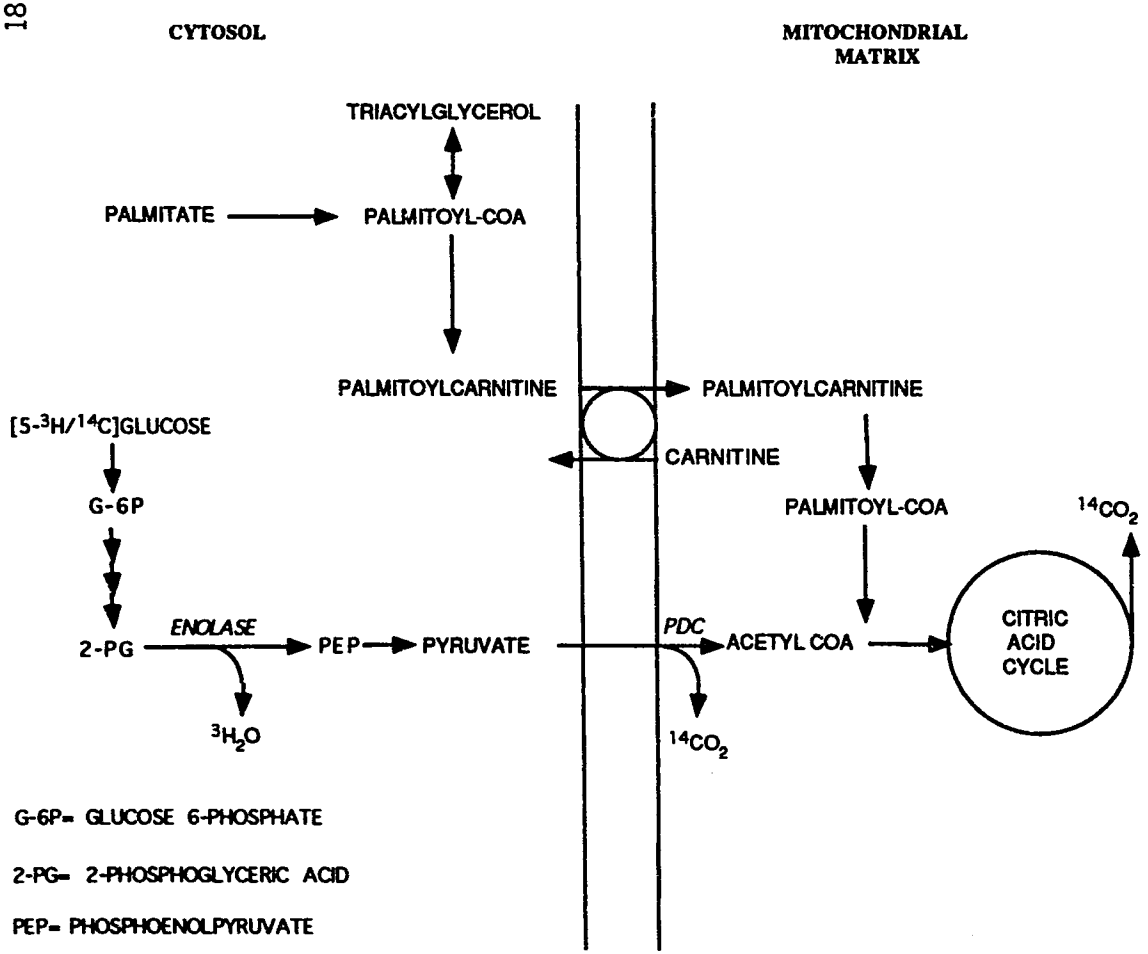


Figure 2. Radio-labeled products of glycolysis and glucose oxidation

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CHAPTER 2

MYOCARDIAL TRIGLYCERIDE TURNOVER AND CONTRIBUTION TO ENERGY SUBSTRATE UTILIZATION IN ISOLATED WORKING RAT HEARTS

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My role in this study involved performing all the experimental part and the writing-up of the paper.

CHAPTER 2

MYOCARDIAL TRIGLYCERIDE TURNOVER AND CONTRIBUTION TO ENERGY SUBSTRATE UTILIZATION IN ISOLATED WORKING RAT HEARTS

Introduction

Circulating fatty acids are an important myocardial energy source that provides 60-70% of the heart's energy requirements (1). Endogenous myocardial triglycerides, through lipolysis, are also a potential source of fatty acids for oxidative metabolism (2,3). The concentration of exogenous fatty acids plays an important role in the regulation of triglyceride lipolysis (3); increasing the concentration of exogenous fatty acids inhibits endogenous triglyceride lipolysis in isolated perfused hearts and stimulates triglyceride synthesis, possibly through stimulating the enzyme PAP (4). Because of the potential importance of triglycerides as an extended substrate source, several studies have addressed the topic of triglyceride lipolysis in myocardial tissue (5-10). For the most part, however, measurements of triglyceride turnover have been indirect, i.e. by measuring glycerol release by the myocardium. To our knowledge, no studies have measured myocardial triglyceride turnover directly. In addition, most of the studies conducted on intact isolated heart preparations have used perfusates devoid of fatty acids.

The other major substrate for the aerobically perfused heart is glucose. It has been well established that high levels of fatty acids interfere with glucose utilization; both glycolytic flux and glucose oxidation are inhibited by fatty acids (1,11,12). This occurs primarily through inhibition of both PFK, the rate limiting enzyme in the glycolytic pathway (13), and PDC, respectively (14). It

has been suggested that glucose contributes from 15% to 55% of oxidative metabolism of human hearts under fasting conditions (15-20), that glucose extraction significantly increases during acute hyperglycemia, and that glucose becomes the major substrate for oxidative metabolism under conditions of hyperglycemia (21-23). However, Wisneski (24) recently demonstrated in humans that despite an increase in glucose uptake during hyperglycemia, only 32% of the glucose extracted was oxidized, suggesting that primary fate of the extracted glucose was not oxidative metabolism. The relationship between both endogenous and exogenous fatty acid oxidation, and both glycolytic flux and glucose oxidation has not been characterized.

In this study, we developed a protocol to measure triglyceride turnover directly in isolated working rat hearts. This was achieved by pre-labeling the triglyceride pool during a "pulse" perfusion with [1-^{14}C]palmitate, and subsequently measuring the rate of endogenous triglyceride fatty acid oxidation during a "chase" perfusion. During "chase", exogenous fatty acid oxidative rates were also determined. By measuring glucose utilization (both glycolysis and glucose oxidation) in a parellel series of hearts perfused under similar conditions, we were able to determine the contribution of endogenous triglycerides to overall myocardial ATP production. This was performed in the absence and presence of low and high concentrations of exogenous fatty acids.

Materials and Methods:

Materials

D-[^{14}C(U)]glucose, D-[2-^{3}H(N)]glucose, [9,10-^{3}H]palmitic acid, and [1-^{14}C]palmitic acid were obtained from NEN (Wilmington, DE). Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1M in methanol solution) was obtained from NEN Research Products (Boston, MA). Dowex 1X-4 anion exchange resin (200-400 mesh chloride form) was obtained from Bio-Rad Laboratories (Richmond, CA). ACS Aqueous Counting Scintillant was obtained from Amersham Canada Ltd (Oakville, Ontario). Baker Si250-PA (19C)-silica gel plates were obtained from Johns Scientific (Toronto, Ontario). Triglyceride assay kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

***Heart Perfusions*:**

Adult male Sprague Dawley rats (200-250 g) were anesthetized with sodium pentobarbital (60 mg/kg). Hearts were quickly excised and placed in ice-cold Krebs Henseleit buffer. Within one minute, the aorta was cannulated, and a retrograde perfusion using Krebs Henseleit buffer initiated, as described previously (25). During this initial perfusion, each heart was trimmed of excess tissue, the pulmonary artery was cut, and the opening to the left atrium was cannulated. Following a 10 minute equilibration period, hearts were switched to the working heart mode, and perfused at a 11.5 mm Hg left atrial preload and a 80 mm Hg aortic afterload, as described previously (11,25). Spontaneously

beating hearts were used throughout the studies, with heart rate and peak sytolic pressure (PSP) being measured by a Gould P21 pressure transducer in the aortic outflow line. All hearts were perfused with Krebs-Henseleit buffer containing 11 mM glucose, 3% albumin, and 2.5 mM free Ca^{2+}. Hearts were also perfused in the presence or absence of palmitate, as indicated in the individual experiments. When used, palmitate was pre-bound to the albumin, as described previously (25). The radioisotopes of glucose or palmitate that were used are also indicated in the individual experiments.

Perfusion Protocols

The protocol for the first series of heart perfusions are shown in Table 1. Initially, all hearts in this series were perfused for 60 minutes with re-circulated Krebs Henseleit buffer containing 1.2 mM [1-^{14}C]palmitate (specific activity = 160 Bq/μmol) to label the endogenous lipid pools (pulse). During this labeling period, exogenous steady state fatty acid oxidation was also measured by quantitative collection of myocardial $^{14}CO_2$ production (as described below). Additional preliminary experiments were also performed in which the "pulse" period was extended, in order to determine the optimal labeling periods. At the end of the "pulse" period, hearts were switched to a retrograde Langendorff drip-out perfusion with unlabeled Krebs-Henseleit buffer. During this 10 minute period, buffer containing [^{14}C]palmitate was removed from the system, and replaced with buffer devoid of fat (no fat), 0.4 mM [9,10-^{3}H]palmitate (low fat) (specific activity = 474 Bq/μmol), or 1.2 mM [9,10-^{3}H]palmitate (high fat)(specific activity = 194 Bq/μmol). One group of hearts (control group) were frozen at the end of this Langendorff perfusion (with Wollenberger tongs cooled to the temperature of liquid N_2) for measurements of endogenous label

and lipid content. Three other groups of hearts were switched back to the working mode, and perfused for a subsequent 60 minute period with the new buffers described above. In order to determine how much [^{14}C]palmitate was left in the system at the very beginning of "chase", and whether there was any back diffusion of fatty acids released from triglyceride pools to the perfusate, perfusate samples were taken at the beginning and at the end of "chase" and their ^{14}C-palmitate content determined.

In the second series of heart perfusions, the same perfusion protocol and perfusion substrates as described above were used, except that palmitate was not labeled during the "pulse" period (Table 2). In addition, the "chase" perfusion contained 11 mM [2-^{3}H/U-^{14}C]glucose (specific activity = 36 Bq of [^{3}H]/μmol glucose and 63 Bq of [^{14}C]/μmol glucose) instead of labeled palmitate.

In all hearts, mechanical function was monitored throughout the entire perfusion. At the end of the Langendorff washout perfusion following the pulse, or at the end of "chase", heart ventricles were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. The frozen ventricular tissue was then 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 ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 hr at 100° C, and weighed. The dried atria weight, frozen ventricular weight, and ventricular dry-to-wet ratio was then used for determination of total dry weight of the heart.

Measurement of exogenous and endogenous fatty acid oxidative rates

During the pulse, steady state exogenous palmitate oxidation rates were determined by quantitatively measuring $^{14}CO_2$ production by hearts, as described in detail previously (11,12,25). Hearts were perfused in a closed system that allowed collection of both perfusate and gaseous $^{14}CO_2$. The $^{14}CO_2$ liberated into the gaseous phase was trapped in a 1 M hyamine hydroxide solution in the air outlet line. Both perfusate and hyamine hydroxide samples were obtained at 20 minute intervals throughout the initial 60 minute pulse period. Perfusate samples (3 ml each) were immediately injected below a 1ml volume of mineral oil to prevent liberation of perfusate $^{14}CO_2$. The $^{14}CO_2$ from the perfusate was subsequently extracted by injecting 1 ml of perfusate into 1 ml of 9N H_2SO_4 contained in sealed metabolic vials, which also contained 400 µl of 1 M hyamine hydroxide in suspended center wells (25). Vials were gently shaken for 1 hr and the center wells removed and counted in ACS scintillant using standard scintillation counting procedures.

During the "chase", $^{14}CO_2$ production was used as a measure of endogenous fatty acid oxidation, while $^{3}H_2O$ production was a measure of exogenous fatty acid oxidation rates. Perfusate and gaseous samples were collected at 10 min intervals during the "chase", and $^{14}CO_2$ production measured as described above. During this period exogenous steady state palmitate oxidation was determined by measuring $^{3}H_2O$ levels in the perfusate samples. $^{3}H_2O$ was separated from [^{3}H]palmitate by treating 0.5 ml buffer samples with a 1.88 ml of a mixture of chloroform:methanol (1:2 vol:vol) and then adding 0.625 ml chloroform and 0.625 ml of a 2M KCl:HCl solution. The aqueous phase was then collected using a Pasteur pipette and was subsequently treated with a mixture of chloroform, methanol, and KCl:HCl with a ratio of 1:1:0.9. Two 0.5 ml

samples of the aqueous phase were then counted for each perfusate sample for total ^3H_2O determination, taking into account the dilution factor. This technique resulted in a greater than 99.7% extraction and separation of ^3H_2O from the [^{3}H]palmitate. Exogenous palmitate oxidation rates during the "chase" were expressed as nmol [^{3}H]palmitate oxidized/min·g dry wt, while endogenous oxidation rates were expressed as nmol [^{14}C]palmitate oxidized/min·g dry wt.

Measurement of glycolysis and glucose oxidation rates

In the second series of hearts, in which the "chase" perfusate contained 11 mM [2-^{3}H/U-^{14}C]glucose, quantitative ^3H_2O production was used to measure steady state glycolytic rates (^3H_2O is liberated at the phosphoglucoisomerase step of glycolysis), while quantitative $^{14}CO_2$ production was used to measure glucose oxidation rates ($^{14}CO_2$ is liberated at the level of PDC and in the TCA cycle). $^{14}CO_2$ production was determined using the same methods described above for palmitate oxidation. To measure glycolysis, ^3H_2O was separated from [^{3}H]glucose and [^{14}C]glucose as described by Kobayashi and Neely (27), using columns containing Dowex 1-X4 anion exchange resin (200-400 mesh) suspended in 0.2 M potassium tetraborate (the volume of resin was 0.5 x 0.5 cm). The Dowex in the columns was extensively washed with H_2O prior to use. A 0.2 ml volume of perfusate was then added to the column and eluted into scintillation vials with 0.8 ml H_2O. Following addition of ACS scintillant, the samples were subjected to standard double isotope counting procedures, with the windows set at 0-300 nm (^{3}H) and 400-670 nm (^{14}C). The Dowex columns were found to retain 98-99.6% of the total [^{3}H]glucose and [^{14}C]glucose present in the perfusate. The ^3H_2O (which passes through the column) was corrected for the small amount of [^{3}H]glucose that passed through the column.

This could be accomplished since an equal amount of [^{14}C]glucose also passed through the column, and could be used as an internal standard for the degree of [^{3}H]glucose contamination in the $^{3}H_2O$ sample. Correction was also made for the degree of spillover of ^{14}C into the ^{3}H counting window, by measuring this degree of spillover using standards containing only [^{14}C]glucose.

Glucose utilization was expressed as nmol glucose metabolised/min·g dry wt.

Measurement of metabolic intermediates:

Tissue lipids were extracted as previously described (11,25). Neutral lipids were separated from phospholipids using the methods described by Bowyer and King (28). Triglycerides were separated from other neutral lipids using Baker Si250-PA (19C)-silica Gel plates and a solvent system that consisted of isooctane:diethyl ether:acetic acid (74:24:2 vol/vol/vol) (25). [1-^{14}C]palmitate and ^{3}H-palmitate incorporation into triglycerides, other neutral lipids, and phospholipids was measured using double radioisotope counting techniques. Label content of all lipids was expressed as μmol palmitate incorporated into these pools/g dry wt. Absolute triglyceride content (μmol free fatty acid equivalents/g dry wt) was determined using Wako enzymatic colorimetric assay kits.

Statistical analysis:

Data are presented as the mean ± SE of the mean. Analysis of variance, followed by the Neuman-Keuls test was used in the determination of statistical difference in groups containing 3 or more sample populations. The unpaired Student's t-test was used to determine statistical significance in groups containing 2 sample populations. A value of $P< 0.05$ was regarded as significant.

Results:

Measurements of heart function

Heart mechanical function in the spontaneously beating working hearts was monitored throughout the perfusion protocol. Table 3 shows the heart rate, peak systolic pressure (PSP) development, and the heart rate-pressure product obtained during the "pulse" perfusion and during the "chase" perfusion. No significant deterioration of heart function was seen throughout the 2 hr perfusion period in hearts perfused with no fat. The presence or absence of either low fat or high fat in the perfusate was also without significant effect on heart function, although the PSP in hearts chased with high fat was slightly but significantly lower. The triglyceride turnover and energy substrate utilization measurements made in this study, therefore, were not complicated by major changes in heart work.

Labeling of endogenous myocardial lipids during the "pulse" perfusion

Initial experiments were performed to determine the optimal conditions for labeling myocardial lipids with [^{14}C]palmitate. A concentration of 1.2 mM [^{14}C]palmitate was used in the perfusate since we have previously observed that perfusion of isolated working hearts with this concentration of fatty acid does not deplete myocardial triglyceride stores (26). Hearts were initially "pulsed" with 1.2 mM [^{14}C]palmitate for either a 60 minute or 120 minute period, followed by a 10 minute dripout perfusion as Langendorff hearts in the absence of labeled fatty acids. [^{14}C]palmitate content and oxidation in hearts

subjected to a 60 minute "pulse" are shown in Table 4. As expected, during the "pulse" the majority of the [^{14}C]palmitate taken up by the hearts was oxidized to $^{14}CO_2$ (63%). The actual steady state oxidation rate during this "pulse" period was 659 ± 51 nmol [^{14}C]palmitate oxidized/min·g dry wt (n=10). The [^{14}C]palmitate taken up by the heart which was not oxidized was primarily incorporated into triglyceride stores (~ 86%), with a small amount being incorporated into phospholipids (~ 10%). If hearts were "pulsed" for 120 minutes, a further increase in [^{14}C]palmitate incorporation into triglycerides was not seen (22.55 ± 4.15 (n=5) vs 20.41 ± 3.22 μmol/g dry wt, in hearts "pulsed" for 120 minutes or 60 minutes, respectively). An increase in [^{14}C]palmitate incorporation into phospholipids was seen in hearts "pulsed" for the longer period (4.7 ± 0.8 vs 2.3 ± 0.5 μmol/g dry wt in hearts "pulsed" for 120 minute or 60 minutes, respectively). Since the objective of this study was to maximally label the triglyceride pool, all further experiments were performed using a 60 minute "pulse" period.

A series of hearts (n=8) were also "pulsed" for a 60 minute period in the presence of 0.4 mM [^{14}C]palmitate. This resulted in a decreased amount of [^{14}C]palmitate incorporated in triglycerides (5.18 μmol [^{14}C]palmitate/g dry wt) compared to hearts perfused with 1.2 mM [^{14}C]palmitate. Overall myocardial triglyceride content also decreased under these perfusion conditions to 12.87 μmol free fatty acid equivalents/g dry wt (normal triglyceride levels in unperfused rat hearts [hearts that are quickly excised and frozen] are 50-60 μmol free fatty acid equivalents/g dry wt). As expected, "pulsing" hearts with 1.2 mM [^{14}C]palmitate did not deplete myocardial triglycerides (Table 4).

Perfusion of hearts for a 60 minute "pulse" period resulted in labeling of 42% of the total myocardial triglyceride stores, without changing the size of the overall pool size (Table 4). The 60 minute "pulse" period with 1.2 mM [^{14}C]palmitate was therefore used in all subsequent experiments involving triglyceride turnover.

Endogenous triglyceride turnover and oxidation during the "chase" perfusion

During the 60 minute "chase" perfusion, [^{14}C]palmitate was not present in the perfusate. Instead, hearts were perfused with buffer containing 11 mM glucose, 3% albumin, in the presence of either no fat, low fat, or high fat (Table 1). To measure exogenous fatty acid oxidation during the "chase" period in the low fat and high fat groups, [9,10-^{3}H]palmitate was present in the perfusate. The production of $^{3}H_2O$ during the "chase" is a measure of exogenous palmitate oxidation. The time course of $^{3}H_2O$ production during the "chase" period is shown in Figure 1. In both the low fat and high fat groups, exogenous [^{3}H]palmitate oxidation was linear throughout the 60 minute "chase" period. The steady state rates of [^{3}H]palmitate oxidation are shown in Table 5. As expected, exogenous fatty acid oxidation rates were significantly greater in hearts perfused with high fat. The validity of using this approach to measure exogenous palmitate oxidation during the "chase" is demonstrated by the comparison of the steady state [^{3}H]palmitate oxidation rates during the "chase" to steady state [^{14}C]palmitate oxidation rates during the "pulse". In hearts perfused with 1.2 mM palmitate (high fat), steady state exogenous [^{14}C]palmitate

oxidation during the "pulse" was 659± 51 nmol [^{14}C]palmitate oxidized/min·g dry wt (n=10), compared to 633 ± 60 nmol of exogenous [^{3}H]palmitate oxidized/min·g dry wt during the "chase" (n=9). In hearts perfused with 0.4 mM palmitate (low fat), steady state exogenous [^{14}C]palmitate oxidation during the "pulse" were 454 ± 45 nmol [^{14}C]palmitate oxidized/min·g dry wt (n=8), compared to 361 ± 68 nmol of exogenous [^{3}H]palmitate oxidized/min·g dry wt during the "chase" (n=6).

Rates of oxidation of myocardial derived [^{14}C]palmitate during the "chase" are shown in Figure 2. Interestingly, a linear rate of [^{14}C]palmitate oxidation occurred within 20 minutes in all three experimental groups, and was maintained throughout the 60 minute "chase" period. This demonstrates that [^{14}C]palmitate contained in the endogenous lipid pools was being released at a constant rate within each experimental group throughout the 60 minute "chase" period.

Table 5 shows the steady state rates of endogenous [^{14}C]palmitate oxidation during the "chase" period in the three experimental groups. The loss of [^{14}C]palmitate from myocardial lipids during this 60 minute "chase" period is shown in Table 4. The vast majority of this endogenously derived palmitate was derived from triglycerides (greater than 90%). The contribution of [^{14}C]palmitate from phospholipids, and of neutral lipids other than triglyceride, to oxidative metabolism during the "chase" was negligible.

The most dramatic utilization of [^{14}C]palmitate from endogenous triglycerides during the "chase" was seen in hearts from the no fat group. Steady state rates of [^{14}C]palmitate were over 3 times those seen in the

low fat and high fat groups (Table 5). This was accompanied by a dramatic loss of [^{14}C]pamitate content from myocardial triglyceride stores (Table 4). Since no exogenous fatty acids were present with the buffer used in the no fat group to replace these fatty acids, a net loss of triglycerides was seen (Table 4). Comparison of the absolute triglyceride loss (from 48.1 to 17.8 μmol free fatty acid equivalents/g dry wt) to the loss of [^{14}C]palmitate from triglycerides (from 20.41 to 6.64 μmol/g dry wt), indicates that 44% of the triglyceride fatty acid loss consisted of [^{14}C]palmitate. Interestingly, this almost equals the portion of the triglyceride pool that was initially labeled with [^{14}C]palmitate (42%). This suggests that the same pool of myocardial triglycerides that was initialy labeled during the "pulse" was being lost during the no fat "chase".

Steady state oxidation rates of endogenous [^{14}C]palmitate during the "chase" were similar in both the low fat and high fat groups (Table 5). In both of these groups, endogenous triglyceride [^{14}C]palmitate made a significant contribution to overall fatty acid oxidation. In the low fat group, 20% of total fatty acid oxidation obtained from exogenous or endogenous labeled fatty acids was obtained from myocardial triglycerides. Even in the presence of high fat, 12% of labeled fatty acid oxidation was obtained from myocardial triglycerides. This demonstrates that myocardial triglycerides are an important source of fatty acids for oxidative metabolism.

The loss of [^{14}C]palmitate from triglycerides during the "chase" in the low fat and high fat groups was similar (Table 4). This would be expected, since steady state endogenous [^{14}C]palmitate oxidation rates

during the "chase" were the same in these two groups. As expected, overall triglyceride content in the high fat group did not signficantly change during the "chase" perfusion (Table 4). This occurred because the loss of [^{14}C]palmitate from triglycerides during the "chase" was accompanied by a similar degree of incorporation of [^{3}H]palmitate from the perfusate into triglycerides (Table 6). Suprisingly, however, a significant loss of overall triglycerides did occur in the low fat group (Table 4). This loss (from 48.1 to 21.7 μmol free fatty acid equivalents/g dry wt) cannot be fully explained by the significant decrease in [^{3}H]palmitate incorporation into triglycerides during the "chase" (Table 6). In addition, the loss of [^{14}C]palmitate during the low fat "chase" (from 20.4 to 12.6 μmol/g dry wt) accounts for only 30% of the total fatty acids released from triglycerides, even though 42% of the pool was initially labeled with [^{14}C]palmitate. This suggests that a greater portion of unlabeled rather than labeled fatty acids are being lost from the triglyceride during the "chase".

Myocardial glucose utilization during the "chase" perfusion

A second series of heart perfusions were performed in order to determine the contribution of glucose as an energy substrate during the "chase" perfusion. This was achieved by using perfusion protocols similar to that described for the first series, except that non-labeled palmitate was used throughout the entire protocol (Table 2). Steady state rates of glycolysis and glucose oxidation were measured simultaneously by [illegible] hearts with 11 mM [2-^{3}H/U-^{14}C]glucose during the 60 minute "chase" [illegible]

Figure 3 shows the steady state rates of glucose oxidation and glycolysis obtained during the "chase" in the no fat, low fat, and high fat groups. An interesting observation is the greater rates of glycolysis compared to rates of glucose oxidation. In the no fat group, only 47% of the glucose that passed through the glycolytic pathway was subsequently oxidized. This greater flux of glucose through glycolysis compared to glucose oxidation in the no fat group parallels what has previously been reported by Kobayashi and Neely in hearts perfused in the absence of fatty acids (27). In our study, if fatty acids were added to the perfusate a dramatic drop in glucose oxidation occurred, with only a slight decrease in glycolytic rates. This effect was most dramatic in the high fat group. In this group of hearts, only 7% of the glucose that passed through glycolysis was eventually oxidized. This observation suggests that in the intact heart, fatty acids are a much more potent inhibitor of flux through PDC than flux through PFK.

Overall myocardial utilization of palmitate and glucose

With the steady state rates of both exogenous and endogenous radiolabeled palmitate during the "chase", as well as the steady state rates of both glycolysis and glucose oxidation, it is possible to calculate overall steady state ATP production by the heart. Table 7 shows the steady state rates obtained from radiolabeled substrates during the 60 minute "chase" perfusion. Since overall triglyceride content of hearts chased with no fat and low fat decreased during the "chase" perfusion, we also calculated ATP production during the 60 minute "chase" perfusion in which non-labeled fatty acid oxidation was considered (Table 8). Values in Table 8 are expressed as overall ATP production during the 60 minute "chase", as opposed to steady state ATP

production, since it cannot be determined from this study whether non-labeled triglyceride contribution to oxidative metabolism was linear during the "chase". Table 8 assumes that all unlabeled fatty acids released from triglycerides were oxidized by the heart. To confirm this assumption we measured [^{14}C]palmitate specific activity in the perfusates at the beginning and at the end of the "chase" perfusion . The activity was higher at the beginning of "chase" (equivalent to 4.3±1.2 μmol [^{14}C]palmitate vs 2.5±0.45 at the end of chase). This suggests that all of the palmitate released from triglycerides during the "chase" was oxidized. It also demonstrates that only 3% of our original"chase" buffers were initially contaminated with [^{14}C]palmitate from the "pulse" perfusion.

An interesting observation seen in Table 7 and 8, is that if fatty acids were omitted from the perfusate a major portion of myocardial ATP production is derived from endogenous triglycerides. If only steady state oxidative rates are considered, endogenous [^{14}C]palmitate oxidation accounted for 42% of the total ATP production (Table 7). Overall, endogeneous triglycerides contributed to 59% of total ATP production during the "chase" period.

As expected, if fatty acids were present in the perfusate, the contribution of glucose to ATP production significantly decreased. The contribution of glucose utilization to steady state ATP production from radiolabeled substrate decreased from 58% in the no fat group to 24% in the low fat group, and 8% in the high fat group (Table 7). If overall ATP production during the "chase" was measured, glucose utilization decreased from 41% in the no fat group, to 15% in the low fat group, to 8% in the high fat group.

Another observation from Table 7 and 8 is the contribution of endogenous triglycerides to ATP production in hearts perfused with low fat and high fat. As expected, exogenous fatty acids were the major source of both

steady state and overall ATP production in both groups. In the low fat group, however, endogenous [^{14}C]palmitate contributed to 15% of steady state ATP production (Table 7), while exogenous fatty acids contributed to 39% of overall ATP production (Table 8). Even in the presence of high fat, 11% of steady state ATP production was derived from triglycerides. A similar contribution to overall ATP production was seen in the high fat group (Table 8), since myocardial triglyceride levels remained constant, due to the similar rates of triglyceride lipolysis and synthesis (Table 5 and 6).

Table 1

Perfusion protocol for measuring exogenous and endogenous fatty acid oxidation in isolated working rat hearts

	Pulse period ---------->	Washout period -------->	Chase period
Conditions	60-min prelabeling with 11 mM glucose and 1.2 mM [^{14}C]palmitate	10-min aerobic retrograde perfusion	60-min perfusion with: a) no fat (11 mM glucose) b) low fat (11 mM glucose, 0.4 mM [^{3}H]palmitate) c) high fat (11 mM glucose,1.2 mM [^{3}H]palmitate)
Procedure	*measure $^{14}CO_2$ production*	*change perfusate*	*measure $^{14}CO_2$ and $^{3}H_2O$*

Table 2

Perfusion protocol for measuring rates of glycolysis and glucose oxidation in isolated working rats hearts.

	Pulse period ---------->	Washout period --->	Chase period
Conditions	60-min with 11 mM glucose and 1.2 mM palmitate	10-min aerobic retrograde perfusion	60-min perfusion with: a) no fat (11 mM [$^{3}H/^{14}C$]glucose) b) low fat (11 mM [$^{3}H/^{14}C$]glucose, 0.4 mM palmitate) c) high fat (11 mM [$^{3}H/^{14}C$]glucose, 1.2 mM palmitate)
Procedure		*change perfusate*	*measure $^{14}CO_2$ and $^{3}H_2O$*

TABLE 3

Heart mechanical functions during "pulse" and "chase" perfusions.

Perfusion Condition	Heart Rate (beats/min)	Peak systolic pressure (PSP) (mm Hg)	Heart rate x PSP $x10^{-3}$
During "pulse" (n=33)	225 ± 4	101.5 ± 2.3	22.8 ± 0.8
During "chase"			
1) with no fat (n=13)	247 ± 6	102.9 ± 4.4	24.6 ± 1.4
2) with low fat (n=10)	241 ± 8	100.8 ± 2.2	24.2 ± 0.9
3) with high fat (n=10)	239 ± 9	84.2 ± 5.0*	20.3 ± 1.7

Values are the mean ±SEM. The number of hearts in each group is indicated between brackets.

*, significantly different from values during the "pulse", from hearts chased with no fat, and from hearts chased with low fat.

TABLE 4

Fate of [^{14}C]palmitate at the end of the "pulse" and "chase" perfusions

Perfusion Condition	[^{14}C]palmitate as triglycerides (μmol/g dry wt)	[^{14}C]palmitate as phospholipids (μmol/g dry wt)	[^{14}C]palmitate as free fatty acid (μmol/g dry wt)	[^{14}C]palmitate as neutral lipids other than triglyceride (μmol/g dry wt)	$^{14}CO_2$ production (μmol [^{14}C] palmitate/g dry wt·60 min)	Trigyceride content (μmol free fatty acid/g dry wt)
At end of pulse (n=6)	20.41 ± 3.22	2.28 ± 0.47	0.80 ± 0.14	0.26 ± 0.03	39.54 ± 3.06	48.1 ± 6.0
Following the 60 minute chase perfusion						
1) no fat (n=6)	6.64 ± 0.30*	3.34 ± 0.40*	0.46 ± 0.03*	0.06 ± 0.02	17.85 ± 3.16*	17.8 ± 3.4*
2) low fat (n=6)	12.58 ± 1.08*	2.59 ± 0.29*	0.28 ± 0.05*	0.20 ± 0.05	5.29 ± 0.82*†	21.7 ± 2.7*
3) high fat (n=7)	11.89 ± 1.30*	1.04 ± 0.38*†a	0.26 ± 0.05*	0.49 ± 0.17†	5.26 ± 0.46*†	56.5 ± 5.9†a

Values are the mean ± S.E.M of a number of hearts indicated in brackets.
Hearts were perfused as described in "Methods" and frozen following the 10 minute washout perfusion (at end of "pulse") or following the 60 minute "chase" perfusion.
*, significantly different from hearts frozen at the end of the "pulse".
†, significantly different from no fat hearts frozen following the "chase".
a, significantly different from low fat hearts frozen following the "chase".

TABLE 5

Steady state exogenous and endogenous palmitate oxidation rates during the chase

Perfusion Condition	Steady State Exogenous Palmitate oxidation rate (nmol/g dry wt/min)	Steady State Endogenous Palmitate oxidation rate (nmol/g dry wt/min)
Chase with no fat (n=6)	--------	279 ± 50
Chase with low fat (n=6)	361 ± 68	88.2 ± 13.6[a]
Chase with high fat (n=7)	633 ± 60[b]	87.6 ± 7.7[a]

Hearts were perfused as described in "Methods". Values are the mean±SEM of a number of hearts indicated in brackets.
[a], significantly different from hearts chased with no fat.
[b], significantly different from hearts chased with low fat.

TABLE 6

Fate of [^{3}H]palmitate at the end of "chase" perfusion

Perfusion Condition	[^{3}H]Palm. in TGs	[^{3}H]Palm. in PLs	[^{3}H]Palm. as t FFA	[^{3}H]Palm. in MG,DG, & CE
		µmol/ g dry wt		
Hearts chased with low fat (n=6)	5.29±0.70	0.88 ± 0.09	0.21 ± 0.05	0.31 ± 0.03
Hearts chased with high fat (n=6)	13.23±1.59*	1.2 ± 0.38	0.70 ± 0.13*	1.29 ± 0.37*

Values are the mean±SEM of six hearts in each group.

*, significantly different from hearts chased with low fat.

TGs= Triglycerides. PLs= Phospholipids. t FFA= Tissue free fatty acid.

MG= Monoacylglycerol. DG= Diacylglycerol. CE= Cholesterylester.

TABLE 7

Steady state myocardial ATP production (μmol/g dry wt·min) during the chase perfusion calculated from steady state rates of glycolysis, glucose oxidation, endogenous palmitate oxidation, and exogenous palmitate oxidation

	ATP Production (μmol/g dry wt·min)		
ATP Source	No Fat (n=5)	Low Fat (n=6)	High Fat (n=7)
Glucose oxidation	45.24 ± 5.68 (52.2%)	12.85 ± 1.43 (17.0%)	4.46 ± 0.95 (4.4%)
Glycolysis	5.39 ± 0.92 (6.2%)	4.96 ± 0.68 (6.6%)	3.36 ± 0.56 (3.3%)
Endogenous [^{14}C]palmitate oxidation	35.99 ± 6.45 (41.6%)	11.38 ± 1.75 (15.0%)	11.3 ± 0.99 (11.2%)
Exogenous [^{3}H] palmitate oxidation	------	46.56 ± 8.81 (61.5%)	81.66 ± 7.74 (81.0%)
TOTAL	86.62	75.75	100.78

Values are the mean ± SEM of a number of hearts indicated in brackets. ATP production was calculated as described under "Experimental procedures". Values in brackets are the percentage of total ATP production for each perfusion condition.

Table 8

Total myocardial ATP production during chase perfusion

ATP Source	ATP Production (mmol/g dry wt to 60 min) No Fat (n=6)	Low Fat (n=6)	High Fat (n=6)
Glucose Oxid.	2.71 ± 0.34 (36.7%)	0.77±0.09 (10.7%)	0.27 ± 0.06 (4.5%)
Glycolysis	0.32 ± 0.06 (4.3%)	0.30 ± 0.04 (4.2%)	0.20 ± 0.03 (3.3%)
Exog. [^{3}H]palmitate oxidation	---------	2.79 ± 0.53 (38.6%)	4.90 ± 0.46 (81.0%)
Endog. [^{14}C] palmitate oxidation	2.16 ± 0.39 (29.2%)	0.68 ± 0.11 (9.4%)	0.68 ± 0.06 (11.2%)
Endog. nonlabeled fatty acid oxidation	2.20 ± 0.56 (29.8%)	2.68 ± 0.34 (37.1%)	------
TOTAL	7.39	7.22	6.05

Values are the mean ± SEM of six hearts in each group. ATP production was calculated as described in "Experimental Procedures" from total glycolysis and oxidative rates over the 60 minute chase period, and from the loss of endogenous triglyceride fatty acid over the 60 minute chase period. Values in brackets are the percentage of total ATP production for each perfusion condition.

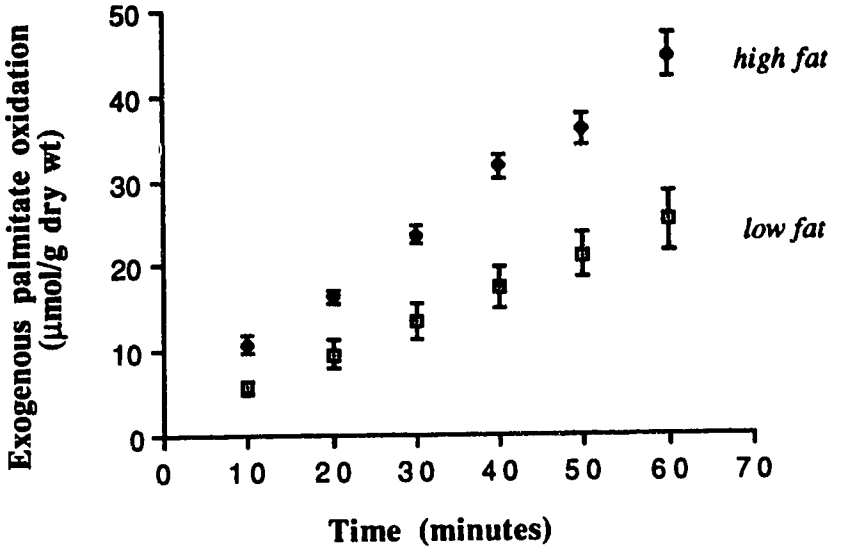

Fig. 1. ^3H_2O production from exogenous [^{3}H]palmitate oxidation during the "chase" perfusion in isolated working hearts perfused with low or high fat.

Values are the mean ± SEM of six hearts in the low fat and 7 hearts in the high fat groups. ^3H_2O production was quantified as described in "Methods".

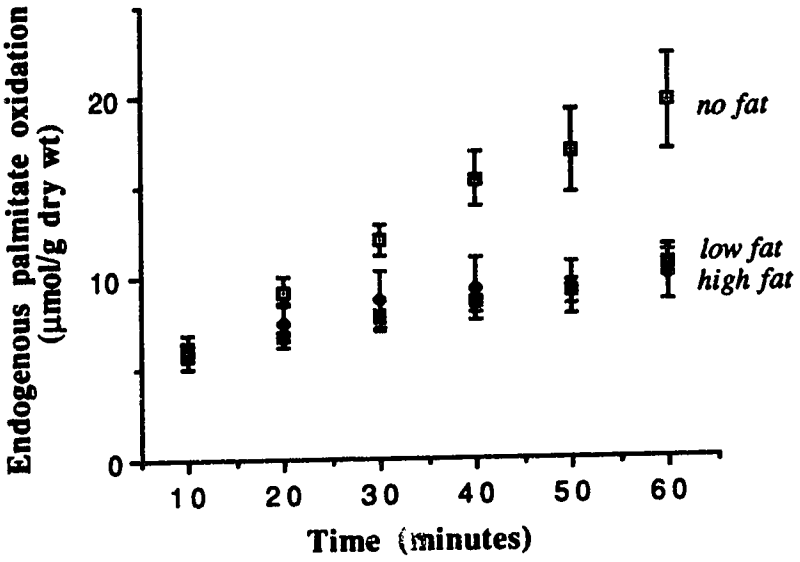

Fig. 2. $^{14}CO_2$ production from endogenous [^{14}C]palmitate oxidation during the "chase" perfusion in isolated working hearts perfused with no fat, low fat or high fat. Values are the mean ± SEM of six hearts in each of the no fat and the low fat groups and seven in the high fat group. Hearts were prelabeled with [^{14}C]palmitate during the "pulse" perfusion as shown in table 1. $^{14}CO_2$ production was quantified as described in "Methods".

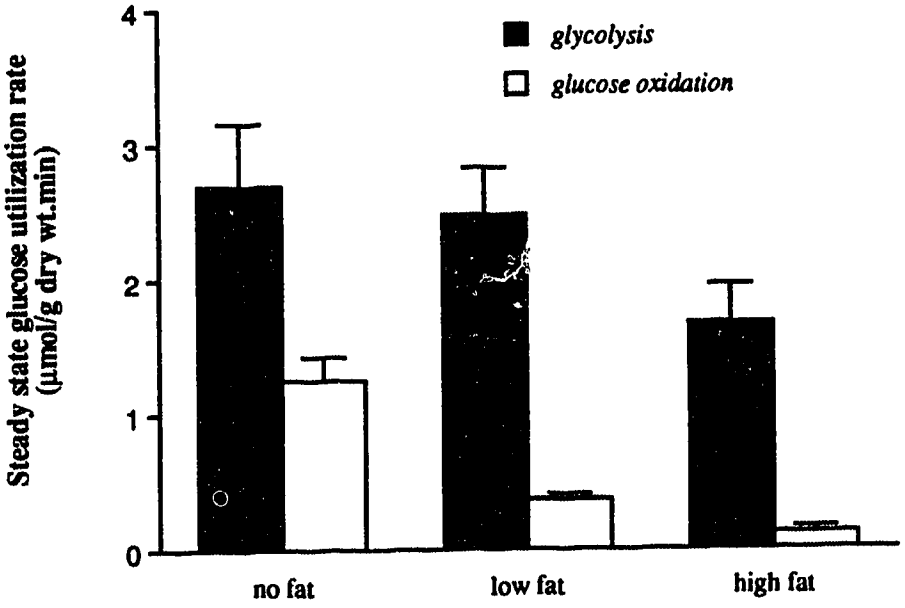

Fig. 3. Steady state rates of glycolysis and glucose oxidation in isolated working hearts chased with no fat, low fat or high fat. Values are the mean ± SEM of six hearts in the no fat group and five hearts in each of the low fat and the high fat groups. Rates of glycolysis and glucose oxidation were determined as described in "Methods".

Discussion:

In this study, we developed and characterized an experimental protocol in which endogenous myocardial triglyceride turnover can be directly measured. This protocol also permits the direct measurement of endogenous triglyceride fatty acid oxidation. During the initial "pulse" perfusion 42% of myocardial triglyceride fatty acids were labeled with [^{14}C]palmitate. In contrast, only a small amount of [^{14}C]palmitate was incorporated into non-triglyceride neutral lipids and phospholipids. During the "chase" perfusion, the [^{14}C]labeled fatty acids released from the triglyceride pool were readily oxidized and were not released into the perfusate. In contrast, the contribution of phospholipids, cholesteryl esters, monoacyl and diacyl glycerol to myocardial oxidative metabolism was very low (5-10%), demonstrating that endogenous triglyceride pools are the major endogenous lipid pools that significantly contribute fatty acids for oxidative metabolism. Using this "pulse-chase" technique, the importance of triglycerides as a source of fatty acids for oxidative metabolism in the heart is clearly demonstrated. In the absence of added fatty acids, the isolated working heart readily uses endogenous triglyceride reserves of fatty acids, with more than 50% of its energy requirements being met by this source. As would be expected, increasing concentrations of fatty acids delivered to the heart decrease the contribution of these pools to myocardial oxidative metabolism. This occurs mainly due to an inhibition of lipolysis, since incorporation of exogenous fatty acids into the triglyceride pool continues. The contribution of myocardial triglycerides to oxidative metabolism was clearly apparent in isolated hearts perfused in the presence of high fatty acid concentrations (1.2 mM palmitate). Under these conditions triglyceride fatty

acids accounted for 11% of total myocardial ATP production, without any change in the size of the endogenous triglyceride pool size. This could be achieved because the rate of triglyceride lipolysis was matched by the rate of incorporation of exogenously derived fatty acids into triglycerides. These observations support the concept that myocardial triglycerides are a readily mobilizable extended substrate source.

It has been previously proposed that the contribution of myocardial triglycerides to energy substrate utilization may be an *in vitro* phenomenon that is seen only in isolated hearts perfused with buffers devoid of fat (15). This suggestion was based primarily on the observation that overall myocardial triglyceride content decreases if hearts are perfused in the absence of fatty acids (1). As expected, a loss of triglyceride content in hearts perfused without fatty acids was also observed in our study. However, under conditions in which triglyceride content was maintained (high fat), we found that substantial triglyceride turnover occurs. This suggests that endogenous myocardial triglyceride pools are never static, and that a continuous lipolysis and synthesis of myocardial triglyceride pools occurs. These pools may serve to maintain the homeostasis of fatty acids in the cytosol. Suprisingly, in our experiments, hearts perfused with physiologic concentrations of exogenous fatty acids (0.4 mM palmitate) did not maintain their endogenous triglyceride content. This loss of overall triglycerides parallels what has previously been reported in isolated rat hearts perfused with a similar concentration of fatty acid (6,29). The reason for this loss of triglycerides at physiological levels of circulating fatty acids is not readily apparent. One potential explanation is that *in vivo*, interstitial fatty acid concentrations may be higher than circulating fatty acid concentrations. *In vivo*, chylomicrons and very low density lipoproteins (VLDL) are potential

sources of fatty acids to the heart, in addition to circulating free fatty acids. Triglycerides in VLDL and chylomicrons particles are subjected to lipolysis by lipoprotein lipase, with the liberated fatty acids then passing to the interstitial spaces prior to uptake by the myocytes. If chylomicron and VLDL triglycerides lipolysis was actively occurring then it is possible that interstitial fatty acid concentrations *in vivo* may exceed the free fatty acid concentration in the blood. In contrast, in isolated hearts perfused with physiological levels of fatty acid as the sole source of exogenous fatty acids (0.4 mM palmitate), interstitial fatty acid levels will quickly equilibrate with the concentration of fatty acid in the perfusate. A reduction in the concentration of fatty acids to which the myocyte itself is exposed may therefore result in the observed depletion of endogenous triglyceride stores. This possibility is supported by the observation that at higher concentrations of exogenous fatty acids (1.2 mM palmitate), endogeneous triglyceride stores were not depleted. Further proof, however, would be obtained by providing chylomicrons or VLDL to isolated perfused hearts. Previous studies have demonstrated that chylomicrons are a significant source of fatty acids for oxidative metabolism in the isolated perfused heart (30-32). To our knowledge, however, no study has been conducted which determines the relative contribution of chylomicrons, VLDL, or plasma free fatty acids to overall myocardial fat uptake and energy requirements.

An interesting observation from this study was the marked differences in the rates of glycolysis and glucose oxidation in the heart. Simultaneous measurement of glycolysis and glucose oxidation in isolated hearts demonstrated that glycolytic rates were twice as high as glucose oxidative rates in hearts perfused in the absence of fatty acids. This observation parallels what has previously been demonstrated by Kobayashi and Neely (27) in hearts

perfused in the absence of fatty acids. In this study, we extended this observation by demonstrating that fatty acids inhibit glucose oxidation to a much greater extent than glycolysis, such that in the presence of high fatty acids, rates of glycolysis are more than 13 times the rate of glucose oxidation. The higher ratio of glycolysis to glucose oxidation in fatty acid perfused hearts is not a result of the heart being artificially subjected to hypoxic or ischemic conditions, since oxidative metabolism is maintained and provides the primary source of ATP (see Tables 7 and 8). This higher ratio in fatty acid perfused hearts supports the concept that fatty acid inhibition of glucose utilization occurs to a greater extent at the level of PDC than at the level of PFK (1,13,14).

It has been proposed that under conditions of hyperglycemia, glucose can become the primary, and even sole, energy substrate to the heart (15,22). Our data would suggest that this cannot occur, however, since even under optimal conditions which cannot be achieved *in vivo* (high glucose, no fat) glucose utilization provided only 40-50% of overall ATP production (Table 8). The differences between our results and the previous studies can be explained by the observation that only a portion of the glucose taken up by the heart was oxidized (Figure 3). Previous studies which have suggested that glucose utilization can serve as the sole energy substrate have primarily relied on arterial and coronary sinus extraction ratios for glucose and oxygen. This oxygen extraction ratio data has suggested that during hyperglycemia glucose can account for up to 100% of oxygen consumption. This approach, however, assumes that the glucose taken up by the heart under aerobic conditions is oxidized, which is clearly not the case (Figure 3). Recent work by Wisneski *et al.* (24), in which myocardial glucose oxidation rates were measured in humans, supports our data. These authors demonstrated that despite a significant

increase in the amount of exogenous glucose oxidized during hyperglycemia, glucose oxidation accounted for only 32% of total glucose extracted.

High levels of cytosolic fatty acids and perhaps more importantly their intermediates can be potentially harmful to the myocardium (11-14,33-35). It has been suggested that hydrolysis of intracellular triglycerides during ischemia enhances cell damage by increasing intracellular concentrations of nonesterified fatty acids (36). During reperfusion of isolated ischemic hearts, an increased incorporation of fatty acids into triglycerides occurs (12). Since lipogenesis is energetically expensive, acceleration of triglyceride turnover in ischemia is regarded by some investigators to be harmful to the ischemic myocardium, the ATP supply of which is already compromized (9). On the other hand, it was also suggested that an accelerated triglyceride turnover may be beneficial during ischemia by scavenging free fatty acids from the cytosol, thus inhibiting their possible detrimental effects (4). In most of these studies however, triglyceride turnover was indirectly measured through measuring triglyceride content and glycerol production, and many of them were conducted on isolated hearts perfused in the absence of added fatty acids. As demonstrated in this study, perfusion of hearts under aerobic conditions in the absence of added fatty acids will markedly accelerate triglyceride lipolysis. This acceleration of triglyeride lipolysis in the presence of non-physiological energy substrates is a potential complication in determining the relative importance of triglyceride fatty acids to ischemic injury. It is important, therefore, to measure triglyceride turnover during ischemia and reperfusion in the presence of high circulating levels of fatty acids, a situation which commonly occurs during and following myocardial infarction in humans.

Cardiac adipocyte triglycerides are probably an important source of fatty acids for oxidative metabolism (5). In the intact hearts used in this study, myocardial triglycerides originating from cardioadipocytes cannot be distinguished from triglycerides originating from cardiomyocytes. In this study, however, most of the $^{14}CO_2$ produced by pre-labeled hearts can be accounted for by total myocardial loss of label from triglycerides. This suggests that if cardioadipocyte triglyceride turnover is occurring, any released labeled fatty acids are oxidized. Therefore, measurement of triglyceride turnover in the intact heart is justified.

Previous studies have demonstrated that individual fatty acids can be preferentially released from triglycerides (37). Therefore, triglyceride turnover experiments need also to consider other preferential turnover of individual fatty acids during both the "pulse" and "chase" perfusions. These experiments have yet to be done. The experimental protocol described in this study, however, should prove useful for determining if preferential turnover does occur (i.e. such as by pulsing and chasing hearts with [^{14}C] and [^{3}H]arachidonic acid or [^{14}C] and [^{3}H]oleic acid).

In conclusion, this study demonstrates that endogenous mobilizable myocardial triglycerides are an important source of fatty acids for oxidative metabolism, and that their contribution is inversely related to the concentration of fatty acids delivered to the heart. Triglyceride fatty acid contribution to overall energy production can range from 11% of myocardial ATP requirements in hearts perfused with high fat, to more than 50%, when the heart is deprived of an exogenous fatty acid supply. This study also demonstrates that glucose oxidation and glycolytic flux cannot meet the total energy demands of the heart, even in the absence of added exogenous fatty acids.

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CHAPTER 3

MYOCARDIAL TRIGLYCERIDE TURNOVER DURING REPERFUSION OF ISOLATED RAT HEARTS SUBJECTED TO A TRANSIENT PERIOD OF GLOBAL ISCHEMIA

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My role in this work involved performing all the experimental part as well as the writing-up of the paper

CHAPTER 3

MYOCARDIAL TRIGLYCERIDE TURNOVER DURING REPERFUSION OF ISOLATED RAT HEARTS SUBJECTED TO A TRANSIENT PERIOD OF GLOBAL ISCHEMIA

Introduction:

High levels of exogenous and endogenous fatty acids are known to be detrimental to the ischemic myocardium. Fatty acids and/or their intermediates have been shown to promote the occurrence of arrythmias (1-3), to interfere with subcellular organelle performance and with membrane integrity (4-7), and to decrease mechanical function (8-11). High concentrations of exogenous fatty acids are also detrimental to the ischemic myocardium during reperfusion (8,9). The exact mechanism of this effect is still unknown, although results from our laboratory (8,12,13) and others (14) have indicated that fatty acids may exert part of their detrimental effect by interfering with myocardial glucose utilization.

Another important potential source of fatty acids in the ischemic and reperfused heart are myocardial triglycerides. The role of myocardial triglycerides in ischemic injury is still not clear. In intact animals, accumulation of triglycerides occurs at the periphery of the infarct zone (15), and an increase in myocardial triglyceride content can be seen in subepicardial (16) and subendocardial regions (17) of the heart. No change in myocardial triglyceride content has also been reported (18). Direct measurments of myocardial triglyceride turnover during and following myocardial ischemia are few. Crass *et al.* (19,20) reported that triglyceride turnover is inhibited during ischemia and hypoxia. However, van Bilsen *et al.* (21) have recently suggested that triglyceride turnover is accelerated during ischemia and ends immediately

after restoration of flow. These authors also suggested that such an acceleration during ischemia could be harmful to the ischemic myocardium, since this turnover is expensive energetically (ATP is required for the esterification of fatty acids). As a result, acceleration of triglyceride turnover will deplete the intracellular ATP levels which are already reduced in the ischemic myocardium (21). In contrast, Schoonderwoerd *et al.* (22) suggested that triglyceride turnover during ischemia is beneficial through scavenging free fatty acids from the cytosol and thus inhibiting the accumulation of fatty acids and their intermediates to toxic levels.

One limitation of many of the previous studies investigating triglyceride turnover is that isolated hearts were perfused with buffer devoid of fatty acids. As we have recently demonstrated (12), this leads to a mark leration of endogenous triglyceride lipolysis even during normoxic p In addition, the suggestion that triglyceride turnover is accelerated during is mia has primarily been based on measurement of glycerol release, with the assumption that the majority of the glycerol released is produced through triglyceride lipolysis. Although this assumption may be valid, it has never been verified (23) and has recently been questioned by Larsen (24) who found that phospholipid breakdown also contributes significantly to glycerol production in isolated rat cardiac myocytes deprived of oxygen and exogenous substrates.

In a recent study we characterized an experimental model in which triglyceride turnover could be directly measured in the intact heart (12). The protocol allows direct measurement of endogenous triglyceride fatty acid oxidation while simultaneously measuring exogenous fatty acid oxidation. The purpose of the present study was to use this model to determine whether triglyceride turnover is accelerated during reperfusion of hearts subjected to a

transient period of global ischemia. This was accomplished by initially prelabeling the myocardial lipid pools during a "pulse" perfusion with 1.2 mM [^{14}C]palmitate. Triglyceride turnover and exogenous fatty acid oxidation were then measured during an aerobic "chase" perfusion following the ischemic period. Our data demonstrates that ischemic hearts reperfused in the presence of a high concentration of fatty acid quickly recover their ability to oxidize exogenous fatty acids. Triglyceride lipolysis is not accelerated during this reperfusion period, although an acceleration of triglyceride synthesis is seen, resulting in an expansion of the total myocardial triglyceride pool.

Materials and Methods:

Materials

[9,10-^{3}H]palmitic acid and [1-^{14}C]palmitic acid were obtained from NEN (Wilmington, DE). Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1M in methanol solution) was obtained from NEN Research Products (Boston, MA). ACS Aqueous Counting Scintillant was obtained from Amersham Canada (Oakville, Ontario). Insulin (regular, CZI) was obtained from Connaught Novo (Ontario, Canada). Baker Si250-PA (19C)-silica gel plates were obtained from Johns Scientific (Toronto, Ontario). Triglyceride enzymatic assay and NEFA C assay kits were obtained from Wako (Osaka, Japan). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

Heart Perfusions:

Adult male Sprague Dawley rats (200-250 g) were anesthetized with sodium pentobarbital (60 mg/kg). The perfusion protocol is shown in Table 1. Hearts were quickly excised, the aorta cannulated, and a retrograde perfusion using Krebs Henseleit buffer initiated, as described previously (12,13,25). During this initial perfusion, hearts were trimmed of excess tissue, the pulmonary artery was cut, and the left atrium cannulated. After a 10 minute equilibration period, hearts were switched to the working mode and perfused with Krebs Henseleit buffer containing 11 mM glucose, 1.2 mM [1-^{14}C]palmitate and 500 μU/ml insulin, at an 11.5 mm Hg left atrial preload and an 80 mm Hg aortic afterload, as described previously (12). We used a high concentration of palmitate (1.2 mM) during the "pulse" perfusion because in a previous study we found that this

concentration of palmitate ensured labeling of endogenous lipid pools during the one hour perfusion without any change in endogenous triglyceride pool size(12). Hearts were spontaneously beating, with heart rate and peak systolic pressure being measured by a Gould P21 pressure transducer in the aortic outflow line. Following the initial one hour "pulse" period, hearts were subjected to a 30 minute period of global no-flow ischemia. During this ischemic period the buffer used during the "pulse" perfusion was drained, the perfusion system was washed thoroughly with distilled water, and the "chase" buffer added to the system. This buffer contained either 11 mM glucose and 500 μU/ml insulin, or 11 mM glucose, 1.2 mM [9,10-^{3}H]palmitate and 500 μU/ml insulin. We used a high concentration of fatty acid during reperfusion of ischemic hearts (1.2 mM palmitate) because such a concentration of fatty acids is usually found in the circulation of patients suffering a myocardial infarction and in patients following coronary bypass surgery. The use of a high concentration of glucose in the presence of a pharmacologic dose of insulin both during "pulse" and "chase" perfusions was chosen so that glucose concentration will not be a limiting factor in myocardial glucose utilization.

At the end of the 60 minute "chase" perfusion, hearts were quickly frozen using Wollenberger clamps that had been cooled to the temperature of liquid N_2. Control hearts were perfused in the same manner except that the 30 minute period of ischemia was replaced by a 10 min aerobic retrograde perfusion, as described previously (12). During this perfusion, the system was washed thoroughly, and an 11mM glucose, 1.2 mM palmitate and 500 μU/ml insulin "chase" buffer was added. Hearts were then perfused for a subsequent 60 minute period.

An additional two series of hearts were also frozen at the end of the aerobic "pulse" perfusion, and at the end of the 30 minute period of ischemia.

Measurement of exogenous and endogenous fatty acid oxidative rates

During the pulse, steady state exogenous palmitate oxidation rates were determined by quantitatively measuring $^{14}CO_2$ production by the hearts, as described in detail previous[illegible]2). Hearts were perfused in a closed system that allowed collection of both perfusate and gaseous $^{14}CO_2$. The $^{14}CO_2$ liberated into the gaseous phase was trapped in a 1 M hyamine hydroxide solution in the air outlet line. Both perfusate and hyamine hydroxide samples were obtained at 20 minute intervals throughout the initial 60 minute "pulse" period. Perfusate samples were immediately injected below a 1 ml volume of mineral oil to prevent liberation of perfusate $^{14}CO_2$. The $^{14}CO_2$ in the perfusate was subsequently extracted by injecting 1 ml of perfusate into 1 ml of 9N H_2SO_4 contained in sealed metabolic vials, which also contained 400 µl of 1 M hyamine hydroxide in suspended center wells. Vials were gently shaken for 1 hour and the center wells removed and counted in ACS scintillant using standard scintillation counting procedures.

During reperfusion, $^{14}CO_2$ production was used as a measure of endogenous fatty acid oxidation, while ^3H_2O production was a measure of exogenous fatty acid oxidation in hearts reperfused in the presence of 1.2 mM ^{3}H-palmitate. Perfusate and gaseous samples were collected at 10 min intervals during reperfusion, and $^{14}CO_2$ production measured as described above. During this period exogenous steady state palmitate oxidation was determined by measuring ^3H_2O levels in the perfusate samples. ^3H_2O was separated from [^{3}H]palmitate by treating 0.5 ml buffer samples with a 1.88 ml of a mixture of

chloroform:methanol (1:2 vol:vol) and then adding 0.625 ml chloroform and 0.625 ml of a 2M KCl:HCl solution. The aqueous phase was then collected using a Pasteur pipette and was subsequently treated with a mixture of chloroform, methanol, and KCl:HCl with a ratio of 1:1:0.9. Two 0.5 ml samples of the aqueous phase were then counted for each perfusate sample for total ^3H_2O determination, taking into account the dilution factor. We have shown previously (12) that this technique resulted in a greater than 99.7% extraction and separation of ^3H_2O from the [^{3}H]palmitate. Exogenous palmitate oxidation rates during the aerobic "chase" in control hearts and during reperfusion of ischemic hearts were expressed as nmol [^{3}H]palmitate oxidized/min·g dry wt, while endogenous palmitate oxidation rates were expressed as nmol [^{14}C]palmitate oxidized/min·g dry wt.

Measurement of metabolic intermediates:

Tissue lipids were extracted as previously described (25). Neutral lipids were seperated from phospholipids using the methods described by Bowyer and King (26). Triglycerides were seperated from other neutral lipids using Baker Si250-PA (19C) silica Gel plates and a solvent system that consisted of isooctane:diethyl ether:acetic acid (74:24:2 vol/vol/vol) (25). [^{14}C]palmitate and [^{3}H]palmitate incorporation into triglycerides, other neutral lipids, and phospholipids was measured using double radioisotope counting techniques. Label content of all lipids was expressed as µmol palmitate incorporated into these pools/g dry wt. Measurement of tissue triglyceride content was conducted as described previously (12) and was expressed as µmol fatty acid equivalent/g dry wt. Total tissue free fatty acids were measured using Wako

NEFA C enzymatic assay kit while buffer glycerol levels were measured using Wako enzymatic assay.

Statistical analysis:

Data are presented as the mean ± SE of the mean. Analysis of variance, followed by the Neuman-Keuls test was used in the determination of statistical difference in groups containing 3 or more sample populations. The unpaired Student's t-test was used to determine statistical significance in groups containing 2 sample populations. Paired Student's t-test was used to compare exogenous palmitate oxidative rates during reperfusion to those seen prior to ischemia in the same heart. A value of $p < 0.05$ was regarded as significant.

Results:

Heart function in aerobic and reperfused ischemic hearts:

Heart rate and peak systolic pressure development was monitored in all hearts throughout the perfusion protocol (Table 2). In aerobic hearts, mechanical function (as measured by the HRxPSP product) was similar in both the "pulse" and "chase" perfusions, demonstrating that the long perfusion protocol did not result in any deterioration of heart function. If hearts were subsequently reperfused during the "chase" with 1.2 mM palmitate, a significant decrease in mechanical recovery was seen compared to hearts not subjected to ischemia. This was the result of both a significant depression of heart rate and peak systolic pressure development. Recovery of function in hearts reperfused in the presence of 1.2 mM palmitate was also depressed compared to hearts reperfused in the absence of fat. This agrees with our previous reports demonstrating that reperfusion of ischemic hearts with high concentrations of fatty acids has a deterimental effect on recovery of mechanical function (8,9,13). Of the 11 hearts reperfused with a high concentration of fatty acid following ischemia, 5 failed to recover. Because of the difficulty in accurately measuring oxidative metabolism in non-recovered hearts, only hearts which recovered some degree of mechanical function were used in the measurement of exogenous and endogenous fatty acid oxidation.

Labeling of the endogenous lipid pools during the "pulse" perfusion

To label the endogenous lipid pools, hearts were perfused for a 60 minute period in the presence of 1.2 mM [^{14}C]palmitate. As shown in Table 3, triglyceride fatty acid content was 56.97 μmol/g dry wt at the end of the 60

minute "pulse" perfusion, which is comparable to values seen in unperfused hearts (hearts which were quickly excised and frozen). During this 60 minute "pulse" perfusion, 65.96 μmol of [^{14}C]palmitate was taken up by the heart, of which 74.2% of this was oxidized (48.96 μmol/g dry wt·60min). The majority of the [^{14}C]palmitate that was not oxidized was incorporated into the endogenous triglyceride pool (14.25 μmol/g dry wt).

Table 3 also shows the label content of endogenous lipids following the 30 minute period of global ischemia. As would be expected, no $^{14}CO_2$ production occurred during this period, due to the absence of oxidative metabolism. In addition, no significant changes in [^{14}C]palmitate content was seen in either myocardial triglycerides or phospholipids. A significant increase in label content of free fatty acids was observed, although overall free fatty acid content decreased from 2.13 ± 0.11 to 1.58 ± 0.17 μmol/g dry wt. During this ischemic period, a small (statistically not significant) increase in total triglyceride pool size was also observed.

Endogenous fatty acid oxidation rates during the "chase" perfusion following ischemia

Following the 30 minute ischemic period, hearts were reperfused under aerobic conditions with perfusate containing 1.2 mM [^{3}H]palmitate. All [^{14}C]palmitate was washed from the perfusion apparatus and from the hearts (chase buffer contained only 0.079±0.033 μmol [^{14}C]palmitate; this means that only 0.07% of the chase buffer was contaminated with [^{14}C]palmitate). As a result, during this "chase" perfusion, any $^{14}CO_2$ production originates from endogenous myocardial [^{14}C]palmitate. During the "chase" perfusion, a total of 3.65 μmol of [^{14}C]palmitate/g dry wt·60 min was oxidized. At the same time

4.35 ± 1.17 μmol of [^{14}C]palmitate/ g dry wt was released to the medium. This means that endogenous triglyceride lipolysis contributed to 8 μmol of [^{14}C]palmitate/g dry wt within the 60 minute period of reperfusion in these hearts. Figure 1 shows the time course for endogenous [^{14}C]palmitate oxidation in reperfused ischemic hearts, and during the "chase" perfusion of hearts not subjected to ischemia. Under both conditions, a linear rate of [^{14}C]palmitate oxidation occured between 10 and 60 minutes of perfusion. However, initial rates of [^{14}C]palmitate oxidation in reperfused ischemic hearts were higher than non-ischemic hearts during the first ten minutes of perfusion. During the subsequent 50 minute "chase" perfusion period, rates of endogenous [^{14}C]palmitate were linear in both aerobic and reperfused/ischemic hearts. Steady state endogenous triglyceride [^{14}C]palmitate oxidation rates in reperfused ischemic hearts were similar to rates seen in non-ischemic hearts (60.87 ± 16.16 vs 72.98 ± 14.47 nmol [^{14}C]palmitate oxidized/g dry wt. min, respectively).

Exogenous fatty acid oxidation rates during the "chase" perfusion following ischemia

During the "chase" perfusion of ischemic hearts, both exogenous palmitate oxidation and the incorporation of [^{3}H]palmitate into endogenous lipid pools were determined (Table 3). The validity of using rates of $^{3}H_2O$ production from [9,10-^{3}H]palmitate as a measure of exogenous palmitate oxidation has been previously demonstrated (12). During the 60 minute "chase" perfusion a total of 75.02 μmol/g dry wt of [^{3}H]palmitate was taken up by the heart. Of this, 80.6% was oxidized (60.45 μmol/g dry wt·60 min) (Table 3). Steady state exogenous fatty acid oxidation during this period was significantly

higher than rates seen prior to ischemia (1008 ± 133 vs 816 ± 136 nmol of palmitate oxidized/min·g dry wt, respectively). This suggests that fatty acid oxidation quickly recovers in hearts reperfused following a transient period of severe ischemia.

Triglyceride turnover in reperfused/ischemic hearts

The majority of [^{3}H]palmitate taken up by the heart which was not oxidized was incorporated into myocardial triglycerides (Table 3). During the 60 minute "chase" period 11.97 ± 0.78 μmol/g dry wt of [^{3}H]palmitate was incorporated into myocardial triglycerides. During the same period, 3.65 ± 0.97 μmol/g dry wt of [^{14}C]palmitate was oxidized and 4.35 ± 1.17 (n=4) μmol/g dry wt of [^{14}C]palmitate was released to the medium. As a result, a significantly greater rate of triglyceride synthesis was observed in reperfused/ischemic hearts compared to lipolysis. This greater rate of triglyceride synthesis compared to lipolysis in reperfused/ischemic hearts resulted in a significant increase in overall triglyceride pool size during the "chase" perfusion (Table 3).

During the "chase" perfusion, no accumulation of free fatty acids was seen in hearts reperfused with high concentrations of fatty acid and myocardial free fatty acid content was similar to that observed in hearts frozen at the end of the "pulse" perfusion.

Myocardial fate of fatty acids in reperfused/ischemic hearts that failed to recover during reperfusion.

As mentioned earlier, 5 ischemic hearts reperfused with high concentrations of fatty acids failed to recover following the 30 minute period of ischemia. The myocardial fate of fatty acids in these hearts is shown in Table 4.

Interestingly, these hearts had significantly higher [^{3}H]palmitate content in endogenous triglycerides (19.71 ± 4.04 μmol/g dry wt) than hearts which recovered (11.97±0.78 μmol/g dry wt, Table 3). In hearts that failed to recover, [^{14}C]palmitate content of triglycerides (Table 4) was not significantly different from that seen in hearts that recovered (Table 3), while endogenous [^{14}C]palmitate oxidation rates were higher in the latter (3.65 ± 0.97 vs 1.12 ± 0.30 μmol [^{14}C]palmitate oxidized/g dry wt·60 min, in those which recovered and those which did not, respectively). This indicates that while triglyceride lipolysis was lower, triglyceride synthesis rates were further accelerated in reperfused/ischemic hearts that did not recover when compared to hearts that recovered.

Unlike the triglyceride pool, [^{14}C]palmitate and [^{3}H]palmitate content of phospholipid and free fatty acids did not differ in the non-recovered hearts, compared to recovered ischemic hearts. Interestingly, measurable rates of both exogenous and endogenous palmitate oxidation occurred in the non-recovered hearts. However, these rates were significantly lower than those seen in hearts which recovered.

Myocardial fate of [^{14}C]palmitate in reperfused/ischemic hearts in which fatty acids were omitted from the "chase" buffer

The majority of previous studies which addressed triglyceride turnover in ischemic hearts were performed in the absence of added fatty acids. We therefore "chased" a series of hearts with perfusate in which fatty acid was not present in the perfusate. Label content and endogenous fatty acid oxidation rates during the "chase" perfusion in hearts perfused under these conditions is shown in Table 5. Loss of [^{14}C]palmitate from the triglyceride pool in these

hearts (from 13.6 to 7.8 μmol/g dry wt) was slightly greater than that seen in hearts "chased" with fatty acids (from 13.6 to 9.6 μmol/ g dry wt). This difference was statistically not significant. However, unlike hearts "chased" with fatty acids, hearts "chased" without fatty acids showed no increase in myocardial triglyceride content following ischemia (Table 3 and 5). This would be expected since no exogenous fatty acids are available for triglyceride synthesis.

Steady state endogenous triglyceride palmitate oxidation rates were significantly higher in these hearts than in hearts reperfused in the presence of palmitate (135.2±24.3 vs 60.9±16.2 nmol/min·g dry wt in the absence and in the presence of palmitate, respectively). [^{14}C]palmitate content in phospholipids and free fatty acids did not differ regardless of whether hearts were "chased" in the presence or absence of fatty acids (Tables 3 and 5). As a result, the increase in endogenous [^{14}C]palmitate oxidation most likely resulted from the the [^{14}C]palmitate released from the endogenous triglyceride pool in these hearts.

Table 1

	Pulse period---------->	Washout period ---------->	Chase period
Conditions	60-min prelabeling with 11 mM glucose and 1.2 mM [^{14}C]palmitate	10-min perfusion or 30-min ischemia	60-min reperfusion with: a) no fat (11 mM glucose) b) high fat (11 mM glucose and 1.2 mM [^{3}H]palmitate)
Procedure	measure $^{14}CO_2$ production	change perfusate	measure $^{14}CO_2$ and $^{3}H_2O$

Table 2

Mechanical function during "pulse" and "chase" perfusions in aerobic and reperfused ischemic hearts.

Perfusion condition	Heart rate (beats/min)	Peak Systolic Pressure (mm Hg)	HRxPSP (x10^{-3})
During aerobic "pulse" perfusion (n=15)	245 ± 6	108.2 ± 4.3	26.2 ± 1.4
During aerobic "chase" perfusion (n=5)	259 ± 10	105.1 ± 14.0	26.9 ± 3.3
During aerobic "chase"following ischemia			
-with fat (n = 11)	134 ± 39*	52.6 ± 15.7*	13.0 ± 4.0*
-without fat (n = 7)	201 ± 35	89.4 ± 15.5	20.9 ± 3.7

-Data are the mean ± S.E.M. of a number of hearts shown in brackets. Hearts were perfused as described in "Methods". HR=Heart rate, PSP= Peak systolic pressure.

*, significantly different from hearts not subjected to ischemia

Table 3

Myocardial fate of [^{14}C]palmitate and [^{3}H]palmitate in hearts aerobically reperfused after 30 minute of ischemia in the presence of high fat

Parameter Measured	At the end of "Pulse" Perfusion (n=7)	At the end of Ischemia (n=7)	At the end of "chase" reperfusion (n=6)
[^{14}C]palmitate content (μmol/g dry wt)			
-as triglyceride	14.25 ± 1.11	13.57 ± 2.33	9.56 ± 1.16
-as phospholipid	2.35 ± 0.23	2.34 ± 0.16	1.55 ± 0.30
-as free fatty acid	0.40 ± 0.09	1.09 ± 0.33*	0.68 ± 0.17
[^{3}H]palmitate content (μmol/g dry wt)			
-as triglyceride	N.A.	N.A.	11.97 ± 0.78
-as phospholipid	N.A.	N.A.	1.55 ± 0.31
-as free fatty acid	N.A.	N.A.	1.05 ± 0.22
$^{14}CO_2$ production (μmol [^{14}C]palmitate/ g dry wt·60 min)	48.96 ± 8.16	N.A.	3.65 ± 0.97
$^{3}H_2O$ production (μmol [^{3}H]palmitate/ g dry wt·60 min)	N.A.	N.A.	60.45 ± 7.95
Free fatty acid content (μmol fatty acid/g dry wt)	2.13 ± 0.11	1.58 ± 0.17*	1.97 ± 0.21
Triglyceride content (μmol fatty acid/g dry wt)	56.97 ± 2.93	66.51 ± 7.48	75.74 ± 4.20*

-Values are the mean ± S.E.M of a number of hearts indicated in brackets.

Hearts were perfused as described in "Methods". *, significantly different from values during "pulse" perfusion. N.A.= not applicable.

TABLE 4

Myocardial fate of labeled palmitate in fatty acid perfused hearts which failed to recover during reperfusion following ischemia

	Label content (µmol/g dry wt)			Total $^{14}CO_2$ production (µmol/g dry wt·60 min)	Total $^{3}H_2O$ production (µmol/g dry wt·60 min)
	As triglyceride	As phospholipid	As free fatty acid		
[^{14}C]palmitat	10.83 ± 1.64	2.09 ± 0.53	0.60 ± 0.05	1.12 ± 0.30	N.A.
[^{3}H]palmitate	19.71 ± 4.04	2.02 ± 0.69	1.40 ± 0.25	N.A.	5.05 ± 1.22

-Data are the mean ± SEM of 5 hearts. Hearts were perfused as described in "Methods".
N.A. = not applicable

Table 5

Myocardial fate of [^{14}C]palmitate and myocardial triglyceride content in hearts reperfused in the absence of fat

Perfusion condition	[^{14}C]palmitate as triglyceride (μmol/ g dry wt)	[^{14}C]palmitate as phospholipid (μmol/ g dry wt)	[^{14}C]palmitate as tissue free fatty acid (μmol/ g dry wt)	Total tissue free fatty acids (μmol /g dry wt)	Triglyceride content (μmol fatty acid/g dry wt)	Steady state endogenous [^{14}C] palmitate oxidation (nmol/min·g dry wt)
During reperfusion	7.80 ± 0.94	1.86 ± 0.46	0.74 ± 0.17	1.41 ± 0.11	59.44 ± 5.43	135.24±24.32

Data are the mean ± S.E.M. of six hearts. Hearts were perfused as described in "Methods".

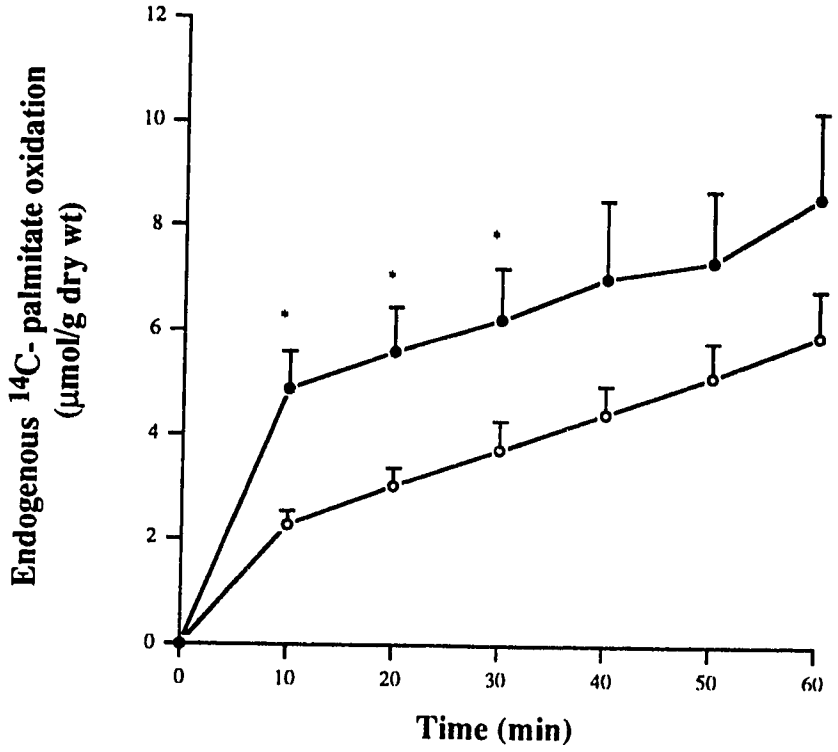

Fig. 1. Total endogenous [^{14}C]palmitate oxidation in fatty acid perfused hearts during the "chase" perfusion of non-ischemic hearts, and during reperfusion following 30 minutes of ischemia.

Values are the mean ± SEM of 6 hearts in each group. Hearts were perfused as described in "Methods". Cumulative endogenous palmitate oxidation is shown. Open circles, control. Filled circles, reperfusion following ischemia.

*, significantly different from control (non-ischemic) hearts.

Discussion:

The role of myocardial triglycerides in ischemic injury is still controversial. Controversy also exists as to the extent of myocardial fatty acid utilization during reperfusion of ischemic hearts (27-30). In this study, we directly measured myocardial triglyceride synthesis and lipolysis rates during reperfusion of ischemic hearts, while simultaneously measuring exogenous fatty acid oxidation. Our data demonstrate that no acceleration in steady state triglyceride lipolysis occurs during reperfusion of ischemic hearts perfused in the presence of high concentrations of fatty acids. However, an increase in triglyceride synthesis is seen, with a resultant increase in myocardial triglyceride content following reperfusion. Accompanying this increase in triglyceride synthesis was a 20% increase in exogenous fatty acid oxidation. The reason for this increase in triglyceride synthesis and exogenous fatty acid oxidation rates during reperfusion of ischemic hearts is not clear. However, our data clearly indicates that in reversibly injured ischemic hearts, fatty acid uptake increases during aerobic reperfusion.

Several studies have dealt with alterations in fatty acid metabolism during ischemia and reperfusion. van Bilsen *et al.* (21) using isolated ischemic rat hearts found that total tissue free fatty acids only started to increase after a 45 minute period of ischemia, at a time when ATP and creatine phosphate levels were the lowest. These authors suggested that this increase in levels of tissue free fatty acids during ischemia is related to the depletion of energy nucleotides which results in impaired acylation of fatty acids and thus impaired triglyceride synthesis. In that study, tissue free fatty acids were shown to rise further during reperfusion, however, the rise was only significant after a period of ischemia exceeding 45 minutes. In our study we found a significant increase in labeled

free palmitate level after a 30 minute period of ischemia. In contrast to van Bilsen *et al.* (21), our tissue free fatty acid levels decreased after ischemia and returned to pre-ischemic levels during reperfusion. In addition, steady state lipolysis rates during reperfusion were not different from those seen in non-ischemic hearts. This indicates that endogenous triglyceride lipolysis is not accelerated during reperfusion of ischemic hearts in the presence of high concentrations of fatty acids. However, an acceleration of triglyceride synthesis does occur in these hearts. Triglyceride synthesis is absent in hearts reperfused in the absence of relevant concentrations of fatty acids. This contrasts with the suggestion of van Bilsen *et al.* that triglyceride turnover stops soon after reperfusion (21). However, their suggestion was built on measuring glycerol production during reperfusion, while in our study a direct measurement of triglyceride lipolysis was conducted. We measured glycerol levels in perfusates of hearts reperfused in the presence or absence of palmitate and found that glycerol release was slightly (but not significantly) lower in hearts reperfused in the absence of palmitate (3.99 ± 0.50 μmol/g dry wt in hearts reperfused in the absence of palmitate vs 5.61 ± 0.56 in those reperfused in its presence). This contrasts with our observation that endogenous [^{14}C]palmitate oxidation rates were significantly higher in hearts reperfused in the absence of fat than those perfused in its presence. Since [^{14}C]palmitate release to the medium was only slightly (but significantly) lower in hearts reperfused in the absence of palmitate, one would expect a higher glycerol release in hearts reperfused in the absence of fat which is not the case. This clearly indicates that glycerol levels *per se* are not an accurate measure of triglyceride lipolysis and that other sources of glycerol may contribute significantly to glycerol release. A previous study by

Larsen (24) has also shown that a significant portion of glycerol in hypoxic hearts originates from phospholipids.

During the early minutes of aerobic reperfusion of ischemic hearts, an increase in endogenous fatty acid oxidation occurred (Figure 1). The reason for this acceleration is not clear. Paulson *et al.* (31) suggested that this difference in lipolysis rates may be a result of the existence of more than one triglyceride pool. However, it is also possible that the heart uses the tissue free fatty acids during early perfusion as well as fatty acid reserves in the endogenous triglyceride pool. It is well known that under stressful conditions such as exercise, a higher rate of endogenous triglyceride lipolysis occurs. Therefore, it is possible that during the initial ten minute period of perfusion when the heart is recovering from ischemia, a greater proporation of its energy requirements will be derived from oxidation of fatty acids delivered from myocardial triglycerides. Following the stabilization period, the heart then shifts to exogenous fatty acids which contributes to the majority of energy requirement of the heart.

The ability of the heart to regain its ability to oxidize exogenous fatty acids during reperfusion following a 30 minute period of no-flow ischemia is clearly demonstrated in this study. There was almost a 20% increase in exogenous fatty acid oxidative rates during this period. This agrees with what has been reported by Liedtke *et al.* (27) who showed an increase in exogenous fatty acid oxidation during reperfusion of swine hearts. It was suggested that this increase in fatty acid oxidative rates during reperfusion is probably related to the ATP debt which has already developed during ischemia. More recently Pauley *et al.* (32) found that the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA was lower in isolated mitochondria from ischemic cardiac cells compared to those from intact cardiac cells. These authors

suggested that a modification in protein folding of the enzyme may take place during ischemia. This could possibly explain the higher oxidative rates of exogenous fatty acid during reperfusion. In contrast to our results (13) and those of Liedtke *et al.* (27), other investigators (28-30) have suggested that a decrease in exogenous fatty acid clearance occurs during reperfusion. These results are based primarily on a delayed clearance of tracer amounts of [^{11}C]palmitate infused into the heart. These apparently contradictory results may be explained by our obervation that triglyceride synthesis is accelerated in reperfused/ischemic hearts. As a result, cycling of tracer amounts of [^{11}C] palmitate through endogenous triglycerides could lead to a decreased rate of clearance of the label, without any parallel decrease in the rate of fatty acid oxidation.

Interestingly, in reperfused/ischemic fatty acid perfused hearts, a tissue free fatty acid concentration of almost 2 μmol/g dry wt could be seen in hearts which recovered greater than 50% of their pre-ischemic ventricular performance (Table 3). Van Bilsen *et al.* (21) has shown that heart function could also be maintained at tissue free fatty acid levels higher than 1 μmol/g dry wt. These observations contrast with the observations of Piper *et al.* (33) who calculated that a myocardial non-esterified fatty acid content of greater than 0.1 μmol/g wet wt (> 0.4-0.6 μmol/g dry wt) is damaging. These values are in fact lower than those found in normal hearts. Their results may be explained by the fact that isolated cardiac mitochondria were used and incubated in the presence of unbound non-esterified fatty acids. This may be unrelated to the actual situation in the functioning myocardium where fatty acid binding proteins may decrease the potential toxic effects of cytosolic fatty acids.

Why hearts reperfused in the absence of fat showed a markedly higher recovery rate when compared to those reperfused in the presence of fat is not well understood. We have previously suggested that high concentrations of fatty acids exert a detrimental effect during reperfusion by inhibiting glucose oxidation (8,13). Agents which stimulate glucose oxidation have also been shown to have a favourable effect on recovery rates in hearts reperfused in the presence of high concentrations of fatty acids (13,34). The question remains as to whether fatty acids orginating from endogenous triglyceride can contribute to reperfusion injury by inhibiting glucose oxidation. This may be particularly relevant in the myocardium of uncontrolled diabetic rats in which the endogenous triglyceride pool is markedly expanded. This remains to be determined.

It has been suggested that triglyceride turnover is accelerated during ischemia with a resultant waste of energy in the release of fatty acids from triglycerides and subsequent ATP dependent incorporation back into triglycerides (21). Such an acceleration could be detrimental to the ischemic myocardium since the ATP supply is already compromised. This cycle of increased lipolysis and synthesis of myocardial triglyceride was termed the "futile cycle" and was attributed to the mass action of alpha glycerophosphate which accumulates in the ischemic myocardium as a result of accelerated glycolysis during ischemia. There are a number of doubts, however, about the concept of a possible detrimental role of triglyceride turnover in ischemic injury. One problem being that acceleration in triglyceride turnover was only inferred from measuring myocardial glycerol levels with the assumption that glycerol originates mainly from triglyceride lipolysis, which is not necessarily true (24). In addition, Crass (19,20) demonstrated that there was a marked reduction in

endogenous triglyceride utilization during a 4 hour period of ischemia in *in vivo* dog hearts. Heathers *et al.* (35) using isolated ischemic rat hearts also found that both triglyceride lipase and glycerol-3-P acyltransferase were inhibited during ischemia. Furthermore, triglyceride lipase has been clearly shown to be inhibited by high cytosolic acyl-CoA levels (36-38) which occur during ischemia (36). Similarly, glycerol 3-phosphate acyltransferase is inhibited by high intracellular Ca^{2+} (39) and catecholamines (40,41), both of which increase during myocardial ischemia.

Accordingly, we suggest that triglyceride turnover is a physiological process which serves as a potential source of energy substrate and as an important reservoir for fatty acids that inhibits their accumulation in the cytosol to dangerous levels. Our suggestion is in agreement with what was proposed previously by Hülsmann *et al.* (22). We speculate that early during ischemia, an acceleration in triglyceride lipolysis takes place (possibly due to the effect of noradrenaline released from cardiac sympathetic nerve endings that accompanies myocardial ischemia) with a resultant increase in tissue free fatty acid levels. However, these levels will be prevented from increasing to very high levels during the initial phase of ischemia since free fatty acids will soon be incorporated into endogenous triglyceride pool (as was seen in this study where total free fatty acids decreased with a concomitant increase in triglyceride content during ischemia). More importantly, the high levels of these free fatty acids and their intermediates will inhibit further triglyceride lipolysis by their own. During prolonged severe ischemia, however, tissue free fatty acid levels will start to rise further due to other factors like excessive phospholipid deacylation and severe depletion of energy nucleotides with a resultant impaired fatty acid esterification. As we mentioned earlier, hearts which were

reperfused and failed to recover maintained their cytosolic concentrations of labeled palmitate at the expense of an increase in their labeled triglyceride pool (Table 4). This adds further evidence to the physiologic protective role of endogenous triglyceride pool in maintaining cytosolic free fatty acid levels within the normal range.

An important observation in this study is back-diffusion of fatty acids to the medium. Although questionable, back-diffusion of fatty acids from myocardium to perfusion medium has been reported previously (31,42) and our data clearly support these reports.

Limitations of this study:

It should be mentioned that our perfusion protocols were conducted in the absence of lactate and ketones, both of which are present in various concentrations *in vivo*. However, both of these substrates are known to be minor energy substrates to the heart. Another limitation of this study is the absence of circulating catecholamines in our perfusates. High levels of circulating catecholamines are known to accompany myocardial infarction and during any form of surgery including coronary bypass surgery.

Some studies have suggested that under experimental conditions in which an equilibration of the experimental system has not occurred (e.g., in hepatocytes and cell free systems), $^{14}CO_2$ production is an inaccurate measure of, and can underestimate, fatty acid oxidation due to the accumulation of label in fatty acid intermediates and release as ketone bodies (43,44). This is unlikely to occur in this study since steady state conditions in the heart are reached very quickly, and $^{14}CO_2$ production has been previously shown to be a good parameter of fatty acid oxidation in the intact working heart model (45) which correlated with myocardial O_2 consumption. In addition, we have recently

shown that in working hearts fatty acid oxidation rates determined by measuring $^{14}CO_2$ production from 1.2 mM [^{14}C]palmitate is identical to fatty acid oxidation rates determined by measuring ^3H_2O production in hearts perfused with 1.2 mM [9,10-^{3}H]palmitate (12).

Summary

In summary, an increase in cytosolic labeled fatty acid concentration was seen during a 30 minute period of no-flow ischemia associated with a lower total tissue free fatty acid concentration. No significant changes in labeled triglyceride or phospholipid content was seen. This indicates a slight increase in triglyceride lipolysis and synthesis rates during ischemia. During reperfusion, steady state triglyceride lipolysis is not accelerated in hearts reperfused in the presence of 1.2 mM palmitate, although triglyceride synthesis does significantly increase. This is accompanied by a 20% increase in exogenous fatty acid oxidation rates. The increase in myocardial triglyceride synthesis with the absence of any acceleration in triglyceride lipolysis results in an expansion of the triglyceride pool during reperfusion.

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CHAPTER 4

MYOCARDIAL TRIGLYCERIDE TURNOVER AND CONTRIBUTION TO ENERGY SUBSTRATE USE IN ISOLATED WORKING HEARTS OF STREPTOZOTOCIN-INDUCED ACUTELY DIABETIC RATS

A version of this chapter has been submitted for publication

My role in this study involved conducting all the experimental part and the writing-up of the paper

CHAPTER 4

MYOCARDIAL TRIGLYCERIDE TURNOVER AND CONTRIBUTION TO ENERGY SUBSTRATE USE IN ISOLATED WORKING HEARTS OF STREPTOZOTOCIN-INDUCED ACUTELY DIABETIC RATS

Introduction

Mitochondrial ß-oxidation of fatty acids is the primary source of energy which sustains contractile function in the aerobically perfused heart (1,2). Although the majority of fatty acids are obtained from the circulation, we and others have demonstrated that endogenous myocardial triglycerides are also a potentially important source of fatty acids for ß-oxidation (2-5). The rate of lipolysis of myocardial triglyceride, and its contribution to overall myocardial ATP production, is inversely related to the concentration of exogenous fatty acids (2,5). As exogenous fatty acid concentrations decrease, the contribution of triglyceride fatty acids to mitochondiral ß oxidation increases (2).

The other major energy substrate used by the heart is glucose. A key determinant of glucose utilization is the levels of circulating fatty acids (1,6). In the presence of high concentrations of fatty acids both glycolysis and glucose oxidation rates decrease (1,2,6-8). An example of this is diabetes, in which plasma levels of both free fatty acids and triglyceride-rich lipoproteins increase. This can lead the heart to become almost entirely dependent on fatty acid oxidation to meet its energy requirements (9,10). However, Kreisberg (11) reported a decrease in oxidation of exogenous triglycerides in diabetes. A previous work in Dr. Lopaschuk's lab showed no change (12) in exogenous fatty acid oxidation in diabetes when corrected for differences in heart function.

Myocardial triglyceride content can be markedly increased in diabetes (12-15). However, the relative contribution of this pool to overall myocardial energy requirements, and the relationship between endogenous and exogenous fatty acid oxidation and glycolysis and glucose oxidation in hearts of diabetic animals has not been well characterized. In most studies the contribution of myocardial triglycerides to oxidative metabolism was inferred from changes in triglyceride content or by measuring glycerol release. Myocardial triglyceride content is so variable among hearts that measuring the content alone will not give a precise measure of lipolysis and will overlook triglyceride synthesis. Glycerol release *per se* is not a good measure of triglyceride lipolysis since it can originate from other sources such as phospholipids (16). Another compounding factor of previous studies is the fact that most of these studies were performed on isolated hearts perfused in the absence of any exogenous fatty acids, despite the fact that levels of plasma free fatty acids and triglyceride rich lipoproteins increase in diabetes.

Cardiac performance is known to be impaired in diabetes and has been attributed to changes in myocardial energy substrate use (17-20). Agents which affect myocardial metabolism have also been shown to improve myocardial performance of isolated diabetic hearts when added to the perfusing medium (10,21). It is important therefore to have a better understanding energy substrate use in diabetic hearts, particularly myocardial triglyceride turnover, since it may serve as an important source of fatty acids for ß-oxidation.

We have previously developed methodology to measure myocardial triglyceride turnover directly in the isolated perfused rat heart (2,3). We therefore used this methodology to measure the contribution of triglyceride fatty acid to mitochondrial oxidative metabolism in isolated working hearts from

streptozotocin-diabetic rats. This was achieved by pre-labeling the triglyceride pool during a "pulse" perfusion with 1.2 mM [1-^{14}C]palmitate, and subsequently measuring the rate of endogenous triglyceride fatty acid oxidation during a "chase" perfusion. During the "chase", exogenous fatty acid oxidative rates were also determined. By measuring glucose utilization (both glycolysis and glucose oxidation) in a parallel series of hearts perfused under similar conditions we were able to determine the contribution of endogenous triglycerides to overall myocardial ATP production. This contribution was determined both in the presence and absence of a high concentration of exogenous fatty acid.

Materials and Methods:

Materials

D-[^{14}C(U)]glucose, D-[5-^{3}H(N)]glucose, [9,10-^{3}H]palmitic acid, and [1-^{14}C]palmitic acid were obtained from NEN (Wilmington, DE). Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1M in methanol solution) was obtained from NEN Research Products (Boston, MA). Dowex 1X-4 anion exchange resin (200-400 mesh chloride form) was obtained from Bio-Rad Laboratories (Richmond, CA). ACS Aqueous Counting Scintillant was obtained from Amersham Canada Ltd (Oakville, Ontario). Baker Si250-PA (19C)-silica gel plates were obtained from Johns Scientific (Toronto, Ontario). Triglyceride assay, plasma glucose assay (Glucose-C) and free fatty acid assay (NEFA-C) kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Urine glucose and ketones were determined using Keto-Diastix reagent strips from Miles Canada Inc. (Etobicoke, Ontario, Canada). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

Heart Perfusions:

Adult male Sprague Dawley rats (250-300 g) were rendered diabetic by injecting them with 110 mg/kg streptozocin (dissolved in 50 mM sodium citrate, pH 4.5) into the tail vein. Age and weight matched control animals were injected with an equal volume of sodium citrate. After 48 hours, the animals were reweighed and plasma samples obtained for blood glucose and fatty acid levels determination, while urine samples were taken for glucose and ketone levels. Animals were then anesthetized with sodium pentobarbital (60 mg/kg)

intraperitoneally. Hearts were quickly excised and placed in ice-cold Krebs-Henseleit buffer. Within one minute the aorta was cannulated, and a retrograde perfusion using Krebs-Henseleit buffer initiated, as described previously (2). During this initial perfusion, each heart was trimmed of excess tissue, the pulmonary artery was cut, and the opening to the left atrium was cannulated. Following a 10 minute equilibration period, hearts were switched to the working heart mode, and perfused at an 11.5 mm Hg left atrial preload and an 80 mm Hg aortic afterload. Spontaneously beating hearts were used throughout the studies, with heart rate and peak sytolic pressure (PSP) being measured by a Gould P21 pressure transducer in the aortic outflow line. All hearts were perfused with Krebs-Henseleit buffer containing 11 mM glucose, 3% albumin, and 2.5 mM free Ca^{2+}. When used, palmitate was pre-bound to albumin, as described previously (2). The radioisotopes of glucose or palmitate that were used are indicated in the individual experiments.

Perfusion Protocols:

The protocol for the first series of control and diabetic rat heart perfusions is as discussed above. Initially, all hearts in this series were perfused for 60 minutes with re-circulated Krebs Henseleit buffer containing 1.2 mM [1-^{14}C]palmitate to label the endogenous lipid pools (pulse). During this labeling period, exogenous steady state fatty acid oxidation was also measured by quantitative collection of myocardial $^{14}CO_2$ production (as described below). At the end of the "pulse" period, hearts were switched to Langendorff drip-out perfusion with unlabeled Krebs-Henseleit buffer. During this 10 minute period, buffer containing [^{14}C]palmitate was removed from the system, and replaced

with buffer containing either 11 mM glucose or 11 mM glucose and 1.2 mM [9,10-^{3}H]palmitate. A group of hearts were also frozen at the end of this washout perfusion (with Wollenberger tongs cooled to the temperature of liquid N_2) for measurements of endogenous label and lipid content. Two additional groups of control and diabetic rat hearts were switched back to the working mode, and perfused for a subsequent 60 minute period with the new buffers described above. In order to determine how much [^{14}C]palmitate from the "pulse" remained in the perfusion system at the very beginning of "chase", and what amount of [^{14}C]palmitate was released from the heart during the "chase", buffer samples were taken at the beginning and at the end of "chase" and their [^{14}C]palmitate content determined.

A second series of control and diabetic rat hearts perfusions were also performed to measure glycolysis and glucose oxidation during the "chase" perfusion. In this series, palmitate was not labeled during the "pulse" period and the "chase" perfusion contained 11 mM [5-^{3}H/U-^{14}C]glucose ± unlabeled palmitate. Glycolysis and glucose oxidation was measured as described below.

In all hearts, mechanical function was monitored throughout the entire perfusion. At the end of the Langendorff washout perfusion following the "pulse", or at the end of the "chase", heart ventricles were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. The frozen ventricular tissue was then 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 ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 hr at 100° C, and weighed. The dried atrial weight, frozen ventricular weight, and ventricular dry-to-wet ratio were then used to determine total dry weight of the heart.

Measurement of exogenous and endogenous fatty acid oxidation rates

During the "pulse", steady state exogenous palmitate oxidation rates were determined by quantitatively measuring $^{14}CO_2$ production by the hearts, as described in detail previously (2). Hearts were perfused in a closed system that allowed collection of both perfusate and gaseous $^{14}CO_2$.

During the "chase", $^{14}CO_2$ production was used as a measure of endogenous fatty acid oxidation, while $^{3}H_2O$ production from [^{3}H]palmitate was used as a measure of exogenous fatty acid oxidation rates (2,3). Perfusate and gaseous samples were collected at 10, 20, 40 and 60 minutes during the "chase". $^{3}H_2O$ was separated from [^{3}H]palmitate as described in detail previously (2). Exogenous palmitate oxidation rates during the "chase" were expressed as nmol [^{3}H]palmitate oxidized/min·g dry wt, while endogenous oxidation rates were expressed as nmol [^{14}C]palmitate oxidized/min·g dry wt.

To determine whether there was release of [^{14}C]palmitate from the hearts to the buffer, two buffer samples were taken for each hearts, one immediately before and the other just after the "chase" perfusion. A duplicate (0.2 ml each) of each sample were then acidified with acetic acid and dried in the fumehood for 24 hours (to eliminate any remaining $^{14}CO_2$ that was still present in the buffer). Samples were then counted for [^{14}C]palmitate content using standard radio-isotope counting techniques.

Measurement of glycolysis and glucose oxidation rates

In the second series of hearts, in which the "chase" perfusate contained 11 mM [5-^{3}H/U-^{14}C]glucose, quantitative $^{3}H_2O$ production was used to measure steady state glycolytic rates ($^{3}H_2O$ is liberated at the enolase step of glycolysis), while quantitative $^{14}CO_2$ production was used to measure glucose oxidation

rates ($^{14}CO_2$ is liberated at the level of the PDC (PDC) and in the tricarboxylic acid (TCA) cycle). $^{14}CO_2$ production was determined using the same methods described above for palmitate oxidation. To measure glycolysis, ^3H_2O was separated from [^{3}H]glucose and [^{14}C]glucose as described by Kobayashi and Neely (22), using columns containing Dowex 1-X4 anion exchange resin (200-400 mesh) suspended in 0.4 M potassium tetraborate (2 ml per column) (2,3). Glucose utilization was expressed as nmol glucose metabolised/min·g dry wt.

Measurement of metabolic intermediates:

Tissue lipids from frozen ventricular tissue were extracted as previously described (2). Neutral lipids were separated from phospholipids using the methods described by Bowyer and King (23). Triglycerides were separated from other neutral lipids using Baker Si250-PA (19C)-silica Gel plates and a solvent system that consisted of isooctane:diethyl ether:acetic acid (74:24:2 vol/vol/vol) (11). [1-^{14}C]palmitate and [^{3}H]palmitate incorporation into triglycerides, other neutral lipids, and phospholipids was measured using double radioisotope counting techniques. Label content of all lipids was expressed as μmol palmitate incorporated into these pools/g dry wt. Absolute myocardial triglyceride content (μmol free fatty acid equivalents/g dry wt) was determined using Wako enzymatic colorimetric assay kits.

Calculation of ATP production from glycolysis, glucose oxidation, and exogenous and endogenous fatty acid oxidation

Myocardial ATP produced from each substrate was calculated from the steady state rates obtained during the "chase" perfusion. The calculation assumed that each mole of glucose which passes through glycolysis produced 2

moles of ATP , and that each mole of glucose oxidized produced 36 moles of ATP. Each mole of palmitate oxidized was assumed to produce 129 moles of ATP.

Statistical analysis:

Data are presented as the mean ± SE of the mean. Analysis of variance, followed by the Neuman-Keuls test was used in the determination of statistical difference in groups containing 3 or more sample populations. The unpaired Student's t-test was used to determine statistical significance in groups containing 2 sample populations. A value of $p < 0.05$ was regarded as significant.

Results:

Characteristics of acutely diabetic rats

Plasma fatty acids, glucose, and triglycerides are shown in Table 1. In rats injected 48 hours earlier with a single i.v. dose of 110 mg/kg streptozotocin, plasma levels of these substrates were all significantly elevated in the diabetic rat, compared to control rats. Myocardial triglyceride content was also significantly elevated in diabetic rats. These rats were also positive for ketones in their urine.

Heart function in control and diabetic rat hearts

Heart function in the spontaneously beating working hearts was monitored throughout the perfusion protocol. Table 2 shows the heart rate, peak systolic pressure (PSP) development, and the heart rate-pressure product obtained during both the "pulse" perfusion and during the "chase" perfusion. Similar to earlier studies, a lower heart rate was seen in the diabetic rat hearts (10). No deterioration in mechanical function was seen throughout the 2 hr perfusion period in either control or diabetic rat hearts perfused in the absence of fatty acids. The presence of palmitate in the perfusate during the "chase" did cause a slight, but significant, deterioration in heart function in control hearts but not in diabetic rat hearts. Overall, Table 2 demonstrates that triglyceride turnover and energy substrate utilization measurements made in this study were not complicated by major changes in heart function.

Triglyceride turnover in control and diabetic rat hearts

Myocardial triglycerides were initially labeled by perfusing hearts for a 60 minute period with buffer containing 1.2 mM [^{14}C]palmitate. These conditions were chosen since we have previously demonstrated that this results in optimal labelling without any depletion of the myocardial triglyceride stores (2,3). Incorporation of [^{14}C]palmitate into myocardial triglyceride is shown in Table 3. Both control and diabetic rat hearts incorporated 22.6 μmol/g dry wt of [^{14}C]palmitate into triglycerides, resulting in labeling of 58% and 35% of the total myocardial triglyceride stores, respectively. Incorporation of [^{14}C]palmitate into triglycerides represented the majority of the [^{14}C]palmitate taken up by the heart that was not oxidized. At the end of the "pulse" perfusion, phospholipids contained 2.54 ± 0.59 and 3.05 ± 0.26 μmol [^{14}C]palmitate/g dry wt in control and diabetic rat hearts, respectively. A small fraction of [^{14}C]palmitate was also present as free palmitate (0.85 ± 0.13 and 0.91 ± 0.22 μmol/g dry wt, respectively). During the 60 minute "pulse" perfusion, control hearts oxidized 40.62 ± 3.97 μmol/g dry wt of [^{14}C]palmitate compared to 31.32 ± 4.79 in diabetes ($p>0.05$). Therefore, during the "pulse" the majority of the [^{14}C]palmitate taken up by the hearts was oxidized to $^{14}CO_2$ (61% in control hearts and 54% in diabetics). The steady state oxidation rates during the "pulse" period were 682 ± 41 nmol [^{14}C]palmitate oxidized/min·g dry wt in control hearts vs 522 ± 80 nmol [^{14}C]palmitate oxidized/min·g dry wt in the diabetic rat hearts ($p>0.05$). The lower fatty acid oxidation rates in the diabetic rat hearts is probably related to the lower heart function seen in these hearts compared to control.

In hearts perfused in the absence of fatty acids a significant loss in triglyceride [^{14}C]palmitate content was seen in both control and diabetic rat hearts. This was accompanied by a significant loss in overall myocardial triglyceride content. The largest decrease in triglyceride content was seen in diabetic rat hearts (20.75 vs 40.68 μmol fatty acid/g dry wt, in control and diabetic rat hearts, respectively). This demonstrates that in hearts perfused in the absence of exogenous fatty acids triglyceride lipolysis is accelerated in diabetic rat hearts compared to control hearts.

If hearts were perfused during the 60 minute "chase" period in the presence of 1.2 mM [^{3}H]palmitate, the loss of [^{14}C]palmitate from triglycerides was significantly greater in diabetic rat hearts compared to control (a loss of 17.8 vs 9.02 μmol [^{14}C]palmitate/g dry wt, respectively) (Table 3). In contrast, during the "chase", [^{3}H]palmitate incorporation into myocardial triglycerides in control hearts was significantly greater then in diabetic rat hearts (13.8 vs 6.17 μmol [^{3}H]palmitate/g dry wt, respectively). In control hearts, the higher amount of [^{3}H]palmitate incorporated in triglycerides compared to [^{14}C]palmitate loss resulted in an small expansion of the overall triglyceride pool during the "chase". In contrast, a small loss of overall triglyceride content was seen in diabetic rat hearts during the "chase", due to a greater loss of [^{14}C]palmitate compared to the gain of [^{3}H]palmitate. This suggests that in the presence of 1.2 mM palmitate, triglyceride synthesis is greater in control hearts, but triglyceride lipolysis is greater in diabetic rat hearts.

[^{14}C]palmitate and [^{3}H]palmitate content in other myocardial lipid pools was also measured following the "chase" perfusion. No significant

loss in [^{14}C]palmitate was seen during the "chase" from myocardial phospholipids in either control or diabetic rat hearts. In control hearts, phospholipid content of [^{14}C]palmitate at the end of the "chase" was 3.34 ± 0.40 and 1.83 ± 0.33 μmol/g dry wt in hearts perfused with no fat and high fat, respectively. In diabetic rat hearts, phospholipid content of [^{14}C]palmitate was 3.91 ± 0.53 and 2.90 ± 0.57 μmol/g dry wt in hearts perfused with no fat and high fat, respectively. Incorporation of [^{3}H]palmitate into phospholipids was lower than control (1.39 ± 0.41 μmol/g dry wt vs 3.32 ± 0.45). In control hearts, [^{14}C]palmitate as free palmitate at the end of the chase was 0.53 ± 0.10 μmol/g dry wt and 0.67 ± 0.16 in hearts perfused with no fat and high fat, respectively. In diabetic rat hearts, [^{14}C]palmitate levels were 0.45 ± 0.13 and 0.16 ± 0.06 μmol/g dry wt, in hearts perfused with no fat and high fat, respectively. Only in diabetic rat hearts perfused with high fat was free [^{14}C]palmitate significantly lower than hearts frozen at the end of "pulse". In both control and diabetic rat hearts, [^{3}H]palmitate as free fatty acid was similar at the end of the "chase" (0.20 ± 0.04 and 0.26 ± 0.05 μmol[^{3}H]palmitate /g dry wt, respectively). These data demonstrate that phospholipids and free fatty acids made only minor contributions to fatty acids for oxidative metabolism during the "chase"period.

Exogenous and endogenous palmitate oxidation during the "chase" perfusion

During the 60 minute "chase" perfusion, [^{14}C]palmitate was not present in the perfusate. Instead, hearts were perfused in the presence or absence of 1.2 mM [^{3}H]palmitate. As a result, oxidation of [^{14}C]palmitate during the "chase"

originated from endogenous triglyceride stores. Cumulative [^{14}C]palmitate oxidation rates during the "chase" in control and diabetic rat hearts are shown in Figure 1. Between 10 and 60 minutes $^{14}CO_2$ production was linear in all groups. In hearts containing 1.2 mM [^{3}H]palmitate during the "chase", the production of $^{3}H_2O$ was a measure of exogenous [^{3}H]palmitate oxidation. Cumulative $^{3}H_2O$ production during the "chase" perfusion is shown in Figure 2. As with endogenous fatty acid oxidation, exogenous [^{3}H]palmitate oxidation was linear between 10 and 60 minutes.

Steady state rates of endogenous [^{14}C]palmitate oxidation and exogenous [^{3}H]palmitate oxidation between 10 and 60 minutes of the "chase" are shown in Table 4. Rates of exogenous [^{3}H]palmitate oxidation were lower in diabetes (550 ± 81 vs 690 ± 67 nmol/g dry wt·min in the diabetic and control hearts, respectively). These steady state rates were similar to the steady state exogenous [^{14}C]palmitate oxidation rates obtained during the "pulse" perfusion. Endogenous [^{14}C]palmitate oxidation rates were greatest in hearts perfused in the absence of fatty acids. In hearts perfused in the absence of palmitate, [^{14}C]palmitate oxidation was significantly higher in diabetic rat hearts compared to control. In contrast, no significant difference in [^{14}C]palmitate oxidation rates between diabetes and control was seen in hearts perfused in the presence of palmitate. The vast majority of this endogenously derived palmitate was derived from myocardial triglyceride evidenced by the significantly lower [^{14}C]palmitate content in myocardial triglyceride in these hearts and the relatively well preserved [^{14}C]palmitate content in phospholipids.

Not all of the [^{14}C]palmitate loss from myocardial triglyceride during the "chase" (Table 3) could be accounted for by [^{14}C]palmitate oxidation. Therefore buffer samples before and after the "chase" were counted for

[^{14}C]palmitate. In agreement with previous reports we found a significant release of [^{14}C] palmitate to the perfusate in all hearts during the "chase" perfusion, particularly in the presence of exogenous palmitate. This back-diffusion of [^{14}C]palmitate was consistently higher in diabetic rat hearts than control ([^{14}C]palmitate release was 8.43 ± 1.26 μmol/g dry wt in diabetic rat hearts perfused in the absence of palmitate vs 15.71 ± 3.56 in the presence of palmitate; in control, [^{14}C]palmitate release was 4.36 ± 1.24 and 9.79 ± 2.34, respectively).

Myocardial glucose utilization during the "chase" perfusion:

A second series of heart perfusions were performed to determine the contribution of glucose as an energy substrate during the "chase" perfusion. Steady state rates of glycolysis and glucose oxidation were measured simultaneously by perfusing hearts with 11 mM [5-^{3}H/U-^{14}C]glucose during the 60 minute "chase" period. Table 5 shows the steady state rates of glucose oxidation and glycolysis obtained in both control and diabetic rat hearts. In control hearts perfused in the absence of fatty acids, glycolytic rates were approximately 2.5 times higher than glucose oxidation rates, in parallel with previous studies (2,22). Addition of 1.2 mM palmitate to control hearts resulted in a much greater decrease in glucose oxidation than glycolysis, confirming that fatty acids are more potent inhibitors of the PDC than of glycolysis.

In diabetic rat hearts, glucose oxidation rates were significantly depressed compared to control hearts both in the absence and presence of 1.2 mM palmitate. In the presence of fatty acids, glucose oxidation rates were essentially abolished in the diabetic rat hearts. Glycolytic rates in diabetic rat

hearts perfused with 1.2 mM palmitate were not depressed compared to control hearts.

In diabetic rat hearts perfused in the absence of palmitate, only a portion of the glucose that passed through the glycolytic pathway was subsequently oxidized (9.1%). When palmitate was present, only 1.5% of the glucose that passed through glycolysis was eventually oxidized in diabetes.

Contribution of myocardial triglyceride to overall myocardial ATP production in control and diabetic rat hearts:

Using the steady state oxidation rates of both exogenous and endogenous radiolabeled palmitate during the "chase", as well as the steady state rates of both glycolysis and glucose oxidation, overall steady state ATP production by control and diabetic rat hearts was determined. Table 6 shows the steady state ATP production from radiolabeled substrates during the 60 minute "chase" perfusion. Interestingly, if fatty acids are omitted from the perfusate a major portion of myocardial ATP production is derived from endogenous triglycerides. This is most dramatic in diabetic rat hearts. If only steady state oxidative rates are considered, endogenous [^{14}C]palmitate oxidation accounts for 34% and 70.4% of the total ATP production in control and diabetic rat hearts, respectively.

If 1.2 mM palmitate was present in the perfusate, the contribution of glucose to steady state ATP production is significantly decreased. The contribution of glucose utilization to steady state ATP production in control hearts decreased from 66% in the no fat group to 9.3% in the high fat group. Diabetic rat hearts values were 29.6% and 6.2%, respectively. As expected, exogenous fatty acids were the major source of steady state ATP production in

both control and diabetic rat hearts. However, even in the presence of this high concentration of palmitate, 10.6% of steady state ATP production was derived from myocardial triglycerides in control hearts and 10.1% in diabetic rat hearts.

Since overall triglyceride content of hearts perfused in the absence of palmitate markedly decreased during the "chase" perfusion in both control and diabetic rat hearts, we also calculated total ATP production during the 60 minute "chase" perfusion in which non-labeled fatty acid oxidation was considered (Table 7). To do this we assumed that the ratio of $^{14}CO_2$ production to the [^{14}C]palmitate release from triglyceride was the same as the ratio of non-labeled fatty acids oxidized to non-labeled fatty acids released from myocardial triglycerides. The assumption that an average triglyceride fatty acid chain length of 16 was made. Values in Table 7 are expressed as overall ATP production during the 60 minute "chase", as opposed to steady state ATP production, since it cannot be determined from this study whether non-labeled triglyceride contribution to oxidative metabolism was linear during the "chase". An interesting observation from Table 7 is that in diabetic rat hearts perfused in the absence of any exogenous fatty acid myocardial triglycerides contributed to almost 85% of myocardial ATP requirements. In control hearts perfused in the absence of exogenous fatty acids myocardial triglyceride were also a major source of myocardial ATP requirements (>50%) which parallels our previous observations (2).

Table 1

Plasma levels of free fatty acids, glucose, and triglyceride as well as myocardial triglyceride content in control and diabetic rats

Condition	Plasma fatty acids (mM)	Plasma glucose (mg/dl)	Plasma triglyceride (mg/dl)	Myocardial TG content (µmol FFA equivalent /g dry wt)
Control (n=6)	0.21 ± 0.03	140.0 ± 5.0	164.5 ± 20.6	33.67 ± 4.14
Diabetic (n=6)	1.22 ± 0.19*	493.3 ± 18.2*	491.9 ± 110.4*	77.48 ± 4.61*

Values are the mean ± S.E.M of a number of hearts indicated in brackets
Animals were sacrificed 48 hours after being injected with 110 mg/kg streptozocin (diabetic) or vehicle (control).
*, significantly different from control.

TABLE 2

Heart function during the "pulse" and the "chase" perfusions in control and diabetic rat hearts.

Perfusion Condition	Heart Rate (beat/min)	Peak systolic pressure (mmHg)	HRxPSP x10^{-3}
During the"pulse"			
-control (n=19)	235 ± 6	102.8 ± 3.7	24.2 ± 1.1
-diabetic (n=14)	179 ± 8*	116.3 ± 3.1*	20.9 ± 1.1*
During the "chase"			
-control			
In the absence of fat (n=8)	241 ± 7	102.2 ± 4.2	24.6 ± 1.2
In the presence of fat (n=13)	236 ± 9	84.3 ± 4.8†	20.1 ± 1.5†
-diabetic			
In the absence of fat (n=9)	187 ± 8*	103.5 ± 4.4	20.4 ± 1.3*
In the presence of fat (n=13)	195 ± 18*	108.1 ± 4.4*	21.3 ± 2.3

Values are the mean ± SEM of a number of hearts indicated in brackets
PSP= Peak systolic pressure, HR= Heart rate.
Heart function was measured as the product of PSP x HR.
*, significantly different from control hearts perfused under similar conditions.
†, significantly different from control hearts perfused in the absence of fat.

Table 3

Myocardial fate of [^{14}C]palmitate and [^{3}H]Palmitate in triglycerides of control and diabetic rat hearts

Perfusion condition	[^{14}C]palmitate as triglycerides	[^{3}H]Palmitate as triglycerides	Triglyceride content (μmol fatty acid/g dry wt)
	μmol/g dry wt		
Control			
- At end of pulse (n=9)	22.59 ± 0.41	N.A.	38.79 ± 3.08
- Following "chase"			
1) with no fat (n=5)	5.75 ± 1.00*	0	18.04 ± 2.47*[a]
2) with high fat (n=5)	13.57 ± 0.93*	13.38 ± 1.67	44.79 ± 10.26
- Change during "chase"			
1) with no fat	- 16.84	0	- 20.75
2) with high fat	- 9.02	+ 13.38	+ 6.00
Diabetes			
- At end of pulse (n=7)	22.57 ± 4.21	N.A.	64.42 ± 4.54†
- Following "chase"			
1) with no fat (n=7)	7.56 ± 1.98*	0	23.74 ± 2.78*[a]
2) with high fat (n=5)	4.74 ± 2.21*	6.17 ± 1.09[b]	57.24 ± 7.77
- Change during "chase"			
1) with no palmitate	- 15.01	0	- 40.68
2) with palmitate	- 17.83	+ 6.17	- 7.18

-Values are the mean ± SEM of a number of hearts indicated in brackets. Hearts were perfused as described in "Methods".

*, significantly different from comparable hearts frozen at the end of "pulse".
a, significantly different from comparable hearts perfused in the presence of palmitate
†, significantly different from control hearts frozen at the end of "pulse".
b, significantly different from control hearts frozen at the end of "chase".

TABLE 4

Steady state exogenous [^{3}H] and endogenous [^{14}C]palmitate oxidation rates during the "chase" perfusion

Perfusion Condition	Steady State Exogenous [^{3}H] Palmitate oxidation rate (nmol/g dry wt·min)	Steady State Endogenous [^{14}C] Palmitate oxidation rate (nmol/g dry wt·min)
-control		
chase with no fat (n=6)	/	201.2 ± 17.1
chase with high fat (n=9)	689.9 ± 67.3	81.6 ± 10.0
-diabetic		
chase with no fat (n=7)	/	278.7 ± 20.8*
chase with high fat (n=6)	550.4 ± 80.8	67.3 ± 22.7

Hearts were perfused as described in "Methods". Values are the mean ± SEM of a number of hearts indicated in brackets*, significantly different from comparable control hearts.

Table 5

Steady state rates of glycolysis and glucose oxidation during the "chase" perfusion in control and diabetic rat hearts

Perfusion Condition	Steady State Glycolysis Rates (nmol/g dry wt·min)	Steady State Glucose Oxidation Rates (nmol/g dry wt·min)
-control		
- chase with no fat (n=8)	3850 ± 1190	1195 ± 124
- chase with high fat (n=7)	2380 ± 500	126 ± 18†
-diabetic		
- chase with no fat (n=8)	2810 ± 500	256 ± 94*
- chase with high fat (n=5)	2100 ± 610	31 ± 6*†

Hearts were perfused as described in "Methods". Values are the mean ± SEM of a number of hearts indicated in brackets*, significantly different from control hearts perfused under similar conditions.
†, significantly different from comparable hearts perfused in the absence of fat.

TABLE 6

Steady state myocardial ATP production during the "chase" perfusion

	ATP Production (μmol/g dry wt·min)			
	CONTROL		**DIABETIC**	
ATP Source	No Fat (n=8)	High Fat (n=7)	No Fat (n=6)	High Fat (n=6)
Glucose oxidation	43.02 ± 4.44 (56.3%)	4.52 ± 0.66 (4.5%)	9.22 ± 3.38 (18.4%)	1.13 ± 0.20 (1.3%)
Glycolysis	7.42 ± 2.08 (9.7%)	4.77 ± 0.99 (4.8%)	5.62 ± 1.00 (11.2%)	4.19 ± 1.22 (4.9%)
Endogenous labeled FA oxidation	25.95 ± 2.2 (34%)	10.52 ± 1.3 (10.6%)	35.31 ± 3.09 (70.4%)	8.54 ± 3.00 (10.1%)
Exogenous FA oxidation	.....	79.73 ± 7.82 (80.1%)	.....	71.01 ± 10.43 (83.7%)
TOTAL	76.39	99.54	50.15	84.87

Hearts were perfused as described in "Methods". Values are the mean ± SEM of a number of hearts indicated in brackets. ATP production ± SEM is shown for each substrate, together with its percentage of overall ATP production in the heart (between the brackets).

TABLE 7: Total myocardial ATP Production during the "chase" perfusion

	ATP Production (mmol/g dry wt·60 min)			
	CONTROL		**DIABETIC**	
ATP Source	No Fat (n=8)	High Fat (n=7)	No Fat (n=6)	High Fat (n=6)
Glucose oxidation	2.58 ± 0.27 (48.7%)	0.27 ± 0.04 (4.5%)	0.55 ± 0.20 (9.1%)	0.07 ± 0.01 (1.4%)
Glycolysis	0.45 ± 0.13 (8.5%)	0.29 ± 0.06 (4.8%)	0.34 ± 0.06 (5.6%)	0.25 ± 0.07 (4.9%)
Endogenous labeled FA oxidation	1.56 ± 0.13 (29.4%)	0.63 ± 0.08 (10.6%)	2.12 ± 0.19 (34.9%)	0.51 ± 0.18 (10.0%)
Endogenous unlabeled FA oxidation	0.71 ± 0.18 (13.4%)	0	3.06 ± 0.49 (50.4%)	0
Exogenous FA oxidation	.....	4.78 ± 0.47 (80.1%)	.....	4.26 ± 0.63 (83.7%)
TOTAL	5.30	5.97	6.07	5.09

Hearts were perfused as described in "Methods". Values are the mean ± SEM of a number of hearts indicated in brackets. ATP production ± SEM is shown for each substrate together with its percentage of overall ATP production in the heart (between the brackets). For ATP calculation, please refer to "Methods".

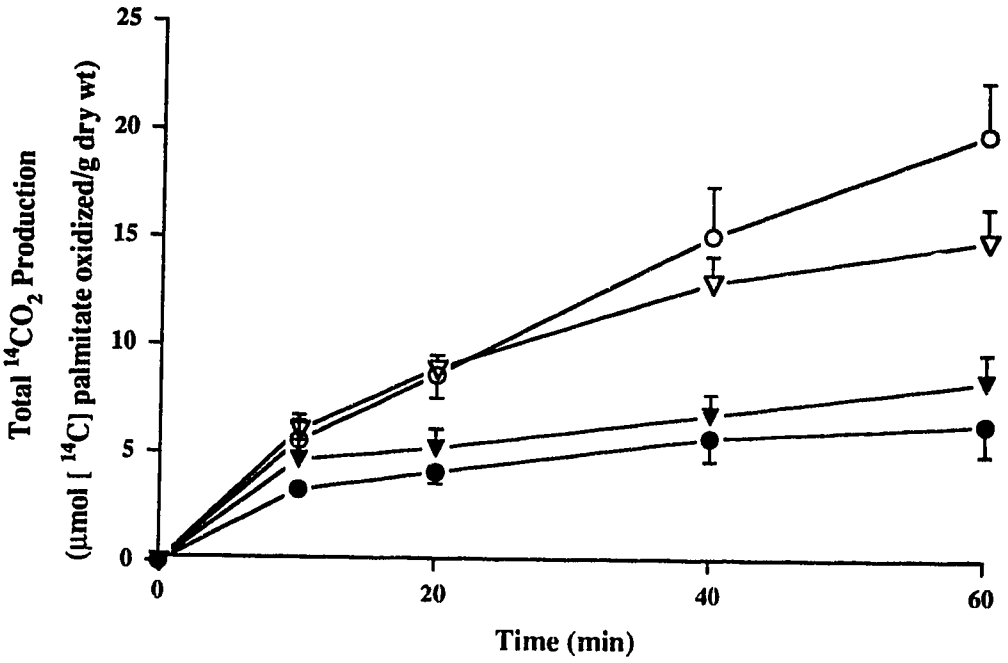

Fig. 1. Cumulative endogenous [^{14}C]palmitate oxidation in diabetic and control rat hearts during the "chase" perfusion.

Hearts were perfused in the absence or presence of palmitate as described in "Methods". Each point represents the mean ± SEM of six hearts in each group except in the control high fat group where n=9. Circles represent diabetic hearts while triangles represent control hearts. Open symbols represent hearts perfused in the absence of palmitate while filled symbols represent hearts perfused in the presence of palmitate.

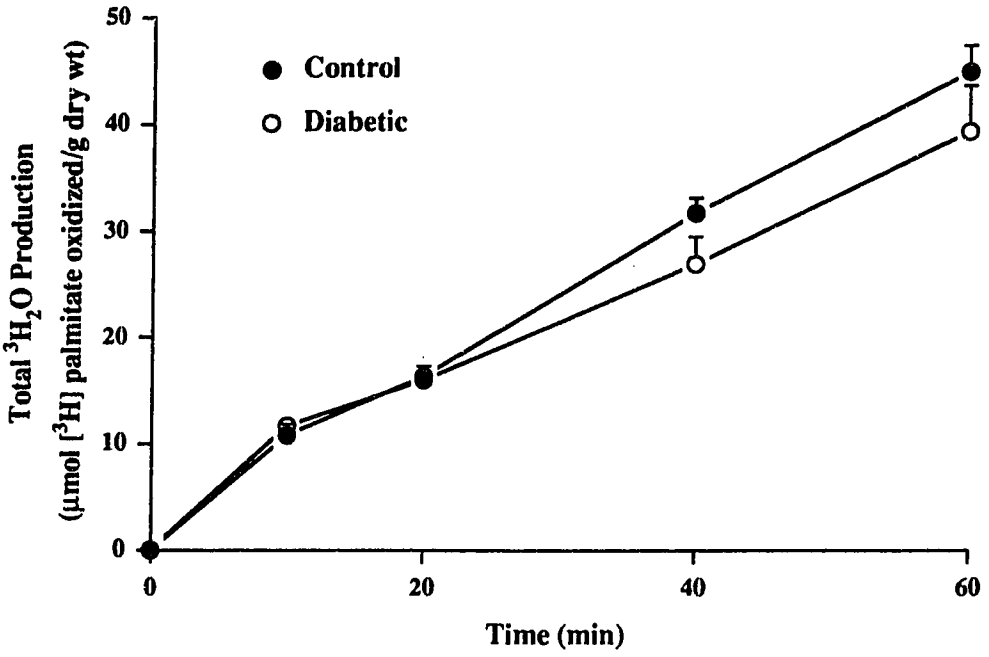

Fig. 2. Cumulative exogenous [^{3}H]palmitate oxidation in diabetic and control rat hearts during the "chase" perfusion.

Hearts were perfused in the presence of [^{3}H]palmitate during the "chase" as described in "Methods". Each point represents the mean ± SEM of seven hearts (control) and six hearts (diabetes).

Discussion:

Myocardial triglycerides are an important source of fatty acids for myocardial oxidative metabolism (2,3). In diabetic animals, myocardial triglyceride content increases (13-15,24), the extent of which depends on the severity of diabetes (15,23). These high myocardial triglyceride levels are in part related to the high concentrations of plasma free fatty acids (15,24) and elevated myocardial CoA levels (12,25) seen in diabetes. This was evident in our study, in which a large increase in triglyceride content was seen in severely diabetic rats which had elevated plasma levels of free fatty acids and total plasma triglyceride concentration compared to control (Table 1). In this study we directly measured the contribution of this expanded triglyceride pool to overall ATP production. Our results demonstrate that myocardial triglycerides can be rapidly mobilized in diabetic rats. In hearts perfused under similar perfusion conditions, triglyceride synthesis was not accelerated in diabetic rat hearts compared to control hearts. However, in both the absence and presence of a high concentration of exogenous fatty acid, triglyceride lipolysis was significantly enhanced in diabetic rat hearts. Myocardial triglyceride stores were also found to be an important source of fatty acids for mitochondrial ß-oxidation. In fact, in hearts perfused in the absence of fatty acids, oxidation of endogenous triglyceride fatty acid oxidation accounted for 85% of overall myocardial ATP requirements (Table 7). This study demonstrates that the expanded myocardial triglyceride stores seen in diabetic rat hearts can be rapidly mobilized, and the released fatty acids can either be readily used for mitochondrial ß-oxidation or can diffuse out of the heart.

Several studies have been conducted to address the issue of myocardial triglyceride turnover (4,24,26,27) and the activity of enzymes involved in

myocardial triglyceride synthesis and lipolysis in diabetes (28,29). In the majority of these studies triglyceride lipolysis was inferred from changes in triglyceride content and/or glycerol release. In hearts perfused in the absence of fatty acids, triglyceride lipolysis was shown to increase (4,24), which is consistent with the results from this study (Table 3). As expected, triglyceride lipolysis in hearts perfused under these conditions resulted in a significant loss of triglyceride content in both control and diabetic rat hearts, since no exogenous fatty acid was available to be incorporated into this store. Most of the fatty acids released from myocardial triglyceride stores in these hearts was oxidized. It has been suggested that this mobilization is an artifact of perfusing hearts in the absence of fatty acids, and that under physiological conditions, myocardial triglyceride turnover does not occur (30). However, our previous studies together with this one do not support this conclusion, and have demonstrated that regardless of circulating fatty acid levels, myocardial triglyceride synthesis and lipolysis can occur at significant rates (2,3). Unfortunately, few studies have looked at triglyceride turnover in diabetic rat hearts perfused with fatty acids. Using triglyceride content changes, Murthy *et al.* (24) suggested that triglyceride lipolysis is completely inhibited in control and diabetic rat hearts perfused in the presence of a physiologic concentration (0.5 mM) of palmitate. One problem with this approach, however is that measuring triglyceride content alone will significantly underestimate triglyceride lipolysis due to the fact that triglyceride synthesis accompanies lipolysis. This is evident from Table 3, in which significant triglyceride lipolysis occurred in both control and diabetic rat hearts perfused with high fat without dramatic changes in overall triglyceride content. Paulson *et al.* (4) measured release of radiolabeled fatty acids from triglyceride stores of hearts from control and

diabetic rats, and suggested that triglyceride lipolysis was lower in diabetic rat hearts perfused in the presence of a high concentration of fatty acid. Our data demonstrates that although $^{14}CO_2$ production from myocardial triglycerides is similar in control and diabetic rat hearts perfused with high fat, lipolysis rates are accelerated in the diabetic rat hearts, as evidenced by the combined measurement of $^{14}CO_2$ production, [^{14}C]palmitate release to the medium, and loss of triglyceride [^{14}C]palmitate content (Table 3). In contrast to hearts perfused in the absence of fatty acids, most of the liberated fatty acid in hearts perfused with high fat was released to the medium rather than being oxidized.

An interesting finding from this study is that despite the increased triglyceride pool size in diabetic rat hearts, triglyceride synthesis rates were not greater in these hearts. Myocardial triglyceride synthesis has been suggested to increase in diabetes (4,13,28). This increase is probably occurring secondary to changes in circulating fatty acid levels in diabetes. Rizza *et al.* (14) found that within hours of administering insulin to diabetic animals both plasma fatty acid levels and myocardial triglyceride content are normalized. However, administration of insulin to the perfusate of isolated perfused hearts had no such effect. The presence of ketone bodies in diabetes may also be involved, by acting as an alternative substrate, resulting in a shunting of fatty acids towards triglyceride synthesis (15, 31). This increase in synthesis of myocardial triglycerides in diabetes was found to be dependent on the availability of cortisol and growth hormone (13). As shown in Table 3, if differences in circulating carbon substrates and hormones are removed, triglyceride synthesis rates are actually lower in the diabetic rat hearts. This suggests that the important factors regulating myocardial triglyceride synthesis in diabetes are extramyocardial in origin.

In this study, the ability of diabetic rat heart to oxidize fatty acids is clearly demonstrated. Rates of exogenous palmitate oxidation in the diabetics were only slightly lower than control hearts and may be related to the poorer function in the diabetics. Kreisberg (11) showed that oxidation of chylomicron tripalmitin-^{14}C was low in isolated hearts of alloxan treated diabetic rats. A previous work in Dr. Lopaschuk's lab, however, showed that diabetic rat hearts can oxidize exogenous fatty acids at rates similar to non-diabetic rat hearts when these rates are corrected for differences in heart functions between the two groups (12). This observation is confirmed in this study. We also demonstrate that even if hearts are perfused in the absence of exogenous fatty acids, a large portion of overall myocardial ATP demands in the diabetic rat hearts are met from fatty acid oxidation (over 85%, Table 7). This requirement of fatty acids is met by the accelerated triglyceride turnover seen in these hearts.

The ability of the heart to utilize glucose is known to be impaired in diabetes mellitus (6, 32-36). However, it has also been shown that activities of hexokinase, PFK and citrate synthase from hearts obtained from acutely and chronically diabetic rats are all comparable to control hearts (35), although an inhibition of PFK has also been reported (34). In contrast, activity of the PDC (key enzyme for glucose oxidation) is markedly inhibited in diabetic rat hearts (33, 36). This inhibition explains the dramatic decrease in glucose oxidation which we observed in the diabetic rat hearts (both in the presence and absence of palmitate), despite only a small decrease in glycolytic rates. This suggests that PDC is inhibited to a disproportionally higher extent than PFK. This difference in rates can be explained by the observation that high levels of fatty acids interfere mainly with glucose oxidation, with only a partial inhibition of glycolysis (2). Inhibition of glucose oxidation in control hearts could be

relieved by the removal of fatty acids from the perfusate, which resulted in a dramatic increase in glucose oxidition (Table 5). A similar increase was not seen in diabetic rat hearts perfused in the absence of fatty acids. This suggests that the relief of PDC inhibition in diabetic rat hearts is either a slow process or does not occur. As a result, the diabetic rat hearts remained more dependent on fatty acid oxidation, resulting in the greater contribution of fatty acid oxidation (from both exogenous and endogenous sources) to meet ATP requirements (Tables 6 and 7).

The issue of fatty acid release from the heart back to the blood or medium (back diffusion) is still controversial. While this phenomenon has been reported by Paulson *et al.* (4) in isolated rat hearts and by Fox *et al.* (37) in dog hearts and by others, Nellis *et al.* reported no significant back diffusion of fatty acid in ischemic/reflow pig hearts (38). We have previously shown that this phenomenon takes place in isolated rat hearts reperfused following a 30 minute period of no-flow ischemia (3). In this study we also found that triglyceride fatty acids back diffused from both control and diabetic rat hearts. However, this back diffusion was higher in diabetic rat hearts compared to control hearts. A higher [^{14}C]palmitate back diffusion explains why myocardial triglyceride [^{14}C]palmitate content in hearts perfused in the presence of palmitate was lower in diabetes compared to control despite a similar [^{14}C]palmitate oxidation. The release of fatty acids from hearts also occurred to a greater extent in hearts perfused in the presence of palmitate than those perfused in its absence. These results suggest that triglyceride lipolysis accelerates in diabetes; when extracellular supply is low most of the fatty acids released from triglyerides are oxidized. If circulating fatty acid supply is not limiting, most of the released

fatty acids diffuse out of the heart so that fatty acid content within the myocardium does not accumulate to high levels.

There are certain points that should be mentioned regarding this study. First, our hearts were perfused in the absence of ketone bodies which may be an important substrate to the heart under certain conditions such as starvation or diabetic ketoacidosis. The relative contribution of ketones to overall myocardial ATP requirements needs to be addressed in future studies. Secondly, both control and diabetic rat hearts in our study were perfused in the absence of insulin. We intentionally omitted insulin from the perfusates since insulin levels are known to be very low or even absent in Type 1 diabetes mellitus, a situation similar to our experimental model. To negate any effects resulting from the lack of insulin, control hearts were perfused in the absence of insulin as well.

In summary, a significant increase in myocardial triglyceride content occurs within 48 hours of induction of a severe diabetes in rats. Turnover of these triglycerides is also accelerated in these hearts. This accelerated turnover provides an alternate source of fatty acids for mitochondrial ß-oxidation in hearts. Diabetic rat hearts oxidize exogenous fatty acids at rates which are only slightly lower than control, although glucose oxidation rates dramatically decrease. This data suggests that myocardial triglycerides are an important energy source for the diabetic rat heart.

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CHAPTER 5

ACETYL-COA CARBOXYLASE REGULATION OF FATTY ACID OXIDATION IN THE HEART

My role in this study involved doing the perfusion aspect of the experimental part in collaboration with James Gamble (another graduate student in the lab) and the writing-up of the paper. James Gamble performed all the tissue workup in hearts frozen at the end of each perfusion protocol.

CHAPTER 5

ACETYL-COA CARBOXYLASE REGULATION OF FATTY ACID OXIDATION IN THE HEART

Introduction

Acetyl coenzyme A carboxylase (ACC) is a key regulatory enzyme in the fatty acid synthetic pathway. It is a biotin containing enzyme which catalyses the carboxylation of acetyl-CoA to form malonyl-CoA, a substrate for fatty acid synthase (1,2). In addition, malonyl-CoA is also a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1) (3-5), which catalyzes the conversion of acyl-CoA to acylcarnitine. Since CPT 1 is a key regulatory enzyme in the mitochondrial uptake of fatty acids, ACC has also been suggested to play a pivotal role in regulating fatty acid oxidation (6). This is supported by studies which show that ACC is widely distributed mammalian tissues, including those where fatty acid synthesis is not prominent; e.g., heart and skeletal muscle (6,8-10). Two isoforms of this enzyme have been identified to date, a 265 kDa and a 280 kDa isoform (6,8), although more isoforms are thought to exist (11,12). The majority of studies have characterized the role of the 265 kDa isoform (ACC-265), which predominates in highly lipogenic tissues such as liver and white adipose tissue. While less is known about the 280 kDa isoform of ACC (ACC-280), the presence of this isoform in tissues with a high fatty acid oxidative capacity suggests a role of this enzyme in regulating fatty acid oxidation (6,8).

Fatty acid oxidation provides 60 to 90% of the ATP production by the heart, which depends to a large extent on circulating fatty acid concentrations. A key regulatory enzyme involved in fatty acid oxidation is carnitine

palmitoyltransferase 1 (CPT 1), which is highly sensitive to malonyl-CoA inhibition in the heart (5). Cook *et al.* (13) have recently suggested that, unlike the liver, it is probably the malonyl-CoA supply, as opposed to the sensitivity of CPT 1 to malonyl-CoA inhibition, that is the key factor regulating myocardial fatty acid oxidation. However, the relationship between malonyl-CoA levels and fatty acid oxidation rates in the heart has not been determined.

Stimulation of the PDC will increase glucose oxidation rates in the isolated fatty acid perfused rat heart, resulting in a parallel decrease in fatty acid oxidation rates (14). We hypothesize that acetyl-CoA derived from PDC can be transported from the mitochondria to the cytosol where it can then serve as a substrate for ACC, increasing malonyl-CoA production and decreasing fatty acid oxidation. This transport could occur via a carnitine acetyltransferase (CAT) and carnitine acetyltranslocase pathway which is active in the heart and transports acetyl groups from intramitochondrial acetyl-CoA to cytosolic CoA (15-17). This hypothesis is supported by a recent study in our lab which demonstrates that stimulating this pathway with carnitine will increase glucose oxidation rates in the heart, which probably occurs secondary to a decrease in the intramitochondrial acetyl-CoA/CoA ratio (18), with a parallel decrease in fatty acid oxidation. Further support to this hypothesis comes from observations by Lysiak *et al.* (16) that most of acetyl-CoA generated from pyruvate by PDC is readily accessible to CAT while that generated from ß-oxidation is more available to tricarboxylic acid (TCA) cycle.

In the intact heart, fatty acids destined for ß-oxidation can be derived from exogenous sources, or from endogenous myocardial triglyceride stores (19). Using a "pulse-chase" technique in the isolated perfused working heart, we can measure the contribution of both exogenous and endogenous fatty

acids to ß-oxidation (19). This technique involves pre-labeling the triglyceride pool with [^{14}C]palmitate and chasing hearts with [^{3}H]palmitate. This study uses this procedure to determine if ACC is involved in the short term regulation of fatty acid oxidation in the heart. To modify fatty acid oxidation rates, hearts were perfused with dichloroacetate (DCA), a stimulator of PDC. This results in a marked stimulation of glucose oxidation with a parallel decrease in the contribution of fatty acid oxidation to overall ATP production (14). Using this approach we demonstrate a strong correlation between increased acetyl-CoA production from PDC and an increase in myocardial malonyl-CoA production. Increases in malonyl-CoA production also correlated with a reduction in overall myocardial fatty acid oxidation rates, suggesting that ACC is an important regulator of fatty acid oxidation in the heart.

Materials and Methods:

Materials

D-[^{14}C(U)]glucose, [9,10-^{3}H]palmitic acid, and [1-^{14}C]palmitic acid were obtained from NEN (Wilmington, DE). Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1M in methanol solution) was obtained from NEN Research Products (Boston, MA). ACS Aqueous Counting Scintillant was obtained from Amersham Canada Ltd (Oakville, Ontario). Triglyceride assay kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). For HPLC analysis of malonyl-CoA, a precolumn cartridge C18, size 3 cm, 7 μm was purchased from Pierce Chemical Company (Rockford, IL) and a Microsorb short-one column type C18, particle size 3 μm, size 4.6 x 100 mm was purchased from Rainin Instruments Company (Emeryville, CA). ECL Western blotting detection reagents were purchased from Amersham International plc (Amersham, UK). IgG_1 monoclonal antibodies (7AD3) to ACC-280 was chosen from a panel of monoclonal antibodies raised against avidin-Sepharose purified fasted/ refed rat liver enzyme (see ref. 6 for more details). A polyclonal antibody to ACC-265 was raised in rabbits against fasted/ refed rat liver ACC where the 265-kDa band was eluted from an SDS gel for use as an immunogen as described previously (6). Secondary antibodies (peroxidase conjugated affinipore goat anti-mouse IgG [H+L] and peroxidase conjugated affinipore goat anti-rabbit IgG [H+L]) were purchased from Jackson Immunoresearch Laboratories Inc./Bio/Can Scientific (Mississauga,Ontario). Peroxidase labeled streptavidin was purchased through Mandel Scientific from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). For streptavidin and immunoblots, Trans-Blot Transfer

Medium (pore nitrocellulose membrane 0.45 μm) was obtained from BioRad (Richmond, CA). X-ray films (X-OMAT AR Film) were purchased from Kodak (Rochester, NY). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO).

***Heart Perfusions*:**

Adult male Sprague Dawley rats (250-300 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Hearts were quickly excised and placed in ice-cold Krebs-Henseleit buffer. The aorta was rapidly cannulated, and a retrograde perfusion using Krebs-Henseleit buffer was initiated as described previously (19). During this initial perfusion, each heart was trimmed of excess tissue, the pulmonary artery was cut, and the opening to the left atrium was cannulated. Following a 10 minute equilibration period, hearts were switched to the working heart mode, and perfused at an 11.5 mm Hg left atrial preload and an 80 mm Hg aortic afterload. Spontaneously beating hearts were used throughout the studies, with heart rate and peak sytolic pressure (PSP) being measured by a Gould P21 pressure transducer in the aortic outflow line. Cardiac output, aortic flow and coronary flow were measured using Transonic in-line ultrasonic flow probes connected to T 101 ultrasonic blood flow meter.

In all hearts, mechanical function was monitored throughout the entire perfusion. Heart work was expressd as the product of peak systolic pressure x cardiac output.

Perfusion Protocols

All hearts were perfused with Krebs-Henseleit buffer containing 1.2 mM palmitate, 3% albumin, 11 mM glucose and 1.25 mM free Ca^{2+}.

Overall Fatty Acid Oxidation Measurements: Table 1 shows the protocol used to measure both exogenous and endogenous fatty acid oxidation in the heart. In this series of perfusions, hearts were initially perfused for 60 min period with re-circulated Krebs Henseleit buffer containing 1.2 mM [1-^{14}C]palmitate to label the endogenous lipid pools (pulse). During this labeling period, exogenous steady state fatty acid oxidation was measured by quantitative collection of myocardial $^{14}CO_2$ production as described previously (19). At the end of the "pulse" period, hearts were switched to Langendorff perfusion with Krebs-Henseleit buffer containing 11 mM glucose. A group of hearts were frozen at the end of this washout perfusion (with Wollenberger tongs cooled to the temperature of liquid N_2). In the remainder of hearts, the buffer containing [^{14}C]palmitate was removed from the system during this 10 min period, and replaced with buffer containing 11 mM glucose and 1.2 mM [9,10-^{3}H]palmitate ± 1 mM DCA. Hearts were then switched back to the working mode, and perfused for a subsequent 60 min period with the new buffer described above.

During the "pulse", steady state exogenous palmitate oxidation rates were determined by quantitatively measuring $^{14}CO_2$ production by the hearts, as described in detail previously (19). Hearts were perfused in a closed system that allowed collection of both perfusate and gaseous $^{14}CO_2$. During the "chase", $^{14}CO_2$ production was used as a measure of endogenous fatty acid oxidation, while $^{3}H_2O$ production from [^{3}H]palmitate was used as a measure of exogenous fatty acid oxidation rates (19). Perfusate and gaseous samples were

collected at 10, 20, 40 and 60 minutes during the "chase". ^3H_2O was separated from [^{3}H]palmitate as described in detail previously (19). Steady state palmitate oxidation rates during the "chase" were expressed as nmol labeled palmitate oxidized/g dry wt·min·unit work.

Glucose Oxidation Measurements: In this series of heart perfusions, glucose oxidation rates were measured during the "chase" perfusion. The same perfusion protocol and perfusion substrates described above were used, except that the palmitate was not labeled during either the "pulse" period or the "chase" period. Instead, during the "chase" period perfusate contained 11 mM [U-^{14}C] glucose and 1.2 mM palmitate. Glucose oxidation was determined by quantitative measurement of $^{14}CO_2$ production ($^{14}CO_2$ is liberated at the level of PDC and in the tricarboxylic acid cycle). $^{14}CO_2$ production was determined using the same methods described above for palmitate oxidation. Glucose oxidation rates were expressed as nmol glucose oxidized/g dry wt·min·unit work.

Tissue Analysis:

At the end of the perfusions, heart ventricles were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N_2. The frozen ventricular tissue was then 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 ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 12 hr at 100° C, and weighed. The dried atrial weight, frozen ventricular weight, and ventricular dry-to-wet ratio were then used to determine total dry weight of the heart.

Measurement of lipid metabolic intermediates: Tissue lipids from frozen ventricular tissue were extracted as described previously (19). Neutral lipids were separated from phospholipids using the method described by Bowyer and King (20). [^{14}C]palmitate and [^{3}H]palmitate incorporation into neutral lipids was measured using double radioisotope counting techniques. Label content of neutral lipids was expressed as µmol labeled palmitate incorporated into this pool/g dry wt. Absolute myocardial triglyceride content (µmol fatty acid equivalents/g dry wt) was determined using Wako enzymatic colorimetric assay kits.

Acetyl-CoA carboxylase assay: Approximately 200 mg of frozen tissue was homogenized with a buffer containing Tris/HCl (50 mM; pH 7.5 at 4° C), 100 mM NaF, 2 mM EDTA, 0.25 M sucrose, 70 µl ß-mercaptoethanol/100 ml and a mixture of seven protease inhibitors (21). Samples were then ultracentrifuged at 180,000 g for 60 minutes. The supernatant was then dialysed overnight at 4° C with a buffer containing 50 mM Tris/HCl (pH 7.5 at 4° C), 100 mM NaF, 2 mM EDTA, 10 mM ß-mercaptoethanol, and 10% v/v glycerol. Dialysate protein content was measured using Bradford's method (22). To measure ACC activity, 25 µl of the dialysate was added to a reaction mixture (final volume 190 µl) containing Tris acetate (11.5 mM, pH 7.5), BSA (2.9 µM), ß-mercaptoethanol (1.9 µM), ATP (0.41 mM), acetyl-CoA (0.21 mM), magnesium acetate (0.97 mM), $NaHCO_3$ (3.5 mM) and 10 mM magnesium citrate, and incubated for either 0, 1, 2, 3, or 4 minutes. Reactions were stopped by adding 10% perchloric acid (PCA). Samples were then spun for 10 min and the malonyl-CoA concentration measured using an HPLC procedure described below. In order to determine citrate dependence, another set of reactions were conducted at citrate

concentrations ranging from 0-10 mM for 4 minutes. ACC activity was expressed as the amount of malonyl-CoA formed/g dry wt·min.

Determination of CoA esters: CoA esters were extracted as described previously (23). The 6% PCA extract was maintained at a pH of 2-3. The CoA esters were measured using a modified HPLC procedure described by King et al (24). Separation was performed on a Beckman System Gold with a UV detector 167. Each sample (100 µl each) were run through a precolumn cartridge (C18, size 3 cm, 7 µm) and a Microsorb short-one column (type C18, particle size 3 µm, size 4.6 x 100 mm). Absorbance was set at 254 nm and flow rate at 1 ml/min. A gradient was initiated using 2 buffers: buffer A consisted of 0.2 M NaH_2PO_4 (pH 5.0) and buffer B was a mixture of 0.25 M NaH_2PO_4 and acetonitrile (pH 5.0) in a ratio of 80/20 (v/v). Buffers were filtered using filter pure, Nylon-66 filter membrane (Pierce Chem. Company, Rockford, IL). Initial conditions (97% A, 3% B) were maintained for 2.5 min. and at 2.5 min. were changed to 18% B over 5 min. using Beckman's curve 3. At 15 min. it was changed to 37% B linearly over 3 min. and at 20 min. it was changed to 90% B linearly over 17 min. At 42 min. the composition was changed back to 3% B linearly over 0.5 min. and at 50 min. column equilibration was complete. Peaks were integrated by a Beckman System Gold software package.

Western blot analysis of ACC: Dialysate samples were subjected to SDS-PAGE using the method of Laemmli (25). Following the gel electrophoresis, a semi-dry transfer to nitrocellulose was performed. Membranes were then probed with either streptavidin or antibodies to either the 280 or the 265-kDa isozymes.

Chemiluminescent detection was performed on the membranes using ECL western blotting detection kit.

Measurement of mitochondrial CPT I activity: Mitochondria from a fresh adult rat heart were isolated as described previously (26) using ice-cold MSE buffer containing 225 mM mannitol, 75 mM sucrose, and 1 mM EGTA, pH 7.5. The mitochondrial preparation was used to determine CPT I sensitivity to inhibition by malonyl-CoA by measuring CPT I activity in the presence of varying concentrations of malonyl-CoA. CPT I activity was determined as described previously by Bremer (27). Briefly, an incubation medium containing 75 mM KCl, 50 mM mannitol. 25 mM HEPES (pH 7.3), 2 mM NaCN, 0.2 mM EGTA, 1 mM DTT and 1% fat free albumin was used. The mitochondrial preparation (35-40 μg protein) was preincubated with this buffer at 30° C in the presence of 75 μM palmitoyl-CoA (dissolved in 25 mM KH_2PO_4, pH 5.3) and varying concentrations of malonyl-CoA ranging from 0-2.5 μM. 1 μCi L-[methyl-^{3}H]carnitine was then added to a final L-carnitine concentration of 200 μM and incubation was continued for further 6 minutes. Reactions were stopped by adding 100 μl of concentrated HCl. The [^{3}H]palmitoylcarnitine formed was measured using *n*-butanol extraction as described previously (27) and then counted using standard radioisotope counting techniques.

Statistical analysis:

Data are presented as the mean ± SE of the mean. The unpaired Student's t-test was used to determine statistical significance in groups containing 2 sample populations. A value of $p< 0.05$ was regarded as significant.

Results:

Effect of dichloroacetate on heart function: Because the work performed by the heart is a key determinant of oxidative rates, mechanical function was continuously monitored throughout the perfusion period. Table 2 shows heart rate, peak systolic pressure and heart work in control and DCA-treated hearts during both the initial "pulse" period, and during the "chase" period. In control hearts, no deterioration of mechanical function was seen throughout the perfusion period. Addition of DCA following the "pulse" period did not have major effects on heart function. A small increase in both heart rate and heart work was seen in the DCA-treated hearts. To rule out any potential effects of the increase in heart mechanical function seen in DCA-treated hearts on oxidative metabolism, all subsequent oxidative rate measurements were corrected for difference in heart work.

Effects of dichloroacetate on palmitate and glucose oxidation rates: Our previous studies have shown that endogenous myocardial triglycerides are an important source of fatty acids for mitochondrial oxidation (19). Therefore, to determine the role of ACC in regulating fatty acid oxidation, both exogenous and endogenous fatty acid oxidation rates were measured. To achieve this we pre-labeled the myocardial triglyceride pool with [^{14}C]palmitate (see Table 1). This resulted in 23.4 ± 2.6 μmol/g dry wt of [^{14}C]palmitate being incorporated into myocardial neutral lipids. During the 60 minute "chase" perfusion, [^{14}C]palmitate was not present in the perfusate, and hearts were perfused in the presence of 1.2 mM [^{3}H]palmitate. As a result, oxidation of [^{14}C]palmitate during the "chase" originated from endogenous triglycerides (19). During the

60 minute chase period, rates of both endogenous and exogenous fatty acid oxidation were linear (data not shown, see ref. 19).

Steady state rates of endogenous [^{14}C]palmitate oxidation and exogenous [^{3}H]palmitate oxidation during the "chase" are shown in Table 3. As expected, addition of DCA to the perfusate resulted in a marked stimulation of glucose oxidation (secondary to a stimulation of PDC activity). This was accompanied by a parallel decrease in exogenous fatty acid oxidation rate. Table 3 also shows the % contribution of these pathways to overall myocardial ATP production. In control hearts perfused with 1.2 mM palmitate, glucose oxidation provided 7.1% of the overall ATP production. DCA treatment resulted in an increase in the contribution of ATP to 30.6%. The 23% increase in the contribution of glucose oxidation to ATP production was accompanied by a parallel 24% decrease in the contribution of overall fatty acid oxidation to ATP production. This demonstrates that DCA treatment resulted in a significant shift away from fatty acid oxidation towards glucose oxidation as a source of ATP.

Myocardial labeled neutral lipid content and triacylglycerol content: Table 4 shows the label content of [^{14}C]palmitate and [^{3}H]palmitate in hearts at the end of the 60 min "chase" period. In both the control and DCA-treated hearts, the amount of [^{14}C]palmitate remaining in the heart was similar. In contrast, a significantly greater amount of [^{3}H]palmitate was incorporated into neutral lipids during the "chase" period in DCA-treated hearts compared to control. This combined with the lower fatty acid oxidation rates, suggests that fatty acids were shunted away from fatty acid oxidation and towards triacylglycerol synthesis.

Acetyl Coenzyme A Carboxylase Activity in Hearts: In liver both 265 kDa and 280 kDa isoforms of ACC are present. Figure 1 shows an immunoblot analysis of ACC extracted from 3 control and 3 DCA-treated hearts. ACC-280 was identified using a monoclonal Ab specific to ACC-280, while ACC-265 was identified using a sheep polyclonal specific to ACC-265. As shown in Figure 1A and 1B, both the 280 and 265 kDa isoforms of ACC are present. Figure 1C shows a streptavidin analysis of ACC content (streptavidin recognizes the biotin containing moiety of carboxylases). Using this approach the relative distribution of the 280 and 265 kDa isoforms of ACC in heart could be determined. As shown in Figure 1C, although both isoforms were present in heart tissue, the 280 kDa isoform of ACC predominates.

In liver, ACC can be markedly stimulated by citrate. The 265 kDa isoform of ACC is highly regulated by phosphorylation (28-30), which can be overcome in the presence of high concentrations of citrate(28,29). Since the 280 kDa predominates in the heart, we measured the citrate dependence of ACC extracted from control hearts. ACC activity was determined by HPLC measurement of malonyl-CoA produced from the reaction. In both control and DCA-treated hearts, malonyl-CoA production was linear over a 4 min incubation period ($r = 0.998$). As shown in Figure 2, there was very little citrate dependence of ACC extracted from these hearts. This lack of citrate dependence persisted if ACC was prepared from fresh hearts, or if hearts were either slowly frozen or frozen and re-thawed (which is thought to increase phosphate content of ACC; ref 31). Furthermore, perfusion of hearts with glucagon (which phosphorylates ACC-265 and inactivates it) also did not increase the citrate dependence of heart ACC (data not shown). In addition, cardiac ACC has been found to exhibit a higher Km for acetyl-CoA compared

to white adipose tissue ACC (data not shown). These data, together, suggest that the 280 kDa isoform has different enzyme kinetics from those of the 265 kDa isoforom and is not highly regulated by phosphorylation.

ACC activity measured in extracts from control and DCA-treated hearts is shown in Table 5. In the presence of 10 mM citrate and saturating concentrations of acetyl-CoA (5 mM), ACC activity did not differ between control and DCA-treated hearts. This suggests that DCA has no direct allosteric effects on ACC activity.

Myocardial levels of CoA esters: Table 6 shows levels of free CoASH and CoA esters measured in hearts frozen at the end of each perfusion. Malonyl-CoA levels were significantly higher in DCA-treated hearts compared to control hearts. Acetyl-CoA levels were also significantly greater in the DCA-treated hearts. This indicates that both substrate (acetyl-CoA) and product (malonyl-CoA) levels increased in DCA-treated hearts.

A significant correlation was seen between myocardial acetyl-CoA levels and malonyl-CoA levels (Figure 3a). This suggests that acetyl-CoA supply to ACC may be an important regulator of malonyl-CoA production. Figure 3b shows the correlation between myocardial levels of malonyl-CoA and overall fatty acid oxidation rates in the heart. A significant negative correlation was also seen between malonyl-CoA levels and palmitate oxidation rates, suggesting that malonyl-CoA levels are an important determinant of myocardial fatty acid oxidation rates. Overall Figure 3 suggests that an increase in myocardial acetyl-CoA levels by DCA increases malonyl-CoA production, resulting in an inhibition of fatty acid oxidation.

Cardiac CPT I sensitivity to malonyl-CoA : Figure 4 depicts the exquisite sensitivity of CPT I in cardiac mitochondria preparation to malonyl-CoA. An IC_{50} of 50 nM malonyl-CoA/mg mitochondrial protein was obtained. Increasing the concentration of malonyl-CoA to 0.5 μM/mg mitochondrial protein almost completely abolished the enzyme activity.

Table 1

Perfusion protocol for measuring overall fatty acid oxidation (exogenous and endogenous) and glucose oxidation in isolated working hearts perfused in the absence or presence of dichloroacetate

	Pulse period ---------->	**Washout period** -------->	**Chase period**
Perfusion Conditions			
A) Fatty Acid Oxidation	60-min prelabeling with: 11 mM glucose and 1.2 mM [^{14}C]palmitate	10-min aerobic retrograde perfusion	60-min perfusion with 11 mM glucose, 1.2 mM [^{3}H]palmitate) with: a) no addition b) 1 mM dichloroacetate
		or	
B) Glucose Oxidation	60-min preperfusion with: 11 mM glucose and 1.2 mM palmitate	10-min aerobic retrograde perfusion	60-min perfusion with 11 mM [^{14}C]glucose, 1.2 mM palmitate with: a) no addition b) 1 mM dichloroacetate
Procedure Performed	*measure $^{14}CO_2$ production in [^{14}C]palmitate hearts*	*change perfusate*	*measure $^{3}H_2O$ and/or $^{14}CO_2$ production*

-In group A, $^{3}H_2O$ and $^{14}CO_2$ production during the chase was a measure of exogenous and endogenous fatty acid oxidation, respectively. In group B, $^{14}CO_2$ production during the chase was a measure of glucose oxidation.

Table 2

Mechanical function of isolated working hearts perfused in the presence or absence of the pyruvate dehydrogenase complex stimulator, dichloroacetate.

Condition	Heart rate (beat/min)	Peak systolic pressure (mm Hg)	HR x PSP $x10^{-3}$ (beat·mmHg/min)	Heart work (mmHg·ml/min x 10^{-3})
During Pulse Perfusion (n=6)	247 ± 10	90.1 ± 1.0	22.28 ± 1.06	2.3 ±0.4
During Chase Perfusion				
-control (n=9)	217 ± 15	97.9 ± 4.2	21.4 ± 1.5	1.5 ± 0.5
-dichloroacetate (1 mM) (n=9)	256 ± 12	96.6 ± 2.8	24.7 ± 1.3	2.4 ± 0.7

-Data represent the mean ± S.E.M of a number of hearts indicated in bracketst. Hearts were perfused as described in "Methods".
Heart work was determined as the product of PSP x cardiac output.
HR=heart rate, PSP= peak systolic pressure.

Table 3

Oxidation rates of glucose and palmitate (A) and contribution to ATP production (B) in isolated working rat hearts perfused in the presence or absence of dichloroacetate.

Condition	Glucose oxidation	Exogenous palmitate oxidation	Endogenous palmitate oxidation
A) Steady state rates		*(nmol/g dry wt·min·unit work)*	
-Control	72.9 ± 10.0	304.9 ± 51	20.1 ± 5.0
-Dichloroacetate (1 mM)	300.0 ± 28.6*	205.5 ± 40.1*	14.4 ± 3.9
B) Contribution to ATP production		*(% contribution)*	
-Control	7.1%	87.5%	5.4%
-Dichloroacetate (1 mM)	30.6%	65.5%	3.9%

-Data are the mean ± SEM of six hearts in each group. Hearts were perfused as described in Table 1, with oxidation rates measured during the "chase" perfusion. Contribution to ATP production was calculated from the absolute oxidative rates, using a value of 129 moles of ATP produced/mole of palmitate oxidized, and 38 moles of ATP produced/mole of glucose oxidized. *, significantly different from control hearts.

Table 4

^{14}C-palmitate and ^{3}H-palmitate content in neutral lipids and triacylglycerol content in isolated working rat hearts perfused in the presence or absence of dichloroacetate.

Condition	^{14}C palmitate as neutral lipids (μmol/g dry wt)	^{3}H palmitate as neutral lipids (μmol/g dry wt)	Triacylglycerol content (μmol fatty acid/g dry wt)
-Control	14.49 ± 2.33	15.80 ± 1.65	22.54 ± 1.26
-Dichloroacetate (1 mM)	14.33 ± 1.87	22.43 ± 1.25*	27.29 ± 1.87

Data represent the mean ± SEM of seven hearts in each group. Hearts were perfused as described in "Methods".
*, significantly different from control.

Table 5

Acetyl CoA carboxylase activity from cytosolic extracts of isolated working hearts perfused in the presence or absence of dichloroacetate.

Condition	Acetyl CoA Carboxylase activity *(nmol/g dry wt·min)*
-Control	89.6 ± 15.3
-Dichloroacetate (1 mM)	84.4 ± 14.0

Data represent the mean ± SEM of four hearts in each group. Hearts were perfused as described in "Methods".

Table 6

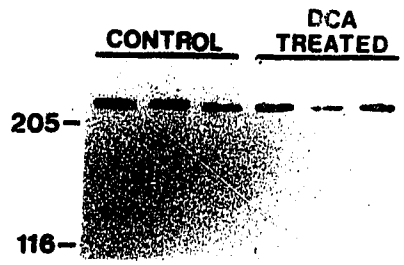
Levels of CoA esters in isolated working rat hearts perfused in the presence or absence of dichloroacetate

Condition	Malonyl CoA	Acetyl CoA	CoA-SH	Succinyl CoA	Glutathione CoA
			(nmol/g dry wt)		
-Control (n=11)	10.0 ± 0.7	32.8 ± 2.9	292.7 ± 37.0	142.1 ± 30.9	85.8 ± 13.5
-Dichloroacetate (1 mM) (n=10)	14.0 ± 0.6*	90.8 ± 6.0*	236.9 ± 22.3	63.8 ± 8.3*	58.3 ± 7.0

Data represent the mean ± SEM of a number of hearts shown in brackets. Hearts were perfused as described in "Methods". *, significantly different from control hearts.

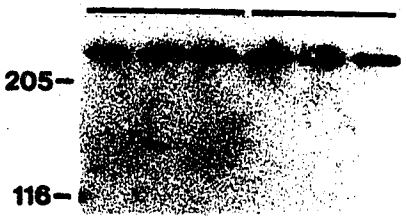
Fig. 1. Immunoblot and streptavidin analysis of acetyl-CoA carboxylase in tissue extracts from isolated perfused hearts. Immunoblots were performed using; A) a monoclonal Ab to the 280 kDa isoform of acetyl-CoA carboxylase (ACC-280), and B) a polyclonal Ab to the 265 kDa isoform of acetyl-CoA carboxylase (ACC-265). Streptavidin analysis (C) was used to determine the relative content of ACC-280 and ACC-265 (streptavidin recognizes the biotin containing groups of carboxylases). Following perfusion, control and DCA-treated hearts were quickly frozen and ACC was isolated as described in "Methods". SDS-PAGE was performed followed by a semi-dry transfer to nitrocellulose membrane. Immunoblot was then performed as described in "Methods". Panels 1-3 represent control hearts while panels 4-6 represent DCA-treated hearts.

ACC 280 Ab



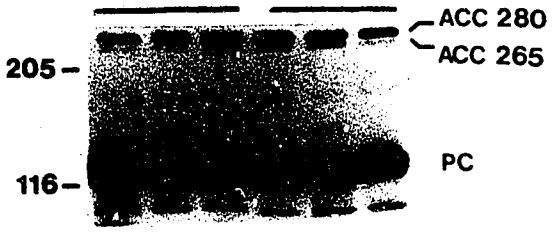
A

ACC 265 Ab



B

STREPTAVIDIN



C

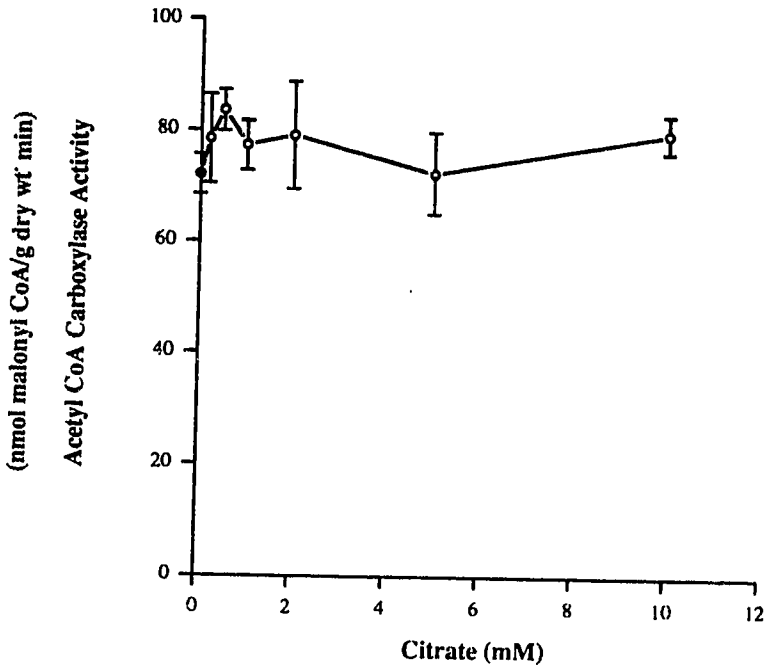


Fig. 2. Citrate dependence of acetyl-CoA carboxylase activity isolated from control or dichloroacetate-treated hearts. Data represent the mean ± SEM of six hearts at each citrate concentration. ACC was extracted from control and DCA-treated hearts frozen at the end of the "chase" perfusion. ACC activity was measured by HPLC analysis of the amount of malonyl-CoA produced per minute per g dry weight of the tissue, as described in "Methods".

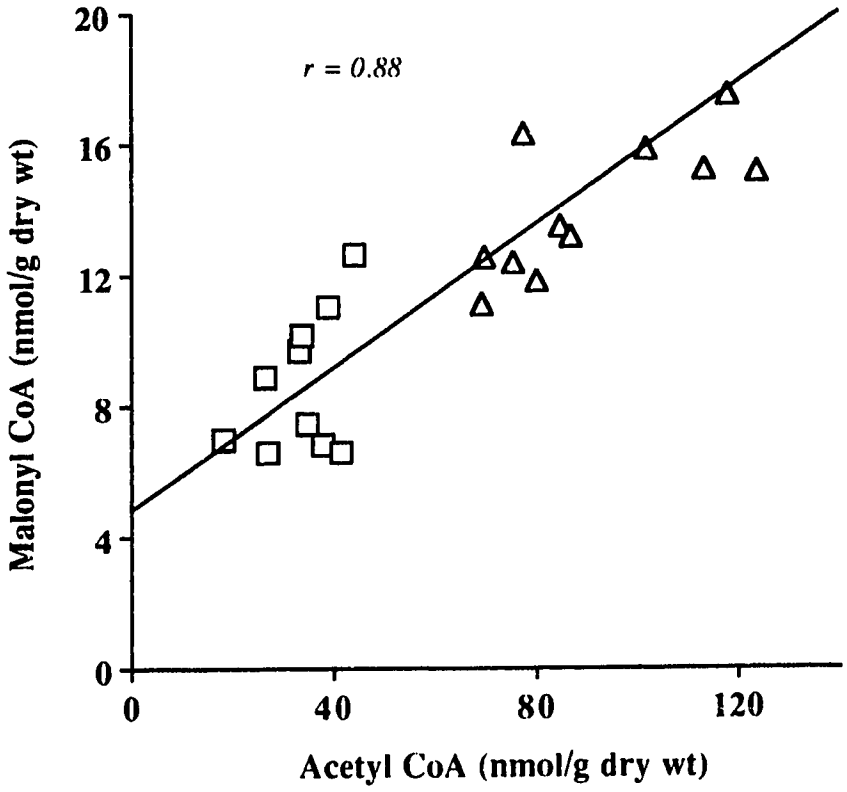


Fig. 3a. Correlation between myocardial total acetyl-CoA and malonyl-CoA levels in isolated hearts perfused in the presence or absence of dichloroacetate. Hearts were perfused as described in Table 1. CoA esters were extracted from hearts using 6% perchloric acid and seperated by HPLC as as described in the "Methods". Triangles, DCA-treated hearts; Squares, control hearts.

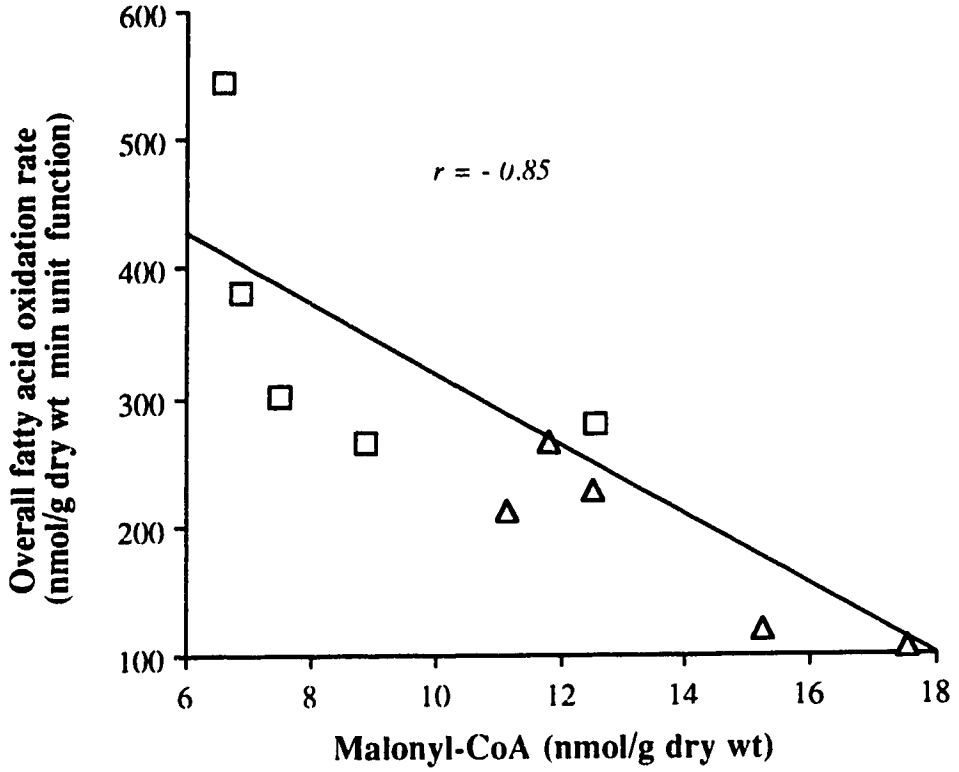


Fig. 3b. Correlation between myocardial total malonyl-CoA levels and total palmitate oxidation rates in isolated hearts perfused in the presence or absence of dichloroacetate. Hearts were perfused as described in Table 1. Palmitate oxidation rates shown are the sum of exogenous and endogenous palmitate oxidation. CoA esters were extracted from hearts using 6% perchloric acid and seperated by HPLC as as described in the "Methods". Triangles, DCA-treated hearts; Squares, control hearts.

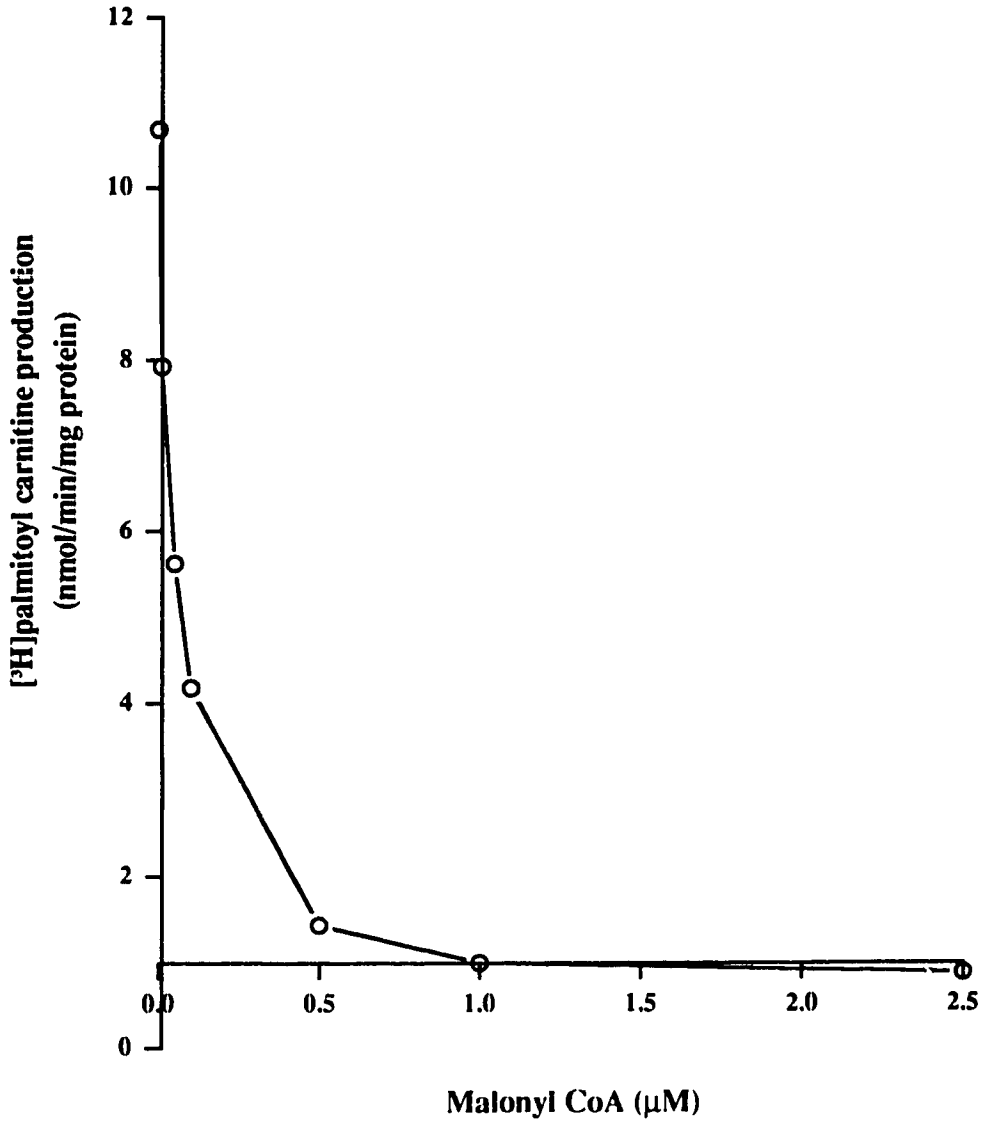
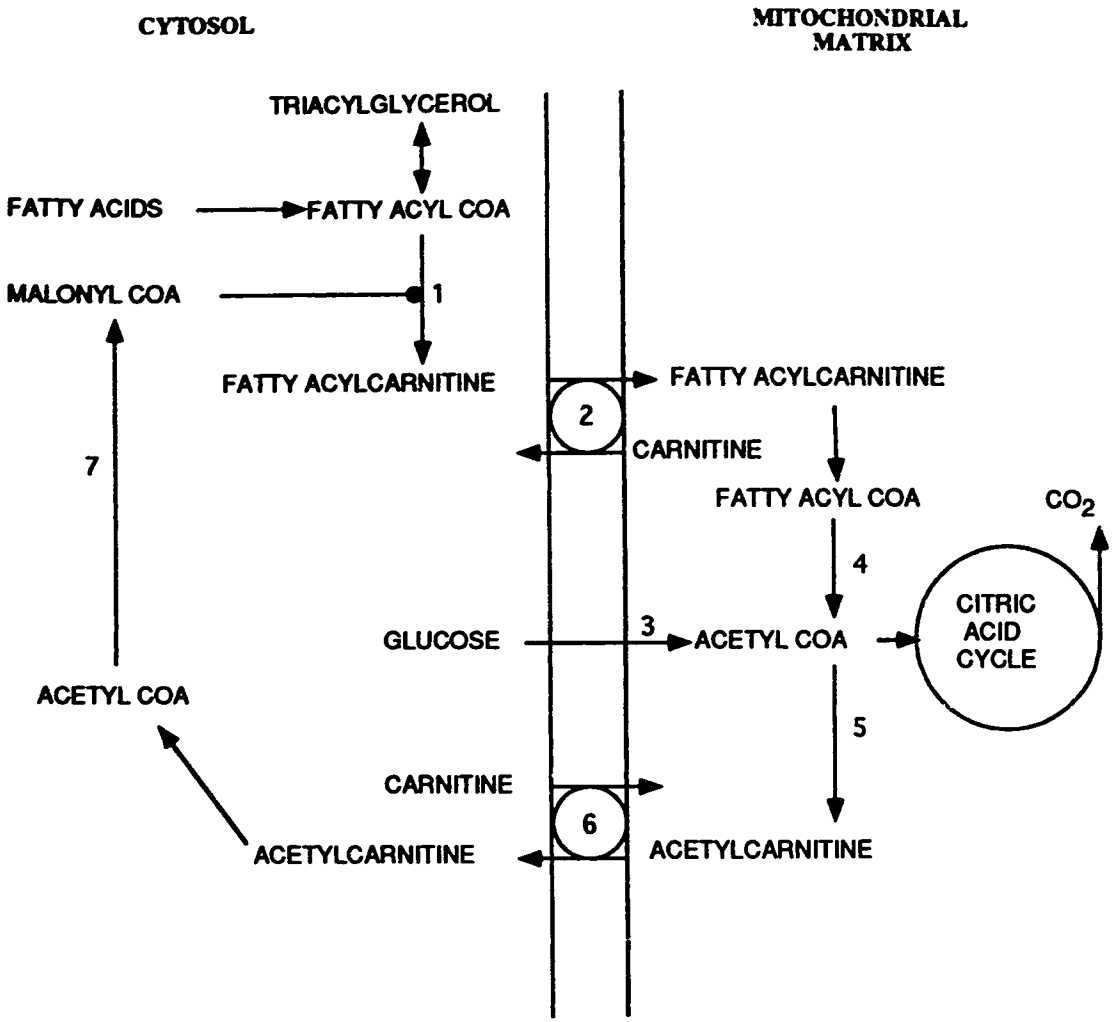


Fig. 4. Sensitivity of cardiac mitochondrial carnitine palmitoyltransferase I to inhibition by malonyl-CoA. Mitochondria were isolated from a rat heart as described in "Methods". CPT I activity in the mitochondrial preparation was determined by measuring the formation of [^{3}H]palmitoylcarnitine from [^{3}H]carnitine, as described in "Methods".

Fig. 5. Proposed relationship between PDC, acetyl-CoA carboxylase, and oxidative metabolism of fatty acids and glucose in the heart (1, carnitine palmitoyltransferase 1; 2, carnitine-acylcarnitine translocase; 3, PDC; 4, ß-oxidation; 5, carnitine acetyltransferase; 6, carnitine-acetylcarnitine translocase; 7, acetyl-CoA carboxylase). Increasing PDC activity (3) will increase the supply of acetyl-CoA for carnitine acetyltransferase (5) and the short chain carnitine carrier system (6). As a result, cytosolic acetyl-CoA levels increase, resulting in an increase in acetyl-CoA carboxylase activity (7). Increased malonyl-CoA production will then inhibit carnitine palmitoyltransferase 1 activity (1), resulting in a decrease in fatty acid oxidation (4).


CYTOSOL
MITOCHONDRIAL MATRIX
TRIACYLGLYCEROL
FATTY ACIDS
FATTY ACYL COA
MALONYL COA
1
FATTY ACYLCARNITINE
FATTY ACYLCARNITINE
2
CARNITINE
7
FATTY ACYL COA
CO_2
4
CITRIC ACID CYCLE
GLUCOSE
3
ACETYL COA
ACETYL COA
5
CARNITINE
6
ACETYLCARNITINE
ACETYLCARNITINE

Discussion:

The role of ACC-265 as the rate limiting enzyme of fatty acid biosynthesis in liver and adipose tissue has been well documented. A 280-kDa isoform of ACC has also recently been identified and is immunologically distinct from the 265-kDa isoform. The observation that ACC-280 is present in the heart (6, Figure 1), and that malonyl-CoA is a potent inhibitor of mitochondrial CPT 1 (3-5) has led to the suggestion that ACC-280 may have an important role in regulating fatty acid oxidation in the heart. However, direct evidence for this role of ACC-280 has not been provided. Our data not only demonstrate that ACC is important in regulating fatty acid oxidation in the heart, but also that the acute regulation of ACC-280 activity differs from ACC-265; i.e., ACC-280 is regulated primarily by acetyl-CoA supply to the enzyme as opposed to regulation by phosphorylation. To address the role of ACC in the heart we used isolated working rat hearts in which overall fatty acid oxidation was directly measured. If hearts are perfused with 1.2 mM palmitate, 95% of ATP production originates from fatty acid oxidation (exogenous and endogenous). Stimulation of glucose oxidation with DCA decreased the contribution of palmitate oxidation to 65% of myocardial ATP production. This decrease in fatty acid oxidation was closely correlated with an increase in cardiac malonyl-CoA levels. The increase in malonyl-CoA levels in the heart were also closely correlated with a DCA induced increase in cardiac acetyl-CoA levels, suggesting that an increase in acetyl-CoA supply from PDC stimulates ACC activity.

Despite the increase in malonyl-CoA levels in DCA-treated hearts, ACC activity measured in extracts obtained from frozen cardiac tissue was similar in both the DCA-treated and control hearts. This lack of difference in ACC

activity is unlikely to be due to non-specific phosphorylation during isolation, since isolation conditions were chosen which are known to preserve the non-phosphorylated state of the enzyme (31). Furthermore, cardiac ACC activity did not show any significant citrate dependency, which occurs when the 265 kDa isoform of ACC is inhibited by phosphorylation (31). The lack of effect of DCA on ACC activity *in vitro* speaks against a covalent modification of ACC as the mechanism behind the increase in malonyl-CoA levels in the heart. Rather, our data suggests that the increase in malonyl-CoA was caused by the increase in the amount of acetyl-CoA available to the enzyme, i.e. cardiac ACC-280 is a substrate driven enzyme. As depicted in Figure 3a, a significant correlation was seen between acetyl-CoA and malonyl-CoA levels in both DCA-treated and untreated hearts frozen at the end of perfusion. Furthermore, ACC purified from rat hearts exhibits different enzyme kinetics from white adipose tissue (in which ACC-265 predominates) (6). Cytosolic levels of total CoA in the heart are in the range of 15-50 μM (23). Although cytosolic content of acetyl-CoA has not been accurately determined, it is obviously lower than overall CoA levels. As a result, cytosolic acetyl-CoA availability may be an important factor modulating cardiac ACC activity. In contrast, overall cytosolic CoA levels in the liver are much greater (32), and the affinity of ACC for acetyl-CoA is higher (6) suggesting that acetyl-CoA supply is probably less important as a regulator of ACC in the liver.

Although most of the acetyl-CoA in the heart is intramitochondrial, a mechanism exists in the heart for the transport of acetyl groups out of the mitochondria via an carnitine acetyltransferase (CAT), and carnitine acetyltranslocase (15-17). As shown in Figure 5, we propose that as mitochondrial acetyl-CoA levels increase (such as by a stimulation of PDC),

acetyl groups are transfered to acetylcarnitine via CAT, where they are subsequently transported into the cytosol. As a result, increases in intramitochondrial acetyl-CoA production from DCA stimulation of the PDC can result in an increase in cytosolic levels of acetyl-CoA. Unfortunately, technical limitations do not allow for an accurate measure of cytosolic versus intramitochondrial acetyl-CoA levels in our perfused hearts. The export of acetyl groups from mitochondrial to cytosolic CoA presents an attractive hypothesis for the feedback regulation of fatty acid oxidation. Under conditions of low work, a decrease in acetyl-CoA demand by the TCA cycle could result in a shuttling of these groups into the cytosol where acetyl-CoA activates ACC resulting in an inhibition of fatty acid oxidation. In contrast, under conditions of high work, an increase in acetyl-CoA demand would result in a decrease in ACC activity and therefore, an increase in fatty acid oxidation rates.

It cannot be discounted that an increase in acetyl-CoA production from PDC may directly inhibit ß-oxidation of fatty acids via an inhibition of ketoacyl thiolase. This, however, does not explain the increase in malonyl-CoA levels we observed in DCA-treated hearts. Further support for the export of acetyl-CoA to cytosol resulting in ACC activation comes from our recent work with carnitine-supplemented hearts. Increasing intracellular carnitine levels was shown to increase glucose oxidation in fatty acid perfused hearts (18), which presumably occurred due to a stimulation of CAT, and a lowering of intra-mitochondrial acetyl-CoA (relieving inhibition of PDC). This stimulation of glucose oxidation was accompanied by a parallel decrease in fatty acid oxidation, which can not be explained by an inhibition of thiolase (i.e., intramitochondrial acetyl-CoA levels decrease under these conditions. We

hypothesized that carnitine effects can be explained by a stimulation of CAT, resulting in an increased transport of acetyl groups from the mitochondria to the cytosol. Increased cytosolic acetyl-CoA levels could then stimulate ACC and malonyl-CoA production, resulting in a decrease in fatty acid oxidation (Figure 4b). To date, however, we have not determined the effects of carnitine supplementation on myocardial malonyl-CoA levels.

Accompanying the decrease in fatty acid oxidation in DCA-treated hearts was an increased incorporation of fatty acids in neutral lipids. This is consistent with a malonyl-CoA induced decrease in CPT 1 activity, since decreasing fatty acid oxidation can re-direct fatty acids in the form of acyl-CoA towards complex lipid synthesis.

Previous methods for measuring ACC have used CO_2 fixation as a measure of ACC activity. This involves using [^{14}C]bicarbonate and measuring its incorporation into malonyl-CoA. However, the strepavidin blot shown in Figure 2c demonstrates the high concentration of other carboxylases in the heart relative to ACC. We therefore developed an ACC assay based on the end product of ACC, malonyl-CoA (see "Methods"). This assay provides an efficient and accurate measure of ACC activity. It should be pointed out, however, that under the assay conditions used, no difference between the HPLC assay and the measurement of [^{14}C]acid soluble products derived from $H^{14}CO_3$ were observed. This suggests that some of the controversy surrounding the $H^{14}CO_3$ assay method is unwarranted.

Cardiac levels of malonyl-CoA reported in this study are comparable to previously reported values of 4-5 nmol/g wet weight (16-20 nmol/g dry weight) (33,34). Although the increase in malonyl-CoA levels in DCA-treated hearts was significant (compared to untreated perfused hearts), this increase was only

around 40%. However, and as shown in Figure 4, cardiac CPT I is highly sensitive to inhibition by malonyl-CoA. It is possible therefore, that even with a slight change in malonyl-CoA concentrations in the heart, a dramatic change in fatty acid oxidation could be expected. Until recently, the malonyl-CoA that is found in the heart was thought to be formed in the mitochondria by propionyl-CoA carboxylase which is abundant in the heart and has some affinity toward acetyl-CoA as well as to its natural substrate propionyl-CoA. Scholte et al (35) however, have detected a cytosolic CO_2-fixing activity that was dependent on citrate in rat hearts suggestive of ACC activity in the heart. Recently Thampy (8) has shown that ACC is the enzyme involved in the synthesis of malonyl-CoA in the heart. Therefore, the increase in cardiac malonyl-CoA levels seen in DCA-treated hearts in this study could only be explained by an increase in ACC activity resulting from an increase in cytosolic acetyl-CoA concentrations.

ACC was shown to be regulated over two different time frames. A rapid regulation (minutes) involves changes in covalent phosphorylation, allosteric regulation and polymerization (7). A long term (hours-days) regulation involves changes in enzyme mass (36) together with changes in enzyme activity caused by the aforementioned rapid regulation (31). While ACC was reported to be dependent on citrate for activation (28,37), Thampy *et al.* (31) found that it is the phosphorylated form of the enzyme that is citrate dependent; quickly frozen livers yielded a more active, citrate-independent ACC with lower phosphate content. Davies *et al.* (38) recently reported that the higher phosphorylated state of ACC was seen when freeze-clamping was not used and that this higher phosphorylation correlated with a large increase in AMP and decrease in ATP (presumably caused by hypoxia during removal of the liver) and with increased activity of AMP-activated protein kinase. In this study, where hearts were

quickly frozen to the temperature of liquid N_2 during the isolated heart perfusion, no citrate dependence was seen in either control of DCA-treated hearts (Figure 2). If freezing of heart tissue was delayed we did not see any increase in citrate dependence of the enzyme (data not shown). Similarly, if hearts are perfused with glucagon (which activates 5'-AMP-kinase activity) neither ACC activity or myocardial malonyl-CoA levels are altered, nor rates of fatty acid oxidation (data not shown). As a result, the ACC-280 which predominates in the heart may not be regulated by phsophorylation to the same degree as ACC-265.

In summary, this study demonstrates for the first time a direct link between malonyl-CoA production by ACC and a decrease in myocardial fatty acid oxidation rattes. Both the 265- and 280-kDa isozymes of ACC are present in the heart, although ACC-280 predominates. Our data suggests that short term regulation of this isoform of ACC is regulated by acetyl-CoA supply to the enzyme.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

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It should be stressed that oxidative rates for exogenous and endogenous palmitate were determined by dividing the radioactivity recovered in ^3H_2O and $^{14}CO_2$, respectively, by the specific activity of palmitate in the corresponding perfusates. While this method holds valid in measuring exogenous palmitate oxidation, an ideal way to measure endogenous palmitate oxidation would be through dividing $^{14}CO_2$ radioactivity by the specific activity of cardiac triglyceride (μmol [^{14}C]palmitate]/μmol triglyceride). However, $^{14}CO_2$ production was linear in all hearts used in this study. This strongly supports adopting the method used in this study to estimate endogenous palmitate oxidation. In addition, measuring the specific activity of cardiac triglycdrides would not be feasible for the following reasons:

1. There is a continuous loss of [^{14}C]labeled cardiac triglyceride throughout the "chase" perfusion. Total triglyceride content either decreased (in hearts perfused in the absence of exogenous fatty acids or in the presence of 0.4 mM palmitate), did not change (in hearts perfused in the presence of 1.2 mM exogenous palmitate) or increased (in hearts reperfused following global ischemia). Measuring specific activity of cardiac triglyceride would necessitate freezing these hearts at different time points, since this activity is changing throughout the "chase" perfusion period. Technically, this would be rather difficult to perform. One way to measure this activity would be through freezing hearts immediately before and at the end of the "chase" perfusion and the specific activity of their triglyceride will then, be determined. However,

it is not known whether the loss in the labeled and the unlabeled triglyceride was linear throughout the perfusion period.

2. There is a strong evidence for the existence of more than one triglyceride pool in the heart. Olson and Hoeschen (1) found that perfusing hearts in the absence of exogenous energy substrates does not completely deplete the triglyceride pool. They suggested that in addition to a quickly mobilizable triglyceride pool, there is a non-mobilizable (or structural pool). Paulson and Crass (2) also suggested the presence of more than one triglyceride pool in the heart evidenced by the presence of two rates for triglyceride lipolysis. Stein and Stein (3), using autoradiography on isolated hearts pulse-labeled by perfusion with [9,10-^{3}H]oleic acid, observed a slower triglyceride turnover in cellular lipid droplets compared to that in the intracellular organelles. They found that after 15 sec pulse, most of the label was over the sarcoplasmic reticulum (SR) and after 30 sec pulse, the label started to increase over the mitochondria. The label was detected in the lipid droplets after 30 seconds pulse and significantly increased when labeling continued for 20 min, where 35% of the total label existed. However, a significant portion of the label was still detectable over the organelles after 20 min perfusion. They suggested that cardiac triglycerides do not form a single metabolic pool. It is safe, therefore, to believe that during the one hour "chase" perfusion in this study, a significant portion of the label was in the lipid droplets.

Taken together, the multiplicity of cardiac triglyceride pools shown in these studies both complicates and undermines the measurement of

specific activity of cardiac triglyceride, since radio-labeling will not be homogeneous hroughtout the triglyceride pools.

Another limitation of this study is the possibility that a portion of palmitate recycles in the triglyceride pool before being oxidized. For technical reasons, an accurate measurement of this cycling is rather difficult. One way to measure this cycling would be through radio-labeling the glycerol backbone of cardiac triglycerides at the time the heart is perfused with radio-labeled palmitate. Radio-labeled glucose will be ideal for such a purpose. Unfortunately, a poor labeling of triglycerides was seen using radio-labeled glucose (unpublished data). In hearts perfused with glucose as the sole energy substrate, only 0.90 ± 0.55 μmol/g dry wt of [^{14}C]glucose was incorporated into cardiac triglycerides. Label incorporation into phospholipids was, for unknown reasons, almost ten times higher. This contrasts what was seen with labeled palmitate where more than 85% of the label was incorporated into cardiac triglycerides.

Taken together, it is anticipated, therefore, that turnover rates of cardiac triglyceride can be even higher than rates reported in this study. However, the model used in this study, and despite these limitations, represents a good method to investigate directly cardiac triglyceride turnover and energy substrate use. The relative contribution of myocardial triglycerides to overall myocardial ATP requirement was calculated in addition to its turnover under different conditions such as ischemia/reperfusion and in diabetes. Furthermore, the importance of fatty acids as major energy substrates to the heart is highlighted. This study also improved our understanding of the relationship between fatty acid and glucose use in the heart. Up to the time of this study, it was thought by some that this turnover is an *in vitro* phenomenon (4). In addition, measurements of

triglyceride turnover has been indirect, i.e., by measuring glycerol release by the myocardium and/or changes in myocardial triglyceride content. Furthermore, most of these studies were carried on hearts perfused in the absence of fatty acids. My results clearly shows that results obtained from those studies should be interpreted with caution for the following reasons:

1) Myocardial triglyceride content is so variable among hearts that accurate measurement of triglyceride contribution to myocardial energy substrate use can hardly be made by measuring triglyceride content only. In addition, in hearts perfused in the presence of exogenous fatty acids, measuring triglyceride content alone will overlook the fact that substantial triglyceride lipolysis may take place without changing myocardial triglyceride content due the concurrence of triglyceride synthesis.

2) Glycerol production *per se* is not a good measure of triglyceride lipolysis. The assumption that the majority of glycerol released by the heart comes from myocardial triglyceride lipolysis has never been documented (5) and has recently been questioned by Larsen (6) who found that phospholipid breakdown also contributes significantly to glycerol production in isolated rat cardiac myocytes deprived of oxygen and exogenous substrates. My results also show that glycerol release into the perfusates did not correlate with myocardial triglyceride lipolysis rates.

3) This study shows that myocardial triglyceride turnover accelerates in hearts perfused in the absence of exogenous fatty acids. This together

with previous studies (7-9) casts doubt on the validity of results obtained in hearts perfused with buffers devoid of fatty acids.

The major findings of this study can be summarized as follows:

1. In control hearts and during the "pulse" perfusion, one third of [^{14}C]palmitate was incorporated into myocardial lipid pools, mostly triglycerides (85%). Only a small amount of [^{14}C]palmitate was incorporated into non-triglyceride neutral lipids and phospholipids. During the "chase" perfusion, the [^{14}C]labeled fatty acid that was released and oxidized from endogenous lipid pools came mainly from the triglyceride pool with only a small contribution from phospholipids, cholesteryl esters, monoacyl and diacyl glycerol (5-10%), Therefore, myocardial triglycerides are a major endogenous lipid pool which significantly contribute fatty acids for oxidative metabolism. In the absence of added fatty acids, the isolated working heart readily uses endogenous triglyceride reserves of fatty acids, with more than 50% of its energy requirements being met by this source. Increasing concentrations of fatty acids delivered to the heart decrease the contribution of this pool to myocardial oxidative metabolism. This occurs mainly due to an inhibition of lipolysis, since incorporation of exogenous fatty acids into the triglyceride pool continues. The contribution of myocardial triglycerides to oxidative metabolism was still apparent even in hearts perfused in the presence of high fatty acid concentrations (1.2 mM palmitate) where 11% of total myocardial ATP requirement was still met by this pool. These observations support the concept that myocardial triglycerides are a readily mobilizable extended substrate source. In addition, my data suggest that the endogenous myocardial triglyceride pool is never static, and that a continuous lipolysis and synthesis of

new myocardial triglyceride occurs. This pool may serve to maintain the homeostasis of fatty acids in the cytosol. Surprisingly, however, and paralleling previous reports (2,9), hearts perfused with physiologic concentrations of exogenous fatty acids (0.4 mM palmitate) did not maintain their endogenous triglyceride content. One potential explanation for this loss is that *in vivo* where chylomicrons and very low density lipoproteins (VLDL) are potential sources of fatty acids to the heart, in addition to circulating free fatty acids, interstitial fatty acid concentrations may be higher than circulating fatty acid concentrations. Although some studies have demonstrated that chylomicrons are a significant source of fatty acids for oxidative metabolism in the isolated perfused heart (10-12), determination of the relative contribution of chylomicrons, VLDL, or plasma free fatty acids to overall myocardial fat uptake and energy requirements has yet to be studied.

Glycolytic rates were twice as high as glucose oxidative rates in hearts perfused in the absence of fatty acids. Fatty acids inhibit glucose oxidation to a much greater extent than glycolysis, such that in the presence of high fatty acids, rates of glycolysis are more than 13 times the rate of glucose oxidation. This higher ratio in fatty acid perfused hearts supports the concept that fatty acid inhibition of glucose utilization occurs to a greater extent at the level of PDC than at the level of PFK (7,13,14).

2. In ischemic hearts, no change in the labeled myocardial triglyceride pool was seen after 30 minutes of no-flow ischemia. However, when these ischemic hearts were reperfused for 60 minutes, a significant incorporation of labeled palmitate into triglyceride was seen with no acceleration in lipolysis. This led to a significant increase in myocardial triglyceride content. This indicates that

during reperfusion of ischemic hearts a significant increase in myocardial triglyceride synthesis takes place. As mentioned previously, high levels of cytosolic fatty acids and perhaps more importantly their intermediates can be potentially harmful to the myocardium (15-18). In contrast to the suggestion of van Bilsen *et al.* (19), my data clearly show that myocardial triglycerides work as a buffer zone for free fatty acids thus preventing any significant increase in the cysotosolic levels of these free fatty acids and therefore protecting the myocardium from this rather damaging event. In addition, my study clearly demonstrates that these hearts quickly recover their ability to oxidize fatty acids during reperfusion. This last observation agrees with two recent studies by Liedtke *et al.* (20) and Gorge *et al.* (21).

3. In diabetes, a significant increase in myocardial triglyceride content takes place even within a very short period of induction of diabetes (e.g., 48 hours). Yet triglyceride synthesis is not increased in these hearts when they are perfused *in vitro*. This is possibly related to the absence of hormonal factors required for inducing this increase (22) or more probably to saturation of this pool in diabetes. This is supported by the observation that lipolysis (including back-diffusion of fatty acids from the heart to the medium) was accelerated in diabetes compared to control especially in hearts perfused in the presence of a high concentration of exogenous fatty acid. My data also show that these isolated hearts of acutely diabetic animals maintained their ability to oxidize fatty acids which were the predominant source of ATP to these hearts. However, glucose oxidation in these hearts was significantly impaired despite the fact that glycolysis was relatively well maintained. The contribution of myocardial triglyceride to overall myocardial ATP requirement is significantly

higher in diabetic rat hearts perfused in the absence of exogenous fatty acids (~70%) compared to control.

4. Another issue that was addressed in this study was the importance of cardiac ACC as a regulator of fatty acid oxidation in the heart. My data provide a direct evidence for the role of this enzyme in regulating myocardial fatty acid oxidation. An increase in cardiac ACC activity results in an increase in malonyl-CoA levels which inhibits CPT I and therefore, decreases fatty acid oxidation. In addition, the observation that cardiac acetyl-CoA levels can regulate ACC activity in the heart represents a novel short-term mechanism regulating the activity of this enzyme in this organ.

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