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# Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart

by

James Gamble 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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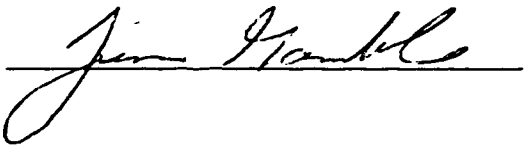

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**Dedication**

To my dad and mom

## Abstract

Fatty acid oxidation typically provides 70% of the heart's energy needs with the remainder being derived from carbohydrate metabolism. In diabetes, this balance is perturbed and fatty acid oxidation can account for over 95% of the hearts energy production. While the pathways of energy metabolism have been extensively studied, the mechanisms underlying their regulation are poorly understood. The purpose herein was to determine the potential role of heart acetyl CoA carboxylase (ACC) isoenzymes in the integrated regulation of carbohydrate and fatty acid metabolism. ACC produces malonyl CoA which inhibits mitochondrial fatty acid uptake. Fatty acid and glucose oxidation rates were directly measured in isolated working rat hearts from normal and from insulin-deficient or insulin resistant rats. In normal hearts ACC activity was positively correlated with levels of malonyl CoA and negatively correlated with rates of myocardial fatty acid oxidation. This relationship could be manipulated by pharmacologically stimulating glucose oxidation or omitting glucose from the perfusate. In insulin-deficient diabetic rats a differential regulation of ACC was found among tissues. In liver and white adipose tissue, the 265 kDa ACC expression was reduced during diabetes. In contrast, in heart (predominant 280 kDa ACC expression), the only change was a reduced enzyme activity with no change in ACC-280 content. In insulin resistance, rat heart expression of ACC isoenzymes was also invariant. However, hearts were more reliant upon fatty acid oxidation for ATP production and exhibited a diminished ACC activity. This could be attributed to an increased 5'-AMP-activated protein kinase (AMPK) activity which phosphorylates and inhibits ACC. Hormonal control of metabolism

and ACC was investigated by perfusing hearts with insulin or glucagon. Insulin supression of fatty acid oxidation was accompanied by a dephosphorylation-dependent activation of ACC and a reduced AMPK activity. Glucagon selectively stimulated glucose oxidation and inhibited ACC activity with no effect on AMPK. In conclusion, myocardial ACC and AMPK are important regulators of fatty acid oxidation in the heart. Therefore, ACC and/or AMPK may be a pharmacological target for optimizing energy metabolism in the heart of diabetics.

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My dad,who has always been a source of inspiration and has been there for me every step of the way no matter how far away from home I am. Thank you.

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## List of Abbreviations:

| | |
|---|---|
| ACC: | acetyl-CoA carboxylase |
| ACC-265: | 265 kilodalton isoenzyme of acetyl CoA carboxylase |
| ACC-280: | 280 kilodalton isoenzyme of acetyl CoA carboxylase |
| AMP: | adenosine monophosphate |
| AMPK: | 5'-AMP activated protein kinase |
| AICAR: | 5-amino-4-imidazolecarboxamide ribonucleoside |
| Ab: | antibody |
| ANOVA: | analysis of variance |
| ATP: | adenosine triphosphate |
| CW: | cardiac work |
| CAT: | carnitine acetyltransferase |
| CPT I: | carnitine palmitoyltransferase I |
| CPT II: | carnitine palmitoyltransferase II |
| CoA: | coenzyme A |
| cDNA: | complementary deoxyribonucleic acid |
| dCTP: | deoxycytidine 5'-triphosphate |
| DCA: | dichloroacetate |
| DEPC: | diethylpyrocarbonate |
| DNA: | deoxyribonucleic acid |
| EGTA: | ethyleneglycol-bis(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid |

| | |
|---|---|
| EDTA: | ethylenediaminetetraacetic acid |
| HR: | heart rate |
| HCl: | hydrochloric acid |
| HEPES: | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| IgG: | immunoglobulin G |
| kDa: | kilodalton |
| mRNA: | messenger ribonucleic acid |
| MW: | molecular weight |
| 7AD3: | monoclonal antibody to acetyl CoA carboxylase 280 kilodalton isoenzyme |
| NAD: | nicotinamide adenine dinucleotide |
| $NADH_2$: | dihydronicotinamide adenine dinucleotide |
| PSP: | peak systolic pressure |
| PCA: | perchloric acid |
| PMSF: | phenylmethylsulfonyl fluoride |
| PC: | pyruvate carboxylase |
| PDC: | pyruvate dehydrogenase complex |
| RPP: | rate-pressure product |
| RNA: | ribonucleic acid |
| SDS: | sodium dodecylsulphate |
| SNF1: | sucrose nonfermenting |
| SAMS: | HMRSAMSGLHVKRR synthetic peptide |
| TOFA: | 5-tetradodecyloxy-2-furancarboxylic acid |
| MEDICA 16: | ß,ß'-tetramethyl hexadecanedioic acid |
| TLCK: | Na-p-Tosyl-L-Lysine chloromethyl ketone |

| | |
|---|---|
| TPCK: | N-Tosyl-L-phenyalanine chloromethyl ketone |
| TCA: | tricarboxylic acid |

# CHAPTER I

## Introduction

### *A) Myocardial energy substrate metabolism*

#### *i) Historical perspective:*

Since the early studies of Bing *et al.* (1953) it has been known that the majority of energy production in the heart is derived from the oxidation of fatty acids. The other important sources of energy are the oxidation of carbohydrates, and to a lesser extent ATP production from glycolysis (Neely and Morgan, 1974). During severe ketoacidosis, ketone bodies may also become an important energy source (Neely and Morgan, 1974). The contribution of the various energy yielding pathways to overall ATP production can vary dramatically, depending to a large extent on the carbon substrate profile delivered to the heart, as well as the presence or absence of underlying pathology within the myocardium. Despite extensive research devoted to the study of the individual pathways of energy substrate metabolism, relatively few studies have been conducted examining the integrated regulation between carbohydrate and fatty acid oxidation in the heart.

The classic studies of Randle's group (Kerbey *et al.* 1976; Randle, 1986) have characterized the mechanisms by which fatty acids inhibit carbohydrate oxidation (i.e. the Randle cycle). However, much less in known as to how carbohydrates regulate fatty acid oxidation in the heart. While it is clear that an increase in intramitochondrial acetyl CoA derived from carbohydrate oxidation (via the pyruvate dehydrogenase complex) can down regulate ß-oxidation of fatty

acids, it is not clear how fatty acid acyl group entry into the mitochondria is down-regulated when carbohydrate oxidation increases. This thesis will focus on the involvement of acetyl CoA carboxylase (ACC) in this regulation of myocardial fatty acid oxidation.

There are a number of important regulatory steps in the fatty acid oxidation pathway and in recent years a number of significant advances have increased our understanding of the complex processes involved in this regulation. This includes a complex process by which fatty acids are delivered to the heart, taken up by the myocyte, transported to the mitochondria, and activated to long chain acyl CoA as outlined in a number of recent reviews (Van der Vusse *et al.*, 1992; Tahiliani (1992), Guzman and Geelen (1993), Schultz (1991;1994), Opie (1992), and Rosenthal (1987).

### *B) Overview of myocardial fatty acid metabolism:*

The main source of fatty acids for the heart is supplied by free fatty acids (primarily oleic and palmitic acid) bound to albumin, and fatty esters present in chylomicrons and very low density lipoproteins. To date, the relative proportion of fatty acids derived from these sources is not clear. The transport of fatty acids across (or around) the endothelial cells, across the interstitial space, and subsequently across the sarcolemmal membrane is presently a source of debate (for reviews see Van der Vusse *et al.*, 1989;1992; Glatz *et al.*, 1993). Recently, Burczynski *et al.*, (1995) have suggested that, unlike the liver, the uptake of palmitate into isolated cardiac myocytes appears to accounted for by a diffusion-reaction mechanism as opposed to a facilitated uptake process. They were also unable to demonstrate any synergism between the endothelial cells and the

cardiomyocyte for the palmitate clearance rate. The explanation as to why the cardiomyocytes, as opposed to liver, would not have a facilitated uptake process when the vast majority of energy production comes from fatty acid metabolism is unclear. One factor may be the fact that these experiments were carried out in quiescent myocytes where the demand for fatty acid metabolism is low as compared to the working heart (as discussed in section **J-ii(b)**).

An overview of many of the processes involved in cellular fatty acid metabolism is provided in Figure 1-1. Once inside the aqueous cytoplasm fatty acids bind to heart-type fatty acid binding proteins (FABPs) and are activated to long-chain acyl-CoA by an acyl-CoA synthetase. The acyl moieties are then transferred into the mitochondria by a complex of enzymes involving carnitine palmitoyltransferase I (CPT I), carnitine:acylcarnitine translocase, and carnitine palmitoyltransferase II (CPT II). Once in the mitochondrial matrix long-chain acyl-CoA passes through the ß-oxidation enzyme system (or spiral) to produce acetyl-CoA. Each successive cycle of the ß-oxidation spiral results in a 2 carbon shortening of the fatty acid and formation of 1 NADH and 1 $FADH_2$. Acetyl-CoA derived from ß-oxidation then enters the tricarboxylic acid (TCA) cycle, resulting in the liberation of 2 $CO_2$, 3 NADH and 1 $FADH_2$. The NADH derived from glycolysis, the pyruvate dehydrogenase complex (PDC), the TCA cycle, and ß-oxidation, as well as $FADH_2$ from the TCA cycle and ß-oxidation, then enter the electron transport chain. The hydrogen on NADH and $FADH_2$ is transferred to $H_2O$ in the presence of $O_2$, and ADP is converted to ATP.

### *C) Aqueous Cytoplasmic Activation of Fatty Acid Oxidation*

#### *i) Uptake of fatty acids by the heart*

The delivery of fatty acids from the blood to the heart is a highly regulated process and has been the source of considerable debate (for reviews see Van der Vusse *et al.*, 1992; Lopaschuk *et al.*, 1994). The specific mechanisms involved in the delivery and transport of fatty acids to and within the cardiac myocyte are somewhat controversial however, the basic concepts are summarized below. Hydrolysis of VLDL and chylomicron triacylglycerols occurs via a lipoprotein lipase present on the luminal side of the endothelial cell. The fatty acids liberated by the lipase then bind to albumin. The contribution of fatty acids bound to albumin already present in the blood, as opposed to fatty acids originating from lipoproteins, as a source of myocardial fatty acids for ß-oxidation has yet to be determined.

While the heart is very efficient at extracting fatty acids, the actual mechanisms involved remain controversial. The pathway(s) by which the fatty acid-albumin complex present in the vascular lumen is transported to the myocyte membrane has not been clearly delineated. Furthermore, whether fatty acids are taken up by the myocytes by a passive diffusion process or by a carrier mediated process is also not clear (Van der Vusse *et al.*, 1992; Burczynski *et al.*, 1995).

#### *ii) Transport within the aqueous cytoplasm*

While the transport of fatty acids between organs is generally thought to be carried out by plasma albumin or as triacylglycerol contained within lipoproteins,

the mediation of intracellular transport and targeting of long-chain fatty acids is thought to occur primarily by fatty acid binding proteins (FABPs). The FABPs are a class of low molecular weight (14-15 kDa) proteins, and are highly abundant in tissues which actively metabolize fatty acids, and are capable of binding hydrophobic ligands (Van der Vusse *et al.*,1989; Glatz *et al.*, 1993). At least five different types of FABPs exist, and are named according to the tissue in which they were first identified. These proteins have all been characterized at both the protein and cDNA level (Van der Vusse *et al.*,1989; Glatz *et al.*, 1993; Veerkamp and van Moerkerk, 1993).

Compelling evidence for a role of FABP's in fatty acid metabolism comes from the high correlation between the content of FABPs in various tissues and the capacity of these tissues to oxidize palmitate under a variety of pathological or pharmacological conditions (Veerkamp and van Moerkerk, 1993). The FABPs may not only be important in directing fatty acids to various metabolic pathways but perhaps, based on their differential binding affinities for fatty acids, may also be important in trafficking different fatty acids to the diverse pathways of fatty acid metabolism (Fournier and Rahim, 1985). They may also play a protective role in buffering intracellular long-chain acyl-CoA's and possibly free radicals which, when elevated, can have actions that are potentially harmful to the integrity of the myocardium, particularly in the setting of reperfusion following ischemia (Glatz *et al.*, 1993; Bass, 1988) .

Another small aqueous cytoplasmic binding protein (10 kDa) which co-purifies with FABP has also been identified in the heart as well as a number of other tissues (Mikkelsen and Knudsen, 1987). However, unlike FABP, this protein specifically binds acyl-CoA esters, hence the name acyl-CoA binding protein (ACBP). It has recently been suggested by Knudsen's group that ACBP's, by

sequestering these acyl-CoA esters, may be important regulators of fatty acid metabolism (Rasmussen *et al.*, 1993; Knudsen *et al.*, 1993). Furthermore, activities of both aqueous cytoplasmic (i.e. acetyl-CoA carboxylase) and mitochondrial enzymes (i.e. adenine nucleotide translocase) are inhibited by the presence of long-chain acyl-CoA esters (Rasmussen *et al.*, 1993), therefore ACBP may act to relieve end-product inhibition of these enzymes by buffering the intracellular pool of acyl-CoA. Like the FABPs, ACBP may also function in the site-specific transport of acyl-CoA esters within the aqueous cytoplasm.

The conversion of the carboxylic head group of fatty acids to the more reactive CoA thioester, a process known as fatty acid activation, is an absolute prerequisite for the subsequent catabolic processes. This conversion of fatty acids to their acyl-CoA esters is catalyzed by a family of acyl-CoA synthetases which differ in their chain length specificity and their subcellular location (Knudsen *et al.*, 1993). In the heart, long-chain acyl-CoA synthetase is localized to the aqueous cytoplasmic face of the mitochondrial membrane (Waku, 1992).

Despite the obligatory role for the acyl-CoA synthetases in the catabolic pathway of fatty acids, their regulatory influence on fatty acid metabolism has yet to be completely elucidated (Normann *et al.*, 1983). Although, the recent cloning of the human enzyme (Abe *et al.*, 1992) has facilitated a number of studies examining the expressional changes of long-chain acyl-CoA synthetase in a variety of physiological states. For example, physical exercise has been found to cause a reduction in long-chain acyl-CoA synthetase activity and mRNA levels in mesenteric fat whereas in skeletal muscle, long-chain acyl-CoA synthetase activity and mRNA levels were elevated following exercise (Shimomura *et al.*, 1993). Both activity and mRNA levels remain unchanged in heart tissue from obese Zucker rats (Shimomura *et al.*, 1992). In addition, a variety of pharmacological

interventions designed to induce carnitine deficiency (Tsoko *et al.*, 1995) or promote hypolipidemia, eg. fibric acid derivatives (Schoonjans *et al.*, 1993), can also enhance the expression of the enzyme and mRNA levels. Complicating this scenario, however, is the recent discovery of multiple promoters for the rat acyl-CoA synthetase gene capable of generating 3 distinct mRNA species which differ in their tissue distribution (Suzuki, *et al.*, 1995).

***Endogenous triacylglycerol as a source of fatty acids***

Acyl groups present on long-chain acyl-CoA can either be targeted for the mitochondria or incorporated in myocardial triacylglycerol stores. This endogenous triacylglycerol pool has been shown to be an important source of fatty acids for oxidative metabolism (Saddik and Lopaschuk, 1991; Crass, 1977; Paulson and Crass, 1982). In the absence of added exogenous fatty acids, the isolated working heart readily uses endogenous triacylglycerol reserves of fatty acids, with more than 50% of its energy requirements being met by this source. As increasing concentrations of fatty acids are delivered to the heart, the contribution of these pools to myocardial oxidative metabolism decreases; mainly due to an inhibition of lipolysis. Even in the presence of high concentrations of fatty acids, however, triacylglycerol-derived fatty acids can account for 11% of total myocardial ATP production (Saddik and Lopaschuk, 1991). This is achieved due to a rapid turnover of the pool, and suggests myocardial triacylglycerols are a readily mobilizable extended substrate source. Although triacylglycerol turnover is significant, only a portion of the fatty acids taken up by the heart pass through the intracellular triacylglycerol pool prior to oxidation. However, even in the presence of high levels of fatty acids about 10% of the fatty acids taken up by the

heart are cycled through triacylglycerol prior to oxidation. Although this futile cycle may have significance in the ischemic heart due to ATP wastage (Van der Vusse *et al.*, 1992), it is not clear if this has significance under aerobic conditions when the extracellular supply of fatty acids is not limiting. It is believed however, that the primary role of triacylglycerol turnover in the aerobic heart is to ensure an adequate supply of fatty acids for ß-oxidation when extracellular fatty acid levels are low.

### *E) The role of carnitine in regulating myocardial metabolism*

L-carnitine is an essential co-factor in the transport of fatty acids into the mitochondria. As a result, any decrease in myocardial carnitine levels has the potential to impair fatty acid oxidation, the major source of energy for the heart. It is also becoming evident, however that carnitine has functions other than facilitating ß-oxidation of fatty acids. Isolated mitochondria studies have shown that carnitine will lower the intramitochondrial level of acetyl-CoA, secondary to a stimulation of carnitine *acetyl*transferase (Pearson and Tubbs, 1967; Lysiak *et al.*, 1988). The decrease in intramitochondrial acetyl CoA/CoA is accompanied by an efflux of acetylcarnitine from the mitochondria. By lowering the ratio of acetyl-CoA to CoA in the mitochondria, PDC is stimulated (Uziel *et al.*, 1988), which should result in an increase in glucose oxidation. In support of this, previous studies from our laboratory have shown that increasing myocardial carnitine content stimulates glucose oxidation in the intact hearts perfused with fatty acids (Broderick *et al.*, 1992; Broderick *et al.*, 1993).

As mentioned, fatty acid oxidation decreases in parallel with the increase in glucose oxidation in response to carnitine treatment (Broderick *et al.*, 1992), an

observation that would not be expected if intra-mitochondrial acetyl-CoA levels decreased (i.e. this normally stimulates ß-oxidation, Schulz, 1994). As a result, the decrease in fatty acid oxidation must occur at a site other than acetyl CoA inhibition of the last enzyme in ß-oxidation, 3-ketoacyl-CoA thiolase. One possibility is that this regulation is occurring at the level of CPT 1, a key enzyme necessary for transport of fatty acids into the mitochondria *i.e.*, the stimulation of glucose oxidation will lead to an increase in malonyl CoA levels due to the shuttling of acetyl CoA from the mitochondria to the cytosol.

***i) Carnitine palmitoyltransferase I in the heart:***

Once activated in the aqueous cytoplasm, long-chain acyl-CoA's are transferred into the mitochondrial matrix by the concerted efforts of three carnitine-dependent enzymes. The first of these enzymes, CPT I, catalyzes the formation of long-chain acylcarnitine from long-chain acyl-CoA. The topographical distribution of this enzyme has been a source of great debate, although it is now generally accepted that it is located on the inner surface of the outer mitochondrial membrane (Murthy and Pande, 1987). The second enzyme, carnitine:acylcarnitine translocase transports long-chain acylcarnitine across the inner mitochondrial membrane. The third enzyme, CPT II, is associated with the inner mitochondrial membrane and catalyzes the reverse reaction ultimately regenerating long-chain acyl-CoA within the mitochondrial matrix.

CPT 1 represents a key regulatory point in the oxidation of fatty acids, and is the rate-limiting step of long-chain acyl-CoA translocation into mitochondria (McGarry *et al.*, 1989). Malonyl-CoA, which is produced by acetyl-CoA carboxylase (ACC), is a potent inhibitor of CPT 1, and acts at a site distinct from

the catalytic site of CPT 1 (McMillin *et al.*, 1994; Murthy and Pande, 1987; Murthy and Pande, 1988; Woeltje *et al.*, 1990). The question as to the exact location of the malonyl CoA binding site, however, has been an area of considerable debate.

Recent studies by Saggerson's group (Ghadiminejad and Saggerson, 1990) and Bieber's group (Chung *et al.*, 1992) have suggested that malonyl CoA binds to a malonyl CoA binding protein that confers the malonyl CoA sensitivity to CPT I. Purification of this protein has suggested that the malonyl CoA binding protein has a MW of 86 kDa (Chung *et al.*, 1992). However, recent cloning of rat liver CPT I by McGarry's group (Esser *et al.*, 1993) has provided strong evidence that the malonyl CoA binding protein is actually CPT 1 itself. The predicted MW of CPT I based upon the cDNA sequence is 88 kDa.

Recent evidence by Bieber's group (Kerner *et al.* 1994), has suggested that the catalytic unit for CPT I is very similar, if not identical, to the catalytic unit of CPT II. It has been suggested that a different isoform of CPT I may be present in the heart compared to the liver (Cook and Lappi, 1992) and indeed, Weiss *et al.*,1994a;1994b) have recently identified 88 and 82 kDa CPT I enzymes designated L-CPT I and M-CPT I respectively, which are expressed simultaneously in heart but exhibit vastly differing kinetics (Brown *et al.*, 1995). It is believed that this form of expression in the heart may underly the intermediate kinetic values for carnitine (K*m*) and malonyl CoA ($IC_{50}$) found in heart with respect to the liver and skeletal muscle enzymes.

In addition, antibodies to liver CPT I which were used in the cloning of the liver enzyme do not react with heart CPT I (Esser *et al.*, 1993a), although, the liver cDNA will recognize a single species of mRNA using Northern Blot analysis of heart mRNA (Weis *et al.*, 1994). Cloning of the 88 kDa CPT I from heart has

revealed a 100% sequence identity with liver CPT I cDNA (Weis *et al.*, 1994). Recently, Yamazaki *et al.*, (1995) using differential screening of a brown adipose tissue cDNA library have identified a clone which repoted to encode a CPT I protein expressed in brown adpose tissue and heart.

Although malonyl-CoA levels are lower in the heart compared to the liver, the sensitivity of CPT I to inhibition by malonyl-CoA is much greater in the heart (Cook, 1984; McGarry *et al.*, 1983). Detailed information regarding the regulation of myocardial CPT I by malonyl CoA, however, is somewhat scarce. Recent work by Cook and Lappi (1992) suggests that absolute changes in malonyl CoA levels in the heart may be the key factor regulating changes in fatty acid oxidation, in contrast to the liver, in which the sensitivity of CPT 1 to malonyl CoA inhibition decreases in diabetic or fasted animals in addition to the reduction in malonyl CoA levels (McGarry *et al.*, 1989).

In addition to responding to absolute levels of malonyl CoA, a diminished sensitivity of liver CPT 1 to malonyl CoA, in the fasting or diabetic state, represents an alternative regulatory mechanism for hepatic fatty acid oxidation. In accordance with this hypothesis, alterations in the sensitivity to inhibition by malonyl CoA in response to dietary or pathological states which are known to occur in liver do not appear to be present in heart (Cook and Lappi, 1992).

In addition to malonyl-CoA, L-carnitine is another important regulator of CPT I. The K*m* for L-carnitine of CPT I in rat heart is 6 times greater than in liver, reflecting, in part, the higher carnitine concentration in heart. On the other hand, the K*m* for the long-chain acyl-CoA of CPT I is reported to be similar in both liver and heart (Cook, 1984). The existence of a tissue specific isoform of CPT I has been suggested from several lines of evidence, such as different sensitivity of CPT I to inhibition by malonyl-CoA (Cook, 1984) or, differential

changes in specific activities of the enzymes, in heart and liver, during alterations in physiological states (Cook, 1984; Paulson *et al.*, 1984; Cook and Lappi, 1992; Brown *et al.*, 1995). At the time research for this thesis was initiated, the relationship between the reliance of the heart on fatty acid oxidation, and the sensitivity of CPT I to malonyl CoA inhibition, had not been directly addressed.

## E) Malonyl CoA in the heart

Historically, 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 (Hulsmann 1966). Scholte *et al.*, (1986) however, have detected a cytosolic $CO_2$-fixing activity in rat heart that was dependent on citrate, suggesting the presence of ACC activity in the heart. Recently, Thampy (1989) has shown that ACC is the enzyme involved in the synthesis of malonyl-CoA in the heart.

The discovery that CPT 1 in the heart is extremely sensitive to inhibition by malonyl CoA ($K_i$ = 50 nM) (McGarry *et al.*, 1983; Cook, 1984; Cook and Gamble 1987; Paulson *et al.*, 1984; McGarry *et al.*, 1989), and that malonyl CoA is present in measurable quantities in the heart (10-20 nmol·g dry $wt^{-1}$) (McGarry *et al.*, 1983; Singh *et al.*, 1984), has led to speculation that malonyl CoA may be an important effector of the entry of long chain acyl CoA's into the mitochondria, and therefore a potentially important regulator myocardial fatty acid oxidation. Interestingly, the dramatic maturation of fatty acid oxidation that occurs in hearts from newborn rabbits (Lopaschuk *et al.*, 1991) is accompanied by parallel decreases in the levels of malonyl CoA (Lopaschuk *et al.*, 1994c).

One of the questions that remains to be answered is the ultimate fate of malonyl CoA in the heart. The fact that acute interventions such as substrate deprivation can cause dramatic yet transient changes in malonyl CoA levels, and that fatty acid oxidation is not persistently inhibited by malonyl CoA, suggests that there must be a mechanism by which malonyl CoA is degraded. One possibility is decarboxylation by malonyl CoA decarboxylase, which appears to be active in heart tissue (Kim and Kolattukudy, 1978) but has not, as of yet, been fully characterized in the heart. However, malonyl CoA decarboxylase is considered to be localized to the mitochondrial matrix and therefore its ability to alter levels of malonyl CoA in the cytosol remain unclear.

A second possibility is that the malonyl CoA produced by ACC in the heart could feed into the fatty acid synthetic or elongation pathway. Although fatty acid synthase has been extensively studied in the liver and in E. coli, there have not been many studies which have looked at whether or not the protein is present in the heart. Although mRNA for fatty acid synthase is present in heart (Semenkovich *et al.*, 1995) fatty acid synthase activity has not been detected in heart (Awan and Saggerson, 1993).

### *F) Acetyl CoA carboxylase in the heart*

Malonyl CoA is synthesized in the cytoplasm from acetyl CoA by the enzyme acetyl CoA carboxylase (ACC) or, as mentioned above, to a lesser extent by the action of propionyl CoA carboxylase in the mitochondria. ACC is a biotin containing enzyme which catalyses the transfer of $CO_2$ from bicarbonate to acetyl-CoA to form malonyl-CoA (Kim *et al.*, 1989). ACC-dependent production of malonyl CoA in liver and white adipose tissue is important as the first committed

step of fatty acid biosynthesis. Indeed, inhibition of hepatic ACC by 5-tetradodecyloxy-2-furancarboxylic acid (TOFA) will dramatically decrease both very low density lipoprotein (VLDL) secretion and triacylglycerol (TG) synthesis (Arbeeny *et al.*, 1992). It appears in heart that the primary role of ACC is in regulating fatty acid oxidation (Bianchi *et al.*, 1990; Saddik *et al.*, 1993). Support for this hypothesis comes from the fact that ACC is present in both heart and skeletal muscle, despite the fact that they have a very low capacity for fatty acid synthesis (Bianchi *et al.*, 1990; Trumble *et al.*, 1991). Based on the tissue distribution of ACC and the metabolic characteristics of the tissues it has been suggested that ACC in the heart, and skeletal muscle may function primarily as a regulator of fatty acid oxidation, due to a potent inhibition of CPT 1 by malonyl-CoA (Cook, 1984; McGarry *et al.*, 1989). It has recently been reported that a 280 kDa isozyme of ACC, which is immunologically distinct from the lower molecular weight isoform, is highly expressed in heart tissue (Bianchi *et al.*, 1990; Thampy, 1989). Thus, the importance of this isoform of ACC in regulating fatty acid oxidation rates in the heart has not been directly addressed.

### *i) Regulation of acetyl CoA carboxylase*

ACC-265 has been shown to be regulated over two different time frames: a rapid regulation (minutes) and long term regulation (hours/days) (Thampy and Wakil, 1985; Pape *et al.*, 1988). The long term regulation of ACC involves tissue-specific use of two distinct ACC gene promoters (PI and PII), and changes in the rate of transcription, synthesis and degradation of ACC (Lopez-Cassilas and Kim, 1989; Luo *et al.*, 1989). The rapid regulation of ACC occurs due to both phosphorylation and allosteric regulation (Jamil and Madsen, 1987a,b; Thampy

and Wakil, 1985). Cloning of a 265 kDa isoform of ACC has provided new information about the structure function relationship of ACC (see Kim *et al.*, 1989 for review) as well as the regulation of expression of the mRNA for ACC. Although the regulation of the 265 kDa isoform has been extensively studied in the liver, little is known about regulation of the isoforms of ACC in the heart.

Citrate is a "feed forward activator" of ACC, (Thampy and Wakil, 1985) while long chain acyl-CoA is a potent inhibitor of ACC (Moule *et al.*, 1992). While liver ACC was reported to be dependent on citrate for activation, (Lane *et al.*, 1989; Thampy and Wakil, 1985), Thampy and Wakil (1988) found that it is the phosphorylated form of the enzyme that is citrate dependent (ie., quickly frozen livers yielded a more active, citrate-independent ACC, with lower phosphate content). Phosphopeptide analysis of ACC-265 has identified a number of potential phosphorylation sites which are acted upon by a variety of kinases (Hardie, 1989; Kim *et al.*, 1989). Inhibition of the enzyme activity in response to hormones, such as epinephrine and glucagon, are thought to be a direct result of enzyme phosphorylation, as opposed to ACC activation, which is associated with enzyme dephosphorylation.

Initial studies suggested that the 280 kDa ACC isozyme was more resistant to phosphorylation than ACC-265. The inability to incorporate $^{32}P$ into ACC-280 in hepatic cell lines, and the lack of citrate dependence in heart (Bianchi *et al.*, 1992) relative to liver, suggested that there may be differential regulation of ACC-280 and ACC-265. Winz *et al.*, (1994), have provided evidence that ACC-280 from rat liver is more readily phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase than the ACC-265 isoenzyme. Along these lines, recent work from our laboratory suggested that heart ACC may be under phosphorylation control by the AMP-activated protein kinase (Kudo *et al.*,

submitted). However, until the ACC-280 has been cloned or sequenced and potential phosphorylation sites identified the phosphorylation control of ACC-280 and whether or not ACC-280 is the same protein in the various tissues remains speculative.

Phosphorylation by cAMP-dependent protein kinase results in inactivation of the ACC enzyme, and is thought to be the mechanism by which glucagon inactivates hepatic ACC-265 (Mabrouk *et al.*, 1990). Recent interest has also focused on the role of 5'AMP dependent protein kinase as an inhibitor of ACC-265 (Davies *et al.*, 1990; Witters *et al.*, 1994). Hardie (1992) has suggested that this kinase may be the primary kinase acting on ACC-265 via phosphorylation at ser-79. It has been suggested that kinase inhibition by insulin and kinase stimulation, by glucagon, can explain the ability of these hormones to activate and inhibit ACC-265, respectively (Mabrouk *et al.*, 1990). Site-directed mutagenesis studies have also provided valuable information as to the phosphorylation sites on ACC-265 (Ha *et al.*, 1994). These authors identified serine-1200 as the critical site for phosphorylation by the cyclic AMP-dependent protein kinase whereas serine-79 appears to be the key phosphorylation site for inactivation of ACC by AMPK.

Another interesting aspect of heart ACC is that it has a very low affinity for acetyl-CoA (Bianchi *et al.*, 1990). Initial characterization of heart ACC has shown that the affinity of ACC for acetyl-CoA, measured in perfused hearts, is approximately 200 μM (Witters, unpublished observations). Since cytosolic acetyl-CoA levels are normally low (less than 50 μM) and less than 5% of the total CoA pool is cytosolic (Robishaw and Neely, 1985), it would suggest that acetyl-CoA levels in the heart can be an important regulator of ACC activity. One way to increase cytosolic acetyl-CoA is via the ATP-citrate lyase pathway whereby intramitochondrial citrate is exported from the mitochondria to form acetyl CoA in

the cytosol. While this pathway is extremely active in liver, the amount of cytosolic acetyl CoA derived from ATP-citrate lyase in the heart is minimal (Robishaw and Neely, 1985).

Alternatively, acetyl CoA in the cytosol can be derived from the carnitine acetyltransferase and translocase carrier in the mitochondrial membrane (Lysiak *et al.*, 1986a,b). As a result, increasing acetyl-CoA supply via the pyruvate dehydrogenase complex (PDC) (i.e. glucose oxidation) could stimulate the transfer of acetyl groups from intramitochondrial acetyl-CoA to cytosolic acetyl-CoA (Lysiak *et al.*, 1988). This combined with the observation that cytosolic CoA levels are low in the heart versus liver, as is the affinity of ACC for acetyl-CoA, would suggest that the cytosolic acetyl-CoA/CoA ratio may be an important determinant of ACC activity in the heart.

***ii) 5' AMP activated protein kinase:***

The 5' AMP activated protein kinase (AMPK), is a multi-subunit protein kinase that exhibits a large degree of homology to the SNF1 protein kinase family in yeast (Mitchelhill *et al.*, 1993; Gao *et al.*, 1995) and appears to be highly conserved throughout eukaryotic evolution (Hardie, 1992b). Unlike most kinases which regulate cell cycle activity or transduce extracellular signals into modulation of intracellular processes, the AMPK responds to changes in intracellular metabolites (Hardie and MacKintosh, 1992) and as such has been termed a "stress" kinase. As the name implies, AMPK is activated by 5'AMP and serves to inhibit ATP utilizing processes when the cellular energy status may be compromised such as with ATP depletion (Witters *et al.*, 1991; Louis and Witters, 1992; Gillespie and Hardie, 1992).

There are a number of known physiological targets for the kinase all of which relate to regulation of cellular lipid metabolism. These cellular targets include HMG-CoA reductase, hormone sensitive lipase, and acetyl CoA carboxylase (Hardie, 1989b) thereby imparting a role for AMPK in modulating the levels of fatty acid and cholesterol synthesis, as wells as the release of fatty acids from intracellular stores of triglyceride and cholesterol ester. Interestingly, the AMPK-mediated phosphorylation of hormone sensitive lipase (at ser-565) does not inhibit the enzyme directly, but rather prevents the phosphorylation by cyclic AMP-dependent protein kinase at an adjacent site (Garton and Yeaman, 1990). Phosphorylation of HMG-CoA reductase by AMPK on ser-872 results in an inhibition of cholesterol biosynthesis (Henin *et al.*, 1995; Carling *et al.*, 1989). The phosphorylation and inactivation of ACC by AMPK occurs at ser-79 (Davies *et al.*, 1990) and this is phosphorylation is thought to be the primary mechanism by which hormones such as insulin and glucagon regulate hepatic ACC activity (Witters and Kemp, 1992; Sim and Hardie, 1988).

Evidence is accumulating that AMPK is a member of a complex protein kinase cascade as, in addition to AMP, the kinase is also activated at least 10-fold by a distinct AMPK kinase (Carling *et al.*, 1987). Also, of interest is that the presence of 5'AMP not only activates the AMPK itself but also facilitates the phosphorylation of the kinase by the AMPK kinase (Moore *et al.*, 1991). A number of recent studies have demonstrated that there is a significant expression of AMPK in heart tissue (Gao *et al.*, 1995; Verhoeven *et al.*, 1995; Aguan *et al.*, 1994; Beri *et al.*, 1994). While a mechanism involving AMPK regulation of ACC has never been demonstrated in heart, the ability of AMPK to phosphorylate and inhibit ACC resulting in an acceleration of fatty acid oxidation is an attractive hypothesis. Indeed, recent work from this author's laboratory has also provided

compelling evidence that AMPK is in fact, capable of phosphorylating and inhibiting ACC in heart tissue and that this, in turn, is accompanied by an acceleration of fatty acid oxidation rates (Kudo *et al.*, 1995a; 1995b).

## *G) Fatty acid metabolism in diabetes:*

### *i) source of myocardial fatty acids in diabetes:*

In diabetes, the delivery of fatty acids to the heart, either as free fatty acids or esterified within chylomicrons and very low density lipoproteins is elevated (Randle *et al.*, 1994; Howard, 1987). Unfortunately, while free fatty acid metabolism in the heart has received considerable attention, relatively little is known regarding the influence of elevated lipoproteins on myocardial fatty acid metabolism. Early work by Kriesberg (1966) has shown a depressed oxidation of chylomicron-derived fatty acids in hearts from diabetic rats. Whether or not this is indicative of a reduced activity of lipoprotein lipase (LPL) in the diabetic heart is somewhat controversial. Nakai *et al.*, (1984) and Nomura *et al.*, (1984) have reported an increase in LPL activity following streptozotocin-induced diabetes. However, a number of studies from Severson's group have suggested heparin-releasable LPL activity in heart is compromised following the induction of diabetes (Rodrigues *et al.*, 1992; Braun and Severson, 1991) and this appears to be due to post-transcriptional mechanisms (Carroll *et al.*, 1995). Thus, although the relative amounts of either free or esterified fatty acids contributing to fatty acid oxidation in the heart has not been delineated, it appears that ultimately the

metabolism of the free fatty acids may be enhanced even in the presence of a potentially reduced LPL activity.

**ii) endogenous triglycerides**

The endogenous triglyceride pool in the heart is greatly expanded in the heart following diabetes (Denton and Randle, 1967; Rizza *et al.*, 1974) and is an important source of fatty acids for oxidative metabolism (Saddik and Lopaschuk, 1991; 1994). Both the high levels of circulating plasma fatty acids (Murthy and Shipp, 1977; Murthy *et al.*, 1983) and the enhanced myocardial levels of CoA in diabetes (Lopaschuk and Tsang, 1987) may be important contributors to this expanded pool.

The contribution of endogenous triacylglycerols to overall energy production in the heart varies inversely with the supply of free fatty acids delivered to the heart most probably reflecting an inhibition of lipolysis at high exogenous fatty acid concentrations (Saddik and Lopaschuk, 1991). For example, in the absence of an exogenous supply of fatty acids, the endogenous triglyceride pool can contribute as much as 42 % of the ATP requirements of the heart whereas supplying hearts with 1.2 mM palmitate reduces the contribution of endogenous triglycerides to 11% of the energy requirements (Saddik and Lopaschuk, 1991).

**iii) myocardial metabolism of free fatty acids in diabetes**

Under normal conditions, fatty acids provide 50-70 % of the energy rquirements of the heart with the remainder derived largely from carbohydrate

metabolism. In diabetes, this coupling between carbohydrate and fatty acid metabolism is disrupted and the heart becomes almost entirely reliant upon fatty acids for ATP generation (Garland *et al.*, 1964; Wall and Lopaschuk, 1989). This altered metabolic profile of energy substrate metabolism in the heart has been found in a number of models of diabetes as outlined in Table 1-1. This is due, in part, to the high levels of circulating free fatty acids and triacylglycerol-rich lipoproteins. However, even if isolated hearts from control and diabetic rats are perfused under similar conditions, myocardial fatty acid oxidation rates are high and glucose oxidation are low in diabetic hearts (Wall and Lopaschuk, 1989). This suggests that alterations in the control of fatty acid and glucose oxidation are occurring within the myocardium.

While the decrease in glucose utilization in the heart following diabetes can be partially explained by a decrease in glucose uptake (Park *et al.*, 1968), a major reason for the decrease is an inhibition of phosphofructokinase (PFK) in the glycolytic pathway and the pyruvate dehydrogenase complex, the rate-limiting enzyme for oxidative metabolism of glucose (Newsholme and Randle, 1962; Kerbey *et al.*, 1985). The consequence of the increased fatty acid oxidation in the diabetic heart is somewhat controversial but may involve an increase in cellular citrate levels resulting in the inhibition of PFK and/or an increase in the acetyl CoA/CoA ratio which activates the PDH kinase thereby inhibiting PDH activity (Patel and Roche, 1990). A relative lack of information exists as to the underlying cause for the increased fatty acid metabolism. Whether or not ACC and AMPK factor into this response in the diabetic heart remains to be determined.

***i) insulin resistance:***

Insulin resistance is a key phenomenon in the pathogenesis of type II diabetes mellitus as well as many other abnormalities of lipid metabolism (Muller-Wieland *et al.*, 1993) and involves a complex interplay between the liver, muscle, pancreatic ß-cells and adipose tissue (Reaven, 1995). Insulin resistance is characterized by marked hyperglycemia as a result of impaired ability of the hormone to promote glucose uptake and storage in muscle cells (De Fronzo *et al.*, 1992). As long as individuals exhibiting insulin resistance are capable of increasing their insulin secretion, the decompensation of glucose homeostasis can be prevented. However, when the insulin secretory response becomes insufficient to maintain normal glucose uptake by various insulin-sensitive tissues, the individuals begin a transition from impaired glucose tolerance to overt diabetes mellitus.

It is becoming increasingly clear that many of the manifestations of insulin resistance can be explained by an altered metabolism in target cells which are most probably occurring independent of insulin's interaction with its receptor (McGarry, 1992). The combination of peripheral insulin resistance and increased hepatic glucose output and a hyporesponsive ß cell to elevated glucose concentrations together lead to the development of type II diabetes. While the general dogma is that insulin resistance is a disorder of glucose metabolism, many of the facets of insulin resistance can be considered a result of an alteration in lipid metabolism (McGarry, 1992; 1994). Indeed, administration of etomoxir, a CPT I inhibitor, has been shown to lower blood glucose levels in a model of NIDDM (Reaven, 1988). Therefore, abnormal fatty acid metabolism seems to be the key factor responsible for many of the metabolic perturbations known to occur in insulin resistance.

Under normal physiological conditions, insulin acts at the liver to inhibit hepatic glucose output i.e. gluconeogenesis and glycogenolysis. Insulin also

serves to stimulate glucose uptake in peripheral tissues, most importantly skeletal muscle, while in adipose tissue, insulin inhibits lipolysis (W. lffenbuttel and van Haeften, 1995). Therefore, in individuals who exhibit insulin resistance, these functions are impaired and metabolic homeostasis is lost. The inability of insulin to suppress lipolysis leads to an increase in the circulating free fatty acid concentrations which inhibit glucose uptake and utilization in muscle, a phenomenon known as the Randle cycle (Newsholme et al., 1962). These high levels of circulating fatty acids, upon reaching the liver, will stimulate fatty acid oxidation, and increase hepatic glucose output which can expand the already enlarged plasma glucose pool. In addition, the enhanced flux of free fatty acids to the liver can also stimulate VLDL secretion and triglyceride synthesis (McGarry, 1992) The function of the ß cell, now exposed to these high levels of glucose and fatty acids can also become compromised further (Reaven, 1995). This series of events eventually lead to a cycle of deleterious responses worsening the overall metabolic status of the individual.

**H) Diabetes-induced alterations in ACC in lipogenic tissues :**

Although the control of ACC in the diabetic heart has not been previously studied, information on diabetes-induced changes in other tissues is available and is discussed below. The knowledge of the hyperlipidemias associated with diabetes has directed a number of studies toward the regulation of fatty acid biosynthesis both in the liver and adipose tissue following the induction of diabetes. Acetyl CoA carboxylase, the enzyme considered rate-limiting for de novo fatty acid synthesis, has been the focus of many of these studies. In addition, ability of the counterregulatory hormones, insulin and glucagon, to

activate and inhibit ACC, respectively, has led to the belief that the hormonal alterations associated with diabetes would in turn influence the activity and/or expression of ACC. Indeed, Pape *et al.*, (1989), using a cDNA probe for ACC-265, have surveyed a number physiological conditions, including diabetes for changes in expression of ACC mRNA in epididymal fat tissue. Following the induction of diabetes there is a marked reduction in ACC mRNA as compared to controls. This drop in message for ACC, however, could be completely reversed if rats were injected with insulin or 6 hours prior to sacrifice. A drop in hepatic and adipose tissue ACC mRNA could also be found in animals that had been fasted and the mRNA levels could subsequently be dramatically increased upon re-feeding a fat-free diet.

A somewhat controversial concept is that ACC may be present in an active cytoplasmic form and an inactive form localized within the mitochondria. Using an acute alloxan-induced diabetic model, Roman-Lopez and Allred, (1987) examined changes in the quantity of hepatic ACC distributed among the cytoplasm and mitochondria. The diabetic state caused a reduction in cytoplasmic ACC that was accompanied by an increase in the mitochondrial form which these authors considered to be an inactive form of ACC. Thus the decreased activity of ACC in the liver following diabetes may result from a translocation of ACC out of the cytoplasm into the mitochondria, a process promoted by the drop in insulin.

These above noted changes in ACC following diabetes are entirely consistent with a mechanism by which a drop in malonyl CoA levels, as a result of a drop in ACC expression and activity, permits an acceleration of fatty acid oxidation in the liver. Further support for this idea was provided by Miethke *et al.*, (1986) who demonstrated that the 40% reduction in ACC activity following non-ketotic streptozotocin-induced diabetes was accompanied by a 2 fold increase

in the activity of CPT I, the enzyme considered to be rate-limiting for fatty acid oxidation. Furthermore, both of these responses were amplified when a ketotic model of diabetes was induced by alloxan.

Despite the potential importance of ACC in regulating fatty acid oxidation in the heart, the effects of diabetes on cardiac ACC expression and activity have not been previously addressed.

## *I) Animal models of diabetes:*

### *i) insulin resistance versus insulin deficiency*

A number of animal models have been developed for the study of insulin resistance leading to non-insulin dependent diabetes mellitus. These include a chemically-induced model where animals are injected with streptozotocin shortly following birth and ultimately develop insulin resistance leading to non-insulin dependent diabetes mellitus in adulthood (Schaffer and Wilson, 1993). Alternatively, a genetic model of insulin resistance has been developed in rats by incorporating the corpulent (*cp*) gene (Russell *et al.*, 1991). One of these strains, the JCR:LA strain, exhibits profound obesity, hyperlipidemia and and insulin resistance when homozygous for the *cp* gene. Animals which are heterozygous for the corpulent gene do not develop insulin resistance and are used as lean controls. The development of overt insulin-resistance in this model appears to occur over a period of approximately 5 weeks. Male rats exhibiting insulin resistance also spontaneously develop myocardial lesions which are believed to be ischemic in origin. The adult male JCR:LA corpulent rat was therefore chosen as the model

for the study of the regulation of myocardial metabolism in insulin resistance in this thesis.

A genetic model of type-I insulin dependent diabetes has been developed and is known as the BB-Wor strain. These animals require daily insulin injections and can provide a model for controlled and uncontrolled diabetes simply by witholding the insulin injections. One goal of this thesis was to study ACC regulation of fatty acid oxidation in this model. However, the animal population made available ultimately exhibited characteristics of the diabetic state whether or not they were administered daily injections of insulin. For this reason, this series of studies was terminated.

Chemically-induced diabetes reminiscent of type 1 insulin-dependent diabetes can be achieved in experimental animals by injection of either alloxan or streptozotocin. Both alloxan, a uric acid derivative, and streptozotocin, which is structurally related to glucose, have fairly short half-lives in the body (approximately 2 and 15 minutes, respectively). As a result, intravenous administration of the drugs is preferred and results in a relatively selective destruction of the pancreatic ß cell. Although both drugs appear to achieve equal ß cytotoxic effects, distribution studies have suggested that streptozotocin may be more specific (Pierce *et al.*, 1988). The mechanism of action also differs between the two drugs. Alloxan appears to achieve its toxic effects by inhibiting phosphate transport across the mitochondrial membrane leading to an inhibition of mitochondrial oxidative phosphorylation. In contrast, streptozotocin is thought to have its effects in the nucleus of the ß cell where it can cause DNA methylation resulting in cellular destruction.

While the chemically induced insulin deficient models of diabetes are relatively simple to produce, they are limited in some respects. Because the onset

of insulin deficiency is rapid, it may no be an accurate reflection of the human type-I diabetes where the development of the pathology may occur over an extended period of time.

### *J) Methodological Considerations:*

#### *i) The isolated working rat heart as a model to study myocardial metabolism:*

In this thesis, I have used the isolated working rat heart as a model to study oxidative metabolism in the heart. This model, in combination with the use of radio-labeled substrates, provides an investigator with the unique ability to study both heart function and the activity of the various metabolic pathways associated with both oxidative and anaerobic metabolism. The isolated working rat heart preparation was originally described by Neely *et al.*, (1967) and is a variation of the Langendorff perfused heart model which was developed more than a century ago. Unlike the Langendorff heart, which employs a retrograde perfusion of the coronary arteries via an aortic cannula, the working heart model consists of a functioning left ventricle which receives fluid in an anterograde fashion. A cannula placed in the left atrium delivers fluid into the atrium which passes into, and is then ejected by, the left ventricle against an afterload pressure provided by an aortic cannula.

The working heart model is therefore amenable to measuring metabolic pathways under situations where the amount of contractile work performed by the heart can be manipulated by altering either preload and afterload pressures, or

simply monitored continuously over the course of experimentation at constant workloads. The fact that any neurohumoral regulation of heart metabolism is absent from this preparation is advantageous in that it allows one to study the influence of various hormones on metabolic parameters of interest, as done in **"CHAPTER VI"**.

On the other hand, there are a number of disadvantages to the isolated working heart model. First, this model is not amenable to studies in which a drug or series of drugs are to be screened for potential effects. Because this model is fairly labour intensive and limited with respect to the number of preparations that can be used in a given day, screening studies would be better performed in isolated myocyte preparations in which dose-response relationships can be more easily determined. Secondly, studies in which the levels of metabolites are to be determined would also be more easily conducted using an isolated myocyte preparation.

### *ii) Other models used to study myocardial metabolism:*

#### *a) in vivo measurements:*

A number of studies have been directed toward measuring myocardial metabolism *in vivo*. These investigations have primarily utilized changes in substrate concentration across the heart as an index of the metabolic pathway activity. As first described by Bing (1953), arterio-venous differences of various substrates, as measured by a catheter placed in the coronary sinus, can provide information as to their uptake and utilization. However, the assumptions made with this method does not take into account the fact that not all of the substrates

taken up by the heart are necessarily oxidized. In addition, endogenous substrates such as lactate and fatty acids may also be released from the heart thereby yielding incorrect measurements of oxidation rates by this method.

### *b) Isolated cardiac myocytes:*

The relative ease of preparation and manipulation that can be performed with myocytes has led to a number of studies using isolated myocytes in order to study energy substrate utilization of the heart. With regard to fatty acid metabolism, isolated myocytes can be a very useful tool to delineate the role of the key regulatory enzymes in these pathways (McMillin *et al.*, 1994). However, while isolated myocytes do exhibit spontaneous contractile activity, the amount of work performed, and thus the metabolic demand, by these cells is much lower than that of a functioning heart. This decreased requirement for ATP production contributes to rates of oxidative metabolism which can be as little as 1/60th those of a working heart (Awan and Saggerson, 1993) and may not be indicative of *in vivo* situations. Therefore, the investigator must be cautious in comparing the regulation of fatty acid metabolic enzymes to the flux through fatty acid oxidation.

## *ii) Preparation of fractions containing acetyl CoA carboxylase and 5' AMP-activated protein kinase :*

A number of techiques have been developed for the isolation of acetyl CoA carboxylase from a variety of tissues. These have included the preparation of a crude cytosolic fraction which contains not only ACC but also a number of cytosolic proteins. While this technique is fairly simple to perform, measurements

of ACC activity in extracts prepared in this manner must take into account the presence of regulatory factors which cannot be controlled by the investigator and may alter the enzymatic activity. A second method involves a partial purification of the enzymes by precipitation with either ammonium sulfate or polyethylene glycol. These techniques allow for the rapid and simultaneous isolation of fractions containing both ACC and the 5' AMP-activated protein kinase (AMPK) . In our experience, we have found that these two precipitation techniques yield fractions with somewhat different characteristics particularly with respect to AMPK. While isolation of AMPK with polyethylene glycol results in higher levels of enzyme activity, western blots on fractions prepared in this fashion are somewhat "messy". On the other hand, fractions blotted from ammonium sulfate precipitation are much "cleaner" although the enzyme activity recovered is relatively low. The final technique used to isolate ACC or AMPK is affinity chromatography. Because ACC is a biotin-containing enzyme, this allows the use of avidin-sepharose columns to partially purify the enzyme. Unfortunately, this does not result in an absolute purification of ACC as other biotin-containing carboxylases such as pyruvate and propionyl CoA carboxylase are also bound by the avidin.

It should be noted that in this thesis, ACC was isolated using either the crude cytosolic fraction (**CHAPTERS III, IV**) or the polyethylene glycol (PEG) precipitation technique (**CHAPTERS V,VI**). These preparations do result in a significant difference in ACC activity (higher activity in the PEG fractionation procedure). The fact the the PEG precipitation procedure results in a partial purification of the enzyme is likely the cause of any disparencies in the activity measured between the two techniques.

***K) Objectives of the work in this thesis:***

The role of ACC in regulating hepatic and adipose tissue metabolism of fatty acids has received considerable attention. The recent identification of isoenzymes of ACC in non-lipogenic tissues such as heart and skeletal muscle has led us to the hypothesis that ACC may also be important in the regulation of fatty acid oxidation in these tissues. We also hypothesized that a decrease in ACC activity or expression is partly responsible for the abnormalities in myocardial fatty acid oxidation seen in diabetes. The objectives of this thesis were therefore as follows:

1) To determine the isoenzyme distribution of ACC in the heart and whether or not myocardial ACC activity could influence fatty acid and glucose oxidation through the production of malonyl CoA.

2) To investigate the influence of acute streptozotocin-induced diabetes on ACC activity and expression from a variety of lipogenic and non-lipogenic tissues of the rat.

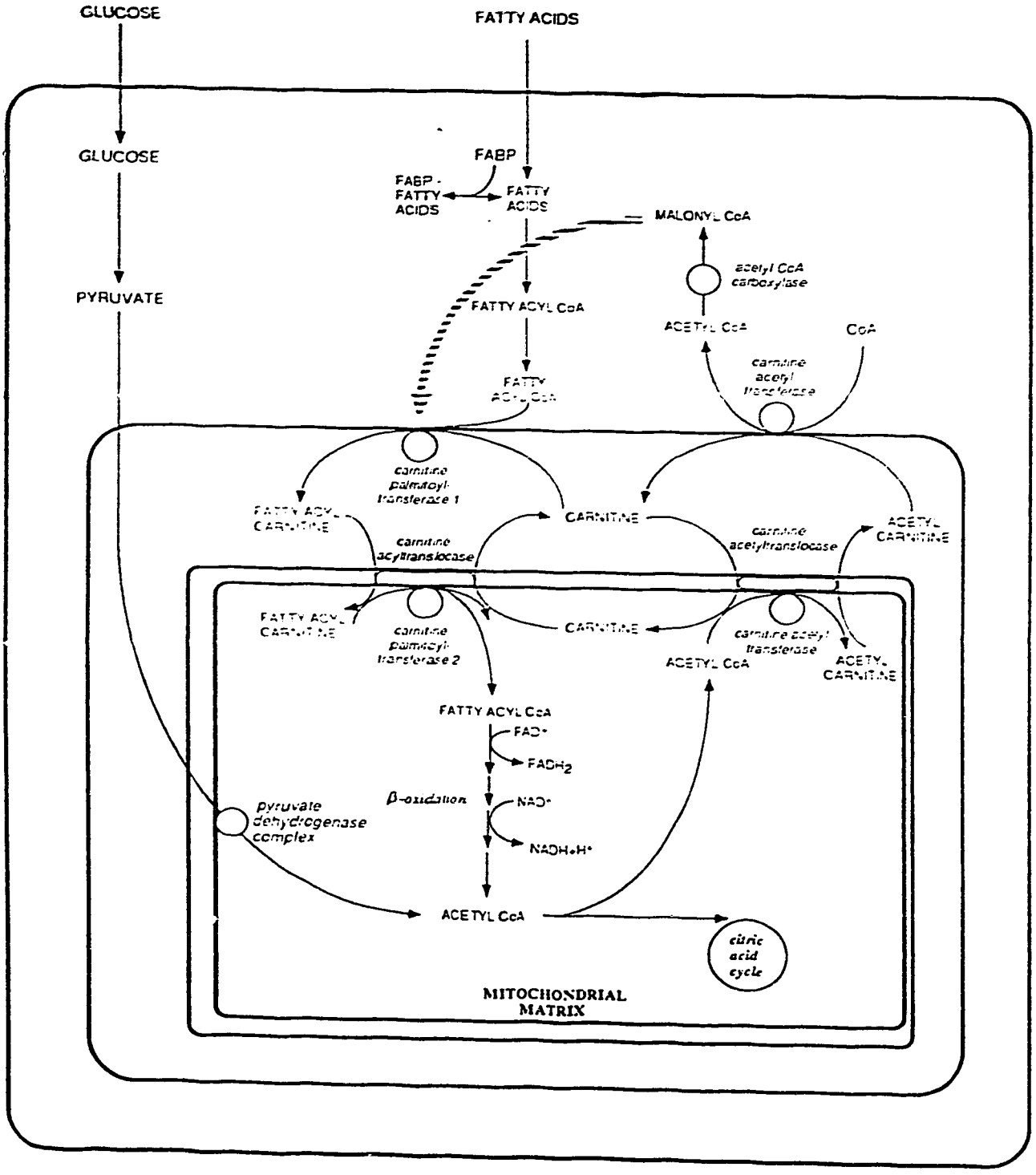
3) To examine the influence of insulin resistance on myocardial fatty acid and glucose oxidation using the the JCR:LA corpulent rat model. Also, to determine if potential alterations in metabolism are associated with changes in ACC and 5'-AMP-activated protein kinase activities.

4) To determine if myocardial glucose and fatty acid metabolism can be regulated by insulin and glucagon secondary to the effects of these hormones on ACC and/or AMPK activity.

5) To determine whether the regulation of myocardial ACC and AMPK by insulin and glucagon is similar to that known to exist in the liver.

**Figure 1-1.** Overview of the pathways involved in fatty acid and glucose oxidation in the heart.[1]

[1] Taken from: Lopaschuk, G.D., Belke, D.B., Gamble, J., Itoi, T., and Schonekess, B.O. (1994) "Regulation of fatty acid oxidation in the mammalian heart in health and disease" Biochim. Biophys. Acta 1213,263-276.


GLUCOSE
FATTY ACIDS
GLUCOSE
FABP
FABP - FATTY ACIDS
FATTY ACIDS
MALONYL CoA
acetyl CoA carboxylase
PYRUVATE
FATTY ACYL CoA
ACETYL CoA
CoA
carnitine acetyl transferase
FATTY ACYL CoA
carnitine palmitoyl transferase 1
FATTY ACYL CARNITINE
carnitine acyltranslocase
CARNITINE
carnitine acetyltranslocase
ACETYL CARNITINE
FATTY ACYL CARNITINE
carnitine palmitoyl transferase 2
CARNITINE
carnitine acetyl transferase
ACETYL CoA
ACETYL CARNITINE
FATTY ACYL CoA
FAD+
FADH2
β-oxidation
NAD+
NADH+H+
pyruvate dehydrogenase complex
ACETYL CoA
citric acid cycle
MITOCHONDRIAL MATRIX

# Chapter II

## General Experimental Methods and Materials

### *A) Animal models:*

A variety of animal models were used in the studies described in this thesis. The following gives a brief outline of the models used throughout the thesis while the specific models used in each study will be noted in each of the pertinent chapters.

#### *i) General:* (CHAPTERS III, IV and VI)

Adult male Sprague Dawley rats weighing 250-300 g were maintained according to the guidelines set forth by the Canadian Council for Animal Care. Animals were housed in the Animal Care Facility at the Heritage Medical Research Center, University of Alberta and maintained by staff of the Health Sciences Laboratory Animal Services. Animals were subjected to a 12 hour light:dark cycle and were provided normal rat lab chow and water *ad libitum*.

#### *ii) Streptozotocin-induced diabetes:* (CHAPTER IV)

Diabetes was induced in enflurane anesthetized male Sprague-Dawley rats via a single tail vein injection of 110 mg/kg streptozotocin (Sigma Chemicals, St. Louis, MO). Streptozotocin was dissolved in a 50 mM citrate buffer, pH 4.5. Control animals were injected with the vehicle alone. Forty-eight hours post-

**Table 1-1.** Comparison of cardiac glucose and fatty acid oxidation rates in various models of diabetes.[2]

| Animal model | Glucose oxidation | Fatty acid Oxidation | Reference |
|---|---|---|---|
| *Stretozotocin Diabetic Rats* | | | |
| -uncontrolled | decreased | normal | Wall & Lopaschuk, 1989<br>Saddik and Lopaschuk, 1994 |
| -islet transplanted | normal | normal | Lopaschuk *et al.*, 1993 |
| *Spontaneously Diabetic BB Rats* | | | |
| -controlled | normal | normal | Lopaschuk and Tsang, 1987 |
| -uncontrolled | decreased | increased | Broderick *et al.*, 1992 |
| *Insulin-resistant JCR:LA Rats* | | | |
| -high insulin | increased | normal | Lopaschuk and Russell, 1991 |
| -low insulin | unknown | unknown | |

[2] Adapted from: Lopaschuk, G.D. Fatty acid metabolism in the heart following diabetes. In: The heart in diabetes. Chatham and Forder (eds) Kluwer Academic Publishers, 1995.

injection, all animals were sacrificed and the heart, liver, white adipose tissue and skeletal muscle (gastrocnemius) were quickly harvested and immediately frozen in liquid $N_2$.

### *iii) Genetically-induced insulin-resistence:* (CHAPTER V)

The JCR:LA corpulent rat used in this study was developed as a genetic model of insulin resistance by incorporating the mutant corpulent gene (*cp*), first isolated by Koletsky (1973) into two strains ie, the LA/N and SHR/N. Following a series of backcrossings with the LA/N strain, a colony was developed and designated the JCR:LA corpulent strain. The JCR:LA corpulent rats, which are homozygous for the corpulent gene, (*cp/cp*) and the lean controls, which are either non-carriers or heterozygous for the gene (+/+ or +/*cp*) were bred in the established colony at the University of Alberta maintained by Dr. J.C. Russell. All animals were subject to a 12:12 hour light cycle and food and water were provided for the animals *ad libitum*.

***B) The isolated working rat heart preparation:***

Adult male Sprague Dawley rats (250-300 g) were anesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally (**CHAPTERS III, IV, VI**) or with halothane delivered via inhalation (**CHAPTER V**). Following thoracotomy, the exposed hearts (and lungs) were quickly excised and placed in ice-cold Krebs-Henseleit solution. Once rinsed, the aorta was rapidly cannulated (< 30 seconds from excision), and a retrograde perfusion using oxygenated Krebs-Henseleit solution, pH 7.4 was initiated as described previously (Saddik and Lopaschuk, 1991). 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. The working heart buffers consisted of a standard Krebs-Henseleit solution containing 11 mM glucose and various concentrations of other components such as palmitate (prebound to 3% bovine serum albumin), calcium, and insulin depending on the protocol employed in each individual study. The specific concentratins of each of these constituents are outlines in the appropriate **Methods** section of each chapter. For all studies, the temperature of the perfusate entering the heart was maintained at 37° C.

Spontaneously beating hearts were used throughout the studies, with heart rate and peak systolic pressure (PSP) being measured by a Gould P21 pressure transducer in the aortic outflow line and visualized on a Gould RS-3600 physiograph. Cardiac output, aortic flow and coronary flow were measured using Transonic in-line ultrasonic flow probes in the atrial inflow and aortic outflow lines, respectively and connected to a T 101 ultrasonic blood flow meter. In all

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

### *C) Determination of Myocardial Glucose Oxidation :*

To measure rates of glucose oxidation, hearts were perfused with buffer that was trace-labeled with [U-$^{14}$C] glucose. Steady-state glucose oxidation rates were determined as described previously (Lopaschuk *et al.*, 1988; Lopaschuk *et al.*, 1990) by quantitative measurement of $^{14}CO_2$ which is liberated from the PDC and the TCA cycle (Figure 2-2). Briefly, hearts were perfused in a closed recirculating system using an oxygenator with a large surface area in constant contact with 95% $O_2$ and 5% $CO_2$ . The gas mixture entered the perfusion apparatus at the top of a closed oxygenation chamber, exited the chamber through an exhaust tube, and was bubbled through a 1 M benzethonium hydroxide trap to collect gaseous $^{14}CO_2$. Perfusate samples, used to determine $^{14}CO_2$ present in the form of bicarbonate ($H^{14}CO_3$), were removed with a syringe directly from the system without exposure to air and were stored under mineral oil. Two 1 ml samples were later injected into a 25 ml stoppered metabolic flask with either a center well containing paper saturated with 0.4 ml benzethonium hyamine hydroxide or a scintillation vial containing filter paper and 0.3 ml benzethonium hyamine hydroxide. The sample of perfusate was acidified by contact with 9N $H_2SO_4$ (1 ml) present in the flask thereby liberating the $^{14}CO_2$ present as $H^{14}CO_3$. The flask was then shaken gently for 1 hour, and the center wells or vials placed in ACS scintillation cocktail to be counted using standard ß-scintillation counting procedures. Glucose oxidation rates were expressed as nmol glucose oxidized·min$^{-1}$ and were normalized either to heart weight (g dry) or an index of heart function, depending on the study.

### *D) Determination of Myocardial Palmitate Oxidation:*

Steady-state palmitate oxidation (**CHAPTERS III, V, VI**) was determined quantitatively from the $^{3}H_2O$ production of hearts perfused with [9,10-$^{3}$H]palmitate (Figure 2-1) as described previously (Saddik and Lopaschuk, 1991). Briefly, chloroform fractions were separated from an acqueous phase in 0.5 ml of perfusion buffer samples that were subjected to 1.88 ml chloroform:methanol (1:2 v/v) mixture followed by 0.625 ml of 2M KCl:HCl solution. The aqueous phase was then removed and re-extractions were carried out on this phase with 1ml chloroform, 1 ml methanol and 0.9 ml of 2M KCl:HCl solution. Duplicate 0.5 ml samples of the aqueous phase were then counted for each perfusate sample for total $^{3}H_2O$ determination. It has been previously determined that this procedure results in 99.7% extraction and separation of $^{3}H_2O$ from [9,10-$^{3}$H]palmitate. Palmitate oxidation rates were expressed as nmol palmitate oxidized·min$^{-1}$ and were normalized either to heart weight (g dry) or an index of heart function, depending on the study.

Alternatively, steady-state palmitate oxidation was determined (**CHAPTER III**) by the detection of $^{14}CO_2$ when the palmitate was labelled with [1-$^{14}$C] palmitate. These determinations were conducted using the benzethonium hyamine hydroxide trap procedure exactly as described above for the determination of glucose oxidation. When both endogenous and exogenous palmitate oxidation were being measured simultaneously as in "**CHAPTER III**", the $^{14}$C-labeled palmitate serves as an internal control when palmitate oxidation is being determined from $^{3}H_2O$ production. Specifically, any $^{3}$H-palmitate which may be present during the separation of $^{3}H_2O$ from the organic phase, can be

determined because an equal amount of $^{14}C$-labeled palmitate would also be present and can be detected using dual-label counting procedures during scintillation counting.

### *E) Calculation of ATP production based on rates of oxidative metabolism:*

Using the steady-state rates of glucose and palmitate oxidation, one can determine the accompanying rates of ATP production. This calculation was carried out as follows. The complete oxidation of 1 mol of glucose results in the fromation of 38 mol of ATP. For every mol of palmitate oxidized there are 129 mol ATP produced.

### *F) Tissue Preparation and Metabolite Determinations:*

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 $N_2$. The powdered ventricular tissue was then transferred to cryovials and stored at -80° C until use. 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.

For studies that did not involve heart perfusions, tissues were harvested quickly from the animals following anesthesia with sodium pentobarbital (60 mg/kg injected intraperitoneally) in the following order: heart, liver, skeletal

muscle (gastrocnemius), and white adipose tissue. Upon excision, all tissues were immediately immersed in liquid $N_2$. Heart, liver and skeletal muscle tissue were later pulverized into a powder state using a mortar and pestle cooled to the temperature of liquid $N_2$, following which they were transferred to cryovials and stored at -80° C until use.

### *ii) Determination of Coenzyme A esters using HPLC*[3]*:*

Coenzyme A esters were extracted as described previously (Idell-Wenger *et al.*, 1978). 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.*, (1988). Separation was performed on a Beckman System Gold with a UV detector 167. Each sample (100 µl each) was run through a precolumn cartridge (C18, size 3 cm, 7 µm particle size) 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 Chemical Company, Rockford, IL). Initial conditions (97% A, 3% B) were maintained for 2.5 min and were changed thereafter to 18% B over 5 min using Beckman's curve 3. At 15 min the gradient was changed linearly to 37% B over 3 min and subsequently to 90% B over 17 min At 42 min the composition was returned linearly back to 3% B over 0.5 min and at 50 min column equilibration was complete. Peaks were integrated by Beckman System Gold software package.

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[3] All HPLC analyses were performed by Mr. Kenneth Strynadka

### *iii) Measurement of lipid metabolic intermediates:*

Tissue lipids from frozen ventricular tissue were extracted as described previously (Saddik and Lopaschuk, 1991). Neutral lipids were separated from phospholipids using the method described by Bowyer and King (1977). [$^{14}$C]palmitate and [$^{3}$H]palmitate incorporation into neutral lipids was measured using a double radioisotope counting technique described previously (Saddik and Lopaschuk, 1991). Label content of neutral lipids was expressed as µmol palmitate incorporated into this pool·g dry wt$^{-1}$. Absolute myocardial triglyceride content (µmol fatty acid equivalents·g dry wt$^{-1}$) was determined using Wako enzymatic colorimetric assay kit.

## *G) Preparation of fractions containing ACC and/or AMPK:*

### *i) Preparation of crude cytosolic fractions:*

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 eight protease inhibitors consisting of soybean trypsin inhibitor (50 µg/ml), PMSF (0.5 mM), benzamidine (0.1 mM), aprotinin (8 µg/ml), leupeptin (4 µg/ml), antipain (50 µg/ml), TPCK (0.1 mM), and TLCK (0.1 mM) (Witters *et al.*, 1988). Samples were then ultracentrifuged at 180,000 g for 60 min. 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 according to Bradford (1976).

*ii) Polyethylene glycol (PEG) fractionation :*

Approximately 200 mg of frozen tissue was homogenized with a buffer containing Tris/HCl (0.05 M; pH 7.5), mannitol (0.25 M), NaF (50 mM), Na-PPi (5 mM), PMSF (1mM), EDTA (1 mM), EGTA (1mM), DTT (1 mM), and the following protease inhibitors: PMSF ( 1mM), soy bean trypsin inhibitor (4 µg/ml) and benzamidine (1 mM). Samples were then centrifuged at 14,000 g for 20 min at 4° C. The supernatant was then brought to 2.5 % PEG with 25 % (w/v) PEG 8000 and shaken for 10 min at 4° C. Samples were then spun at 10,000 g for 10 min at 4° C. The supernatant was then made up to 6 % PEG using the PEG stock described above and shaken once again for 10 min at 4° C. This fraction was then spun at 10,000 g for 10 min and the pellet was then washed with homogenization buffer containing 6% PEG 8000. This was followed by a final centrifugation at 10,000 g and the pellet was resuspended in buffer containing Tris/HCl (0.05 M; pH 7.5), EDTA (1 mM), EGTA (1mM), DTT (1 mM), NaCl (55 mM), glycerol (10% w/v), $NaN_3$ (0.02%), and the following protease inhibitors: soy bean trypsin inhibitor (4 µg/ml) and benzamidine (1 mM). Protein content in the resuspension was measured using the BCA method (Sigma).

## *H) Measurement of enzymatic activities:*

### *i) ACC activity measured using HPLC determination of malonyl CoA*

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 (1.5 µM), ATP (0.41 mM), acetyl CoA (0.21 mM), magnesium acetate (0.97 mM), $NaHCO_3$ (3.5 mM) and 0 or 10 mM magnesium citrate. Samples were incubated at 37° C for either 0, 1, 2, 3, or 4 min, and the reaction stopped by adding 10% perchloric acid (PCA). Samples were then spun for 10 min and the malonyl-CoA concentration in the supernatant measured using the HPLC procedure described (section F ii). In many studies ACC activity was (Bianchi *et al.*, 1990; Thampy, 1989) and is still determined using [$^{14}$C]bicarbonate fixation to acid-stable products. This assay provides a direct estimate of the enzyme activity through measurement of product levels (malonyl-CoA) and has been shown to be accurate (King *et al.*, 1988). It should be noted that both the radioisotopic method and the HPLC methods for determining ACC activity were employed in this thesis.

In order to determine whether cardiac ACC was citrate dependent, another set of reactions were conducted at 7 different citrate concentrations ranging from 0-10 mM for 4 min at 37° C. In all these assay experiments, ACC activity was expressed as the amount of malonyl-CoA produced·g dry $wt^{-1}$·$min^{-1}$.

### *ii) Kinetic analyses of ACC*[4]

To determine the acetyl-CoA kinetics of both ACC-280 and ACC-265, ACC was purified from rat heart and skeletal muscle (predominantly ACC-280) and white adipose tissue (predominantly ACC-265) by monomeric avidin-Sepharose chromatography as described previously (Tipper and Witters, 1982). ACC activity was then measured in the presence of varying concentrations of acetyl-CoA. Lineweaver Burke plots were then generated and $K_a$ values of acetyl-CoA for purified preparations of ACC were determined.

### *iii) ACC activity measured using the "$CO_2$ fixation technique":*

The "$CO_2$ fixation technique" was used to measure ACC activity present in the PEG fraction. Briefly, 12.5 ug protein was added to a reaction mixture (final volume 165 µl) containing Tris acetate (60.6 mM), BSA (1 mg/ml), ß-mercaptoethanol (2.12 µM), ATP (1.32 µM), acetyl CoA (1.06 mM), magnesium acetate (5.0 mM), $NaH^{14}CO_3$ (18.08 mM) and 0 or 10 mM magnesium citrate. Samples were incubated at 37° C for 2, minutes, and the reaction stopped by adding 25 µl of 10% perchloric acid (PCA). Samples were then spun for 20 min at 3500 rpm and 160 µl of supernatant was placed in minivials and dried overnight in a fumehood. 100 µl of $H_2O$ was then added followed by Eco-lite scintillant (4 ml) and the vials were counted for the presence of radiolabelled acid-stable products. ACC activity was expressed as nmol malonyl-CoA produced·min$^{-1}$·mg

[4] Affinity purification and kinetic analyses were performed by Dr. Lee Witters, Dartmouth Medical School.

protein$^{-1}$. A direct comparison of the $CO_2$ fixation technique and the HPLC method revealed no differences in terms of ACC activity.

### *iv) Phosphorylation status of ACC:*

In order to determine the phosphorylation status of ACC, the enzyme activity was measured in fractions that were isolated in a manner that would either; a) maintain the enzyme in its *in vivo* state of phosphorylation, or b) allow for dephosphorylation during the isolation procedure. For these experiments, PEG fractions were prepared by homogenizing the tissue with buffers which included or excluded NaF (50 mM) and NaPPi (5 mM) to inhibit or promote endogenous phosphatase activity, respectively. When NaF and NaPPi were excluded from the homogenization buffers they were replaced with a molar equivalent of NaCl. All fractions were resuspended in the absence of the phosphatase inhibitors since the endogenous phosphatases are removed during the initial precipitation step. ACC assays were then conducted on the paired samples using the $CO_2$ fixation assay as described above.

### *v) 5'-AMP activated protein kinase activity assay:*

AMPK activity was measured in the PEG fractions according to the method of Davies *et al.*, (1989) with slight modification (Kudo *et al.*, 1995). Briefly, 2 µg of the PEG fraction (which was isolated by homogenizing in the presence of NaF (50 mM) and NaPPi (5 mM) and resuspended in their absence) including 0.12% Triton X-100 was added to a reaction mixture (25 µl final volume) composed of HEPES-NaOH (40 mM), NaCl (80 mM), glycerol (8% w/v), EDTA (0.8 mM),

SAMS peptide (200μM), DTT (0.8 mM), [∂-$^{32}$P]ATP (200 μM), $MgCl_2$ (5 mM), and in the absence or presence of AMP (200 μM). The reactions were begun with the addition of the ATP-$MgCl_2$ mixture (2.5 μl). This mixture was then vortexed and incubated for 5 min at 30°C. From this incubation mixture, 15 μl was spotted onto phosphocellulose paper (1.5 cm x 1.5 cm). The paper was then washed 4 x 15 min with 150 mM pyrophosphate followed by a 10 min acetone wash. Papers were then dried and counted for radioactivity incorporated into the SAMS peptide using standard scintillation counting techniques.

### *I) RNA isolation and Northern Blotting:*

Total RNA was isolated from frozen heart, liver, white adipose, and liver tissue using the method described by Cathala *et al.*, (1983) with the following modifications. The concentration of SDS in the solubilization buffer was 1%, the RNA was precipitated using 3.5 volumes of 4 M LiCl per volume of homogenate, and a final precipitation with 4 M sodium acetate was performed to remove glycogen (Sambrook *et al.*, 1989). Approximately 1 g of frozen tissue was homogenized (1 x 1 min, 1 x 30 s) on ice in 7 ml guanidine monothiocyanate (5 M), EDTA (10 mM), Tris-HCl, pH 7.5 (50 mM) and 8% ß-mercaptoethanol (added just prior to use). Samples were spun at 4° C for 15 min @ 4400 rpm and the supernatant was subject to precipitation with LiCl (4 M). Tubes were spun once again @ 4400 rpm for 120 min (4° C) and the precipitate was resuspended in 10 ml 2 M LiCl/4 M urea. A third spin was conducted @ 4400 rpm for 120 min (4° C) and the pellets were resuspended in a total volume of 5 ml with SDS (1%), EDTA (1 mM), Tris-HCl pH 7.5 (10 mM). Phenol (0.5 vol) and chloroform (0.5 vol) were added and the mixture was vortexed for 30 s followed by centrifugation

@ 4400 rpm for 15 min (4° C). The aqueous phase was removed and precipitated overnight @ -20° C with 1/10 vol Na Acetate pH 5.2 (3 M), and 2 vol ethanol. Tubes were centrifuged for 30 min @ 4400 rpm (4° C), the supernatant was decanted, the pellet was rinsed with 10 ml ethanol (70%, -20° C) and mixed by gentle inversion. A final spin was conducted @ 4400 rpm for 15 min (4° C) and the supernatant was discarded and samples were allowed to air dry. Pellets were resuspended in 300 µL DEPC-$H_2O$ and precipitated overnight in a total volume of 900 µl with Na Acetate (4 M, pH 7.0, 4° C). Tubes were spun @ 13000 rpm for 30 min (4° C) and the supernatant was removed and washed with ice cold ethanol (70%). RNA was dried down using a speed vac (no heat) and the pellet was resuspended in 400 µl DEPC-$H_2O$. RNA yield and purity were determined spectrophotometrically by measuring the absorbances at 260 and 280 nm. RNA samples were stored at -80° C until use.

***Northern blotting:***

Analysis of RNA samples on Northern blots was performed essentially as described in Sambrook *et al.*, (1989) except that the agarose gels used for the electrophoretic separation of RNA did not contain formaldehyde (Liu and Chow, 1990). Typically, 20 µg of total RNA was loaded per lane. Gels were run @ 100 volts. RNA was transferred from the gel to the nitocellulose support overnight using the capillary transfer method. Pre-hybridization was carried out for 2 hours @ 42° C in solution containing 10x Denhardt's, 50 % formamide, Tris-HCl (50 mM, pH 7.5), NaCl (1M), and salmon sperm DNA (100 µg/ml). The prehybridization solution was heated to 65° C prior to use. Nitrocellulose filters

were probed with a 0.783 kb insert for rat heart ACC-280 cDNA[5] $^{32}$P-labelled using the Random Primers DNA Labeling System. Hybridization was carried out overnight following which the membranes were washed 2 x 5 min at room temperature, then 3 x 30 min @ 55° C with 2x SSPE, 0.1 % SDS. Membranes were air dried and developed onto film overnight at -70° C.

### *J) Analyses of ACC isoenzymes:*

#### *i) Electrophoresis:*

In order to obtain maximal separation of the ACC-280 and ACC-265, SDS-PAGE was carried out according to the method of Laemmli (1970). The concentration of acrylamide employed was 3% and 5% in the stacking and separating gels, respectively. Ammonium persulfate and TEMED were used as catalysts for polymerization. Once polymerized, the samples were loaded into the wells and the gels were run in sample buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) at 60 V (constant voltage) until the dye front had permeated the separating gel at which time the voltage was increased to 120 V.

#### *ii) Western blotting:*

Following the gel electrophoresis, the protein bands were electroblotted to nitrocellulose membrane. In **CHAPTERS III** the semi-dry transfer method was used. However, because of the high molecular weight of the ACC subunits and the relatively poor transfer efficiency obtained, all of the subsequent studies utilized

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[5] kindly provided by Dr. Lee A. Witters, Dartmouth Medical School

the wet transfer method. The transfer buffer consisted of methanol (20%), Tris (25 mM), glycine (192 mM). For the semi-dry transfer, 0.0005% SDS was also included in the buffer. The semi-dry transfer was performed at 225 mA (constant current) for 2 hours at room temperature. The wet transfer, on the other hand, was conducted at 100 V (constant voltage) for 1 hour @ 4° C. In order to verify complete transfer of the proteins from the gel to the nitrocellulose, all gels were stained following the transfer regardless of the transfer technique employed.

Membranes were then blocked with 5% (w/v) powdered milk for 4 h at room temperature. Immunoreactions (**CHAPTERS III**) employed either a monoclonal Ab (7AD3) specific to ACC-280, or a polyclonal Ab against the N terminal region of ACC-265. Secondary peroxidase-conjugated goat anti-mouse IgG (against the monoclonal Ab) and goat anti-rabbit IgG (against the polyclonal Ab) were used to visualize ACC. In all other studies, membranes were probed with peroxidase-conjugated streptavidin which binds, with high affinity, to the biotin groups contained in carboxylases. The use of streptavidin thereby facilitated the visualization of both ACC isoenzymes simultaneously as well as other carboxylases (i.e., pyruvate carboxylase and propionyl CoA carboxylase) which can serve as controls as in **CHAPTER IV**. Chemiluminescent detection was performed on the membranes using ECL western blotting detection kit.

### *K) Statistical analysis:*

Data are presented as the mean ± standard error of the mean. The paired or unpaired Students t-test was used as appropriate to determine statistical significance in groups containing 2 sample populations. One-way analysis of variance (ANOVA) followed by Students-Newman-Keuls post-hoc or Dunnett

Multiple Comparisons Test analyses were used to compare sample populations of 3 or more. A value of $p < 0.05$ was regarded as significant.

## *H) Materials*

D-[$^{14}$C(U)]glucose, [9,10-$^{3}$H]palmitic acid, and [1-$^{14}$C]palmitic acid were obtained from NEN (Wilmington, DE). $\alpha$-$^{32}$P dCTP and $\gamma$-$^{32}$P ATP were purchased from ICN Biomedicals (Costa Mesa, CA). Bovine serum albumin (BSA fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (1 M 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 $\mu$ was purchased from Pierce Chemical Company (Rockford, IL) and a Microsorb short-one column type C18, particle size 3 $\mu$, size 4.6 x 100 mm was purchased from Rainin Instruments Company (Emeryville, CA). ECL western blotting detection reagents were purchased from Amersham International (Amersham, UK). The GIBCO BRL Random Primers DNA Labeling kit was purchased from (Life Technologies, Burlington, Ontario). $IgG_1$ monoclonal antibodies (7AD3) to ACC-280 were chosen from a panel of monoclonal antibodies raised against avidin-Sepharose purified fasted/ refed rat liver enzyme (Bianchi *et al.*, 1990). 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 (Bianchi *et al.*, 1990). Secondary antibodies (peroxidase conjugated goat anti-mouse IgG [H+L] and peroxidase conjugated 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 micron) 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).

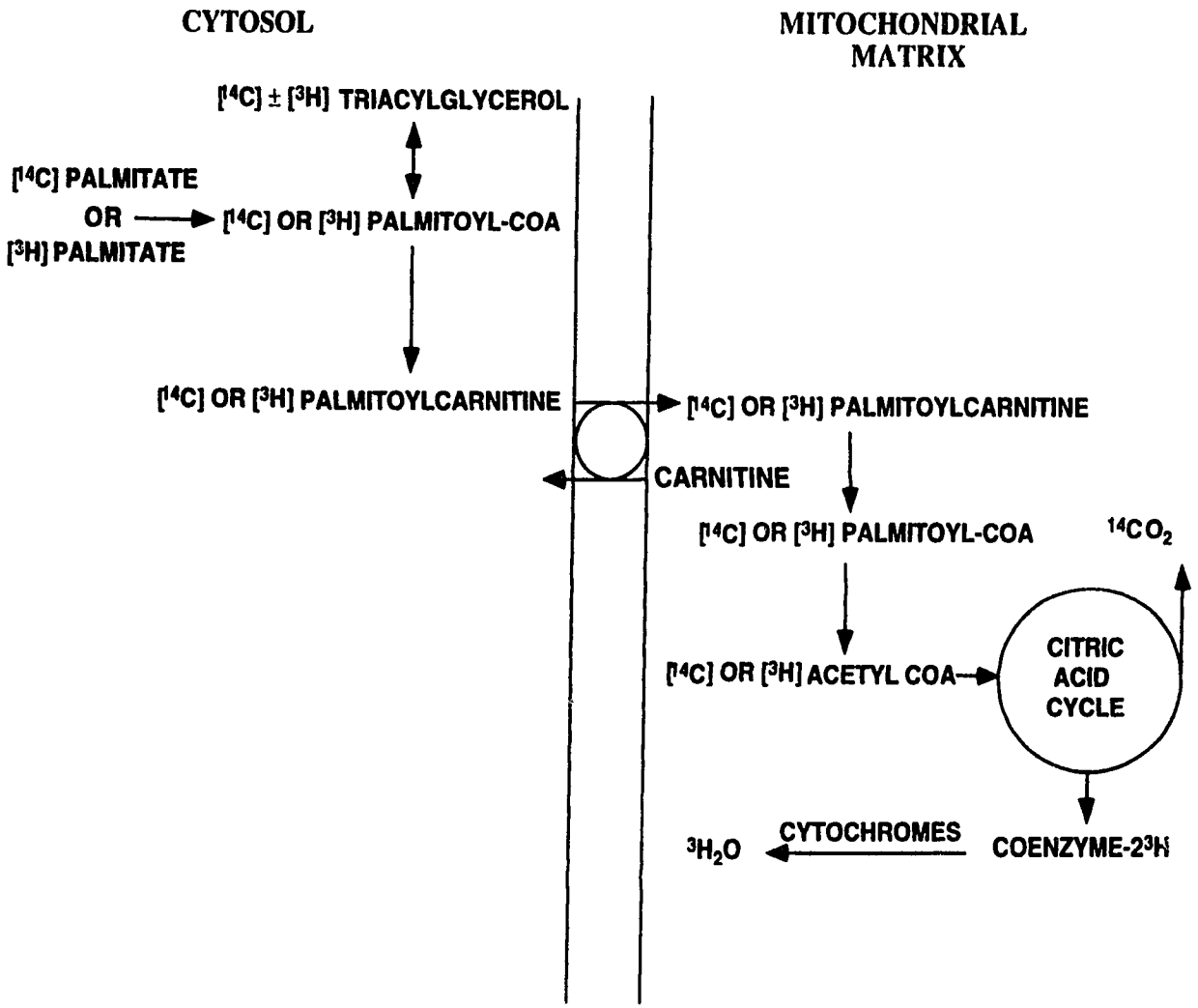


Figure 2-1. Fate of radio-labeled palmitate in the heart

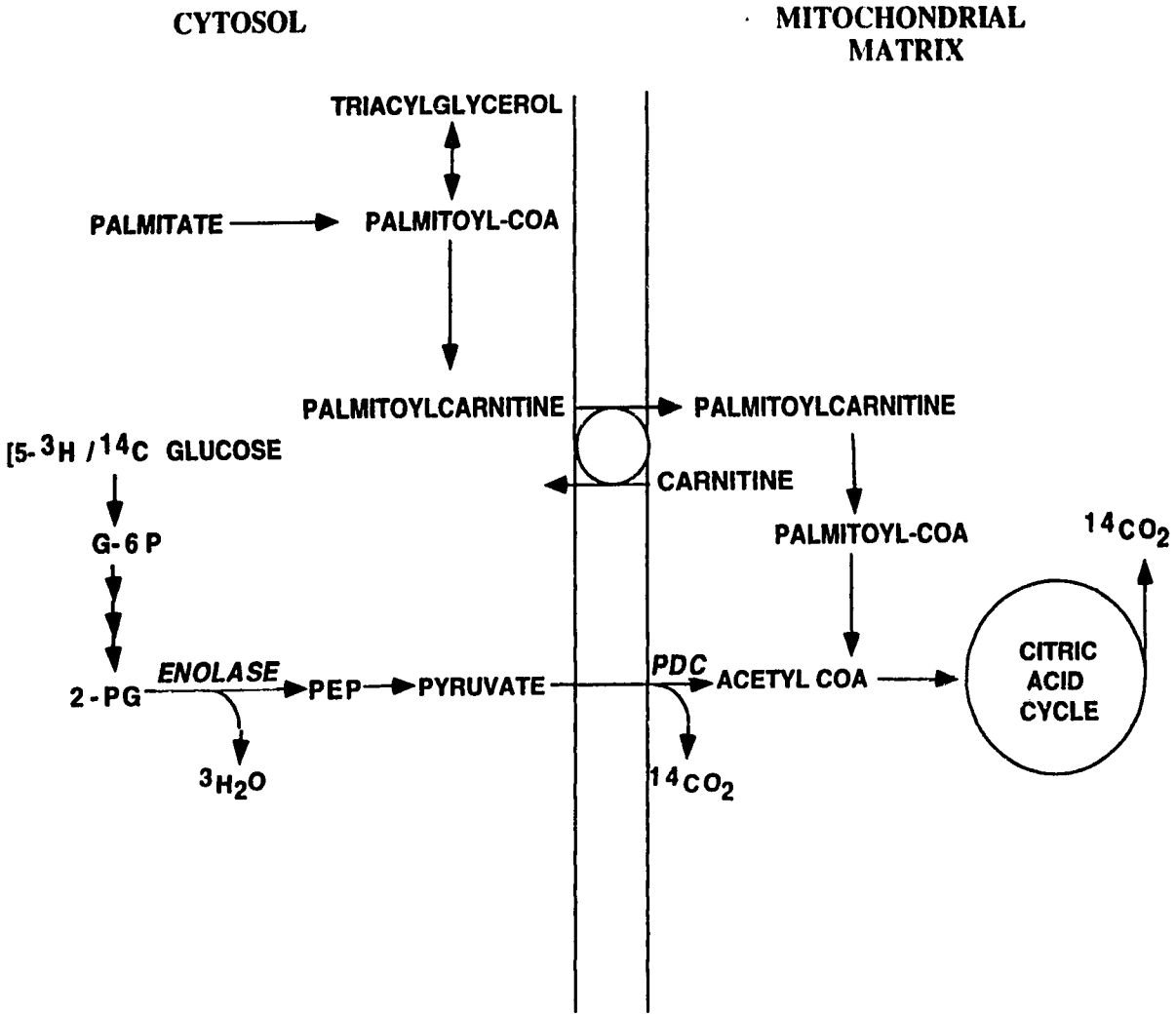


Figure 2-2. Radio-labeled products of glycolysis and glucose oxidation

# CHAPTER III

## Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart[6]

### *A) Introduction*

In the liver, acetyl-coenzyme A carboxylase (ACC) plays an important role in the *de novo* biosynthesis of fatty acids. As with other carboxylases, ACC contains a covalently linked biotin group which catalyses the formation of malonyl CoA via the carboxylation of acetyl-CoA. The malonyl CoA formed serves as a carbon donor for elongation of the acyl chain (Wakil *et al.*, 1958, Mabrouk *et al.*, 1990). In addition to its role in fatty acid biosynthesis, malonyl-CoA is also a potent inhibitor of hepatic carnitine palmitoyltransferase 1 (CPT 1) (Cook, 1984; Cook and Gamble 1987; Paulson *et al.*, 1984; McGarry *et al.*, 1989). The obligatory role of CPT I in the mitochondrial uptake of long chain fatty acids has led to the hypothesis that ACC may also exert a degree of control in fatty acid oxidation (Bianchi *et al.*, 1990). The presence of ACC in a variety of mammalian tissues such as heart and skeletal muscle, both of which are not considered lipogenic in nature (Bianchi *et al.*, 1990; Thampy 1989; Iverson *et al.*, 1990; Trumble *et al.*, 1991), has provided indirect support for this contention.

Two isoenzymes of ACC are known to exist with molecular weights of 265 and 280 kDa (Bianchi *et al.*, 1990, Thampy, 1989). The existence of multiple mRNA species under the control of two distinct promoters has also led to

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[6] A version of this chapter has been published. Saddik, M., Gamble, J., Witters, L.A., and Lopaschuk, G.D. (1993) *J. Biol. Chem.* 268,25836-25845. The perfusion portion of this study was done in collaboration between myself and Dr. Maruf Saddik. The lipid analyses were conducted by Dr. Maruf Saddik. The ACC enzyme and associated work was performed by myself.

speculation that more isoenzymes may exist (Lopez-Casillas and Kim, 1989; Kong *et al.*, 1990). To date, the major focus of investigations pertaining to ACC have been on the characterization of the 265 kDa isoenzyme (ACC-265) in liver and white adipose tissue. Much less is known about the 280 kDa isoform of ACC (ACC-280), particularly with respect to its role in regulating myocardial fatty acid oxidation (Bianchi *et al.*, 1990; Thampy, 1989).

Fatty acid oxidation provides 60 to 90% of the ATP production by the heart, depending to a large extent on circulating fatty acid concentrations. While the CPT I in liver can be regulated by alterations in its sensitivity to inhibition by malonyl CoA, Cook and Lappi (1992) have recently suggested that, unlike the liver, the supply of malonyl-CoA, as opposed to an alteration in sensitivity of CPT I to malonyl-CoA inhibition, may be 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.

Using a "pulse-chase" technique in the isolated perfused working heart, one can measure the contribution of both exogenous and endogenous fatty acids to ß-oxidation (Saddik and Lopaschuk, 1991). This technique involves pre-labeling the triglyceride pool with [$^{14}$C]palmitate and chasing hearts with [$^{3}$H]palmitate as described in **CHAPTER II**. This procedure was employed in the current study 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 the pyruvate dehydrogenase complex (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 (McVeigh and Lopaschuk, 1990).

*B) Methods*

*i) model*

Male Sprague-Dawley rats were used as described in **CHAPTER II**.

*ii) specific perfusion protocol*

Table 3-1 shows the protocol used to measure both exogenous and endogenous fatty acid oxidation in the heart. The perfusion solution consisted of the standard Krebs'-Henseleit solution containing 11 mM glucose, 1.2 mM palmitate bound to 3% bovine serum albumin and 1.25 mM $Ca^{2+}$ . In this series of perfusions, hearts were initially perfused for a 60 min period with recirculated Krebs Henseleit solution containing 1.2 mM [1-$^{14}$C]palmitate to label the endogenous lipid pools (pulse). During this labelling period, exogenous steady state fatty acid oxidation was measured by quantitative collection of myocardial $^{14}CO_2$ production, as previously described (Saddik and Lopaschuk, 1991). At the end of the "pulse" period, hearts were switched to a retrograde Langendorff drip-out perfusion with Krebs-Henseleit solution 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 solution containing [$^{14}$C]palmitate was removed from the system during this 10 min period, and replaced with solution 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 solution.

During the "pulse", steady state exogenous palmitate oxidation rates were determined by quantitatively measuring $^{14}CO_2$ production by the hearts, as

described in **CHAPTER II**. 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 (Saddik and Lopaschuk, 1991). 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 **Chapter II**. Steady state palmitate oxidation rates during the "chase" were expressed as nmol palmitate oxidized·g dry wt$^{-1}$·min$^{-1}$·unit work$^{-1}$.

In one 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" or the "chase" period. Instead, during the "chase" period perfusate contained 11 mM [U-$^{14}$C]glucose and 1.2 mM palmitate, in the presence or absence of 1 mM DCA. Glucose oxidation was determined by quantitative measurement of $^{14}CO_2$ production using the same methods described above for palmitate oxidation. Glucose oxidation rates were expressed as nmol glucose oxidized·g dry wt$^{-1}$·min$^{-1}$·unit work$^{-1}$.

Crude cytosolic fractions were prepared from the frozen ventricular tissue for the analyses of ACC activity and isoenzyme distribution. For the Western blotting procedure, protein bands were transferred from the gel to the nitocellulose membrane using the semi-dry transfer technique. Bands corresponding to the ACC isoenzymes were detected using either streptavidin, which detects all biotin-containing proteins, or antibodies directed against both isoenzymes of ACC. ACC-265 was detected using an antibody generated against the N and C terminal sequences of liver ACC-265. ACC-280 was detected using the monoclonal antibody (7AD3). For acetyl CoA kinetics, ACC was purified from white adipose and skeletal muscle tissue using monomeric avidin-sepharose column

chromatography and ACC activity was determined at varying concentrations of acetyl CoA. Kinetic parameters were subsequently generated by transforming the data for production of Lineweaver-Burke plots.

*C) Results*

*i) Effect of dichloroacetate on heart function:*

Because the work performed by the heart is a key determinant of oxidation rates, mechanical function was continuously monitored throughout the perfusion period. Table 3-2 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, although this increase was statistically not significant. However, to rule out any potential effects of this small increase in function on oxidative metabolism, all subsequent oxidative rate measurements were corrected for differences in heart work.

*ii) Effects of dichloroacetate on palmitate and glucose oxidation rates:*

Previous studies from our laboratory have shown that endogenous myocardial triglycerides are an important source of fatty acids for mitochondrial ß-oxidation (Saddik and Lopaschuk, 1991). 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, the myocardial triglyceride poolwas pre-labelled with [$^{14}$C]palmitate (see Table 3-1). 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 (Saddik and Lopaschuk, 1991).

Addition of DCA to the perfusate had a dramatic effect on myocardial glucose oxidation increasing the absolute rates of glucose oxidation from 110 ± 15 to 722 ± 69 nmol·g dry wt$^{-1}$·min$^{-1}$. Absolute rates of palmitate oxidation oxidation were also increased in the DCA-treated hearts (exogenous palmitate oxidation rates were 401 ± 56 nmol·g dry wt$^{-1}$·min$^{-1}$ in control hearts vs 502 ± 64 in the DCA-treated hearts, while endogenous palmitate oxidation rates were 24.6 ± 6.9 and 27 ± 8.8 nmol·g dry wt$^{-1}$·min$^{-1}$ respectively). As a result, overall acetyl CoA production was increased in hearts treated with DCA. However, DCA treatment also resulted in an improvement in heart function (Table 3-2). Accordingly, oxidative rates were normalized for differences in mechanical function (Table 3-3). Even when corrected for functional differences, DCA treatment resulted in a marked stimulation of glucose oxidation (Table 3-3a) (presumably occurring secondary to a stimulation of PDC activity). This was accompanied by a parallel decrease in both exogenous and endogenous fatty acid oxidation rates. Based on these rates of oxidative metabolism, one can calculate the % contribution of these pathways to overall myocardial ATP production as described in **CHAPTER II**. The results of this calculation are depicted in Table 3-3b. In control hearts perfused with 1.2 mM palmitate, glucose oxidation provided 7.1% of the overall

ATP production whereas in hearts treated with DCA, the contribution of glucose oxidation rose to 30.1% of ATP production. In accordance with the 23% increase in the contribution of glucose oxidation to ATP production, the contribution of overall fatty acid oxidation to ATP production was reduced by 24%. This demonstrates that DCA treatment resulted in a significant shift in the substrate preference of the heart away from fatty acid oxidation towards glucose oxidation as a source of ATP production.

*iii) Myocardial labeled neutral lipid content and triacylglycerol content:*

The label content of [$^{14}$C]palmitate and [$^{3}$H]palmitate in hearts frozen at the end of the 60 min "chase" period are presented in Table 3-4. In both control and DCA-treated hearts, the amount of [$^{14}$C]palmitate remaining in the heart was similar. In contrast, the amount of [$^{3}$H]palmitate incorporated into neutral lipids during the "chase" period was significantly greater (almost 7 μmol·g dry wt$^{-1}$ more) in DCA-treated hearts as compared to controls. This combined with the lower fatty acid oxidation rates, suggests that fatty acids were shunted away from fatty acid oxidation and towards triacylglycerol synthesis.

*iv) Myocardial levels of CoA esters:*

Table 3-5 shows levels of malonyl-CoA and acetyl-CoA 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 indicating that levels of both the

substrate (acetyl-CoA) and product (malonyl-CoA) of ACC is increased in DCA-treated hearts.

The significant correlation between myocardial acetyl-CoA levels and malonyl-CoA levels (Figure 3-1) would suggest that acetyl CoA supply to ACC may be an important regulator of malonyl CoA production. The correlation between myocardial levels of malonyl CoA and overall fatty acid oxidation rates in the heart is depicted in Figure 3-2. A significant negative correlation was seen between malonyl-CoA levels and palmitate oxidation rates, providing support for the notion that malonyl CoA levels are an important determinant of myocardial fatty acid oxidation rates. If this were in fact true, perfusing hearts in the absence of added glucose would be expected to increase fatty acid oxidation and reduce malonyl CoA levels. In order to test this hypothesis, another group of hearts were perfused using the same perfusion protocol as shown in Table 3-1 except that glucose was absent from the perfusate during the chase perfusion. As predicted, absolute rates of exogenous [$^3$H]palmitate oxidation significantly increased in hearts perfused in the absence of glucose (Table 3-6). This was accompanied by a dramatic decrease in malonyl CoA levels. In summation, these data suggest that an increase in myocardial acetyl CoA levels from DCA stimulation of PDC increases malonyl CoA production, resulting in an inhibition of fatty acid oxidation.

*v) Characterization of Acetyl-CoA Carboxylase in Perfused Hearts*:

In order to explore the potential explanations for the DCA-induced alterations in cardiac malonyl-CoA levels, the activity, content and isoenzyme distribution of ACC were determined in crude cytosolic extracts from the perfused

heart tissue. ACC activity, measured in extracts of control and DCA-perfused hearts, in the presence of 10 mM citrate, showed no difference between these two groups (89.6 ± 15 vs 84.4 ± 14 nmol malonyl-CoA produced·g dry $wt^{-1}$·$min^{-1}$ in control and DCA-treated hearts, respectively. The content of the ACC isozymes in these extracts was also invariant between control and DCA-treated groups (Figure 3-3). As shown in this Western blot analysis of ACC extracted from 3 control and 3 DCA-treated hearts, both ACC-280 and ACC-265 isoenzymes are present in these extracts, as determined with isoform-specific antibodies (Figures 3-3A and 3-3B), although the former is the predominant ACC isoenzyme, as determined by probing the blots with streptavidin-peroxidase, which recognizes the biotin moiety of ACC and the other biotin-containing enzymes present (Figure 3-3C).

In liver and adipose tissue, ACC activity (predominantly ACC-265) can be markedly stimulated by the allosteric activator, citrate. Furthermore, this isoenzyme is also highly regulated by variable enzyme phosphorylation (Thampy and Wakil, 1985; Jamil and Masden, 1987a,b). Changes in citrate dependent activity are thought to be indicative of changes in ACC phosphorylation state; for example, highly phosphorylated ACC shows little activity in the absence of citrate and an increased citrate *Km*(Thampy and Wakil, 1985; Jamil and Masden, 1987a). However, as shown in Figure 3-4, cardiac ACC shows little dependence on citrate for activity. These data suggest that, unlike liver and adipose tissue ACC (ACC-265 predominant), heart ACC (ACC-280 predominant) may not be significantly regulated either by the allosteric activator, citrate, or by variable enzyme phosphorylation although further studies are warranted.

Another major distinguishing feature of these two ACC isoenzymes is their affinity for the substrate, acetyl-CoA. ACC was purified from white adipose tissue (which solely expresses ACC-265; see **CHAPTER III**) and from both heart

and skeletal muscle (predominantly ACC-280; **CHAPTER III**) by monomeric avidin-Sepharose chromatography and acetyl-CoA kinetics determined. This Lineweaver-Burke transformation plot (Figure 3-5) illustrates the marked difference in acetyl-CoA K*m* for these isolated isozymes. In this representative parallel isolation of ACC from each tissue, ACC-265 (adipose tissue) displays a K*m* of 67 μM, while ACC-280 (heart and skeletal muscle) has a K*m* of 117 and 109 μM, respectively. Such lower affinity of cardiac and skeletal muscle ACC for acetyl-CoA suggests that substrate regulation of ACC-280 activity could play an important role in these non-lipogenic organs.

*D) Summary*

Based on this series of experiments we can conclude that there is a measureable ACC activity in heart and that myocardial ACC exists as two distinct isoenzymes of 265,000 and 280,000 MW. Malonyl CoA levels where positively correlated with acetyl CoA levels and negatively correlated with rates of fatty acid oxidation thus demonstrating, for the first time, a direct link between ACC activity and fatty acid oxidation rates in the heart. Short term regulation of ACC in the heart involve the supply of acetyl CoA to the enzyme.

**Table 3-1.** 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:<br>11 mM glucose and<br>1.2 mM [$^{14}$C] palmitate | 10-min aerobic retrograde perfusion | 60-min perfusion with<br>11 mM glucose,<br>1.2 mM [$^{3}$H] palmitate)<br>with:<br>a) no addition<br>b) 1 mM dichloroacetate |
| | | **or** | |
| B) Glucose Oxidation | 60-min preperfusion with:<br>11 mM glucose and<br>1.2 mM palmitate | 10-min aerobic retrograde perfusion | 60-min perfusion with<br>11 mM [$^{14}$C]-glucose,<br>1.2 mM palmitate<br>with:<br>a) no addition<br>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 3-2.** Mechanical function of isolated working hearts perfused in the presence or absence of the pyruvate dehydrogenase complex stimulator, dichloroacetate.

| Condition | Heart rate (beat $min^{-1}$) | Peak systolic pressure (mm Hg) | HR x PSP $x10^{-3}$ (beats·mmHg·$min^{-1}$) | Heart work (mmHg·ml·$min^{-1}$ x $10^{-3}$) |
|---|---|---|---|---|
| **During Pulse Perfusion** | 247 ± 10 | [illegible] ± 1.0 | 22.28 ± 1.06 | 2.3 ±0.4 |
| **During Chase Perfusion** | | | | |
| Control | 217 ± 15 | 97.9 ± 4.2 | 21.4 ± 1.5 | 1.5 ± 0.5 |
| Dichloroacetate (1 mM) | 256 ± 12 | 96.6 ± 2.8 | 24.7 ± 1.3 | 2.4 ± 0.7 |

Data represent the mean ± standard error of the mean of six hearts in each group. Hearts were perfused as described in "**CHAPTER II**". Cardiac work was determined as the product of PSP x cardiac output.HR=heart rate, PSP= peak
systolic pressure.

**Table 3-3.** Oxidation rates of glucose and palmitate (A) and contribution to ATP production (B) in isolatedworking rat hearts perfused in the presence or absence of dichloroacetate.

| Condition | Glucose oxidation | Exogenous palmitate oxidation | Endogenous palmitate oxidation |
|---|---|---|---|
| **A) Steady state rates** | *(nmol·ml·mm Hg· g dry wt$^{-1}$)* | | |
| 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% |

*, significantly different from control

Data are the mean ± standard error of the mean of at least six hearts in each group. Hearts were perfused as described in Table 3-1, with oxidation rates measured during the "chase" perfusion. Contribution to ATP production was calculated as described in "**CHAPTER II**".

**Table 3-4** Distribution of $^{14}C$-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$^{-1}$) | $^{3}H$ palmitate as neutral lipids (μmol·g dry wt$^{-1}$) | Triacylglycerol content (μmol fatty acid·g dry wt$^{-1}$) |
|---|---|---|---|
| **-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 ± standard error of the mean of at least six hearts in each group. Hearts were perfused as described in "CHAPTER II".

*, significantly different from control.

**Table 3-5.** Levels of malonyl-CoA and acetyl-CoA in isolatedworking rat hearts perfused in the presence or absence of 1 mM dichloroacetate.

| Condition | Malonyl CoA | Acetyl CoA |
|---|---|---|
| | *(nmol·g dry wt$^{-1}$)* | |
| -Control | 18.0 ± 1.3 | 58.8 ± 2.9 |
| -Dichloroacetate (1 mM) | 25.2 ± 1.1* | 162.9 ± 10.7* |

*, significantly different from control hearts

Data represent the mean ± standard error of the mean of seven hearts in each group. Hearts were perfused as described in "**CHAPTER II**".

**Table 3-6.** Effect of removing glucose from the perfusate on malonyl CoA levels and overall fatty acid oxidation rates in isolated working rat hearts.

| Perfusate Condition | Malonyl CoA | Exogenous palmitate oxidation | Endogenous palmitate oxidation |
|---|---|---|---|
| | *(nmol·g dry wt$^{-1}$.)* | *(nmol·g dry wt$^{-1}$·min$^{-1}$)* | |
| 11 mM glucose 1.2 mM palmitate | 18.0 ± 1.3 | 401.3 ± 56.1 | 24.6 ± 6.9 |
| 1.2 mM palmitate | 2.0 ± 0.9* | 749.5 ± 179* | 69.3 ± 14.6 |

*, significantly different from hearts perfused with 11 mM glucose and 1.2 mM palmitate.

Data are the mean ± standard error of the mean of six hearts perfused with 11 mM glucose, 1.2 mM palmitate, and four hearts perfused with 1.2 mM palmitate alone. Hearts were perfused as described in Table 3-1, with overall fatty acid oxidation rates being measured during the "chase" perfusion as described in "**CHAPTER II**".

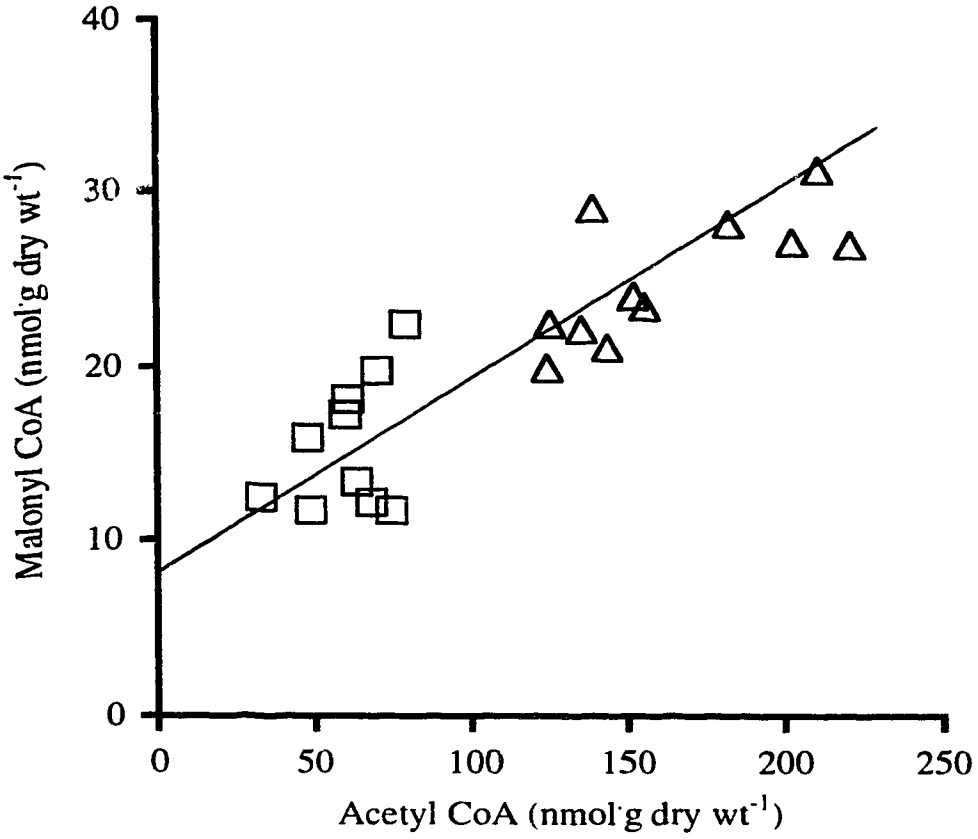



**Figure 3-1** Correlation between myocardial total acetyl-CoA and malonyl-CoA levels in isolated hearts perfused in the presence or absence of dichloroacetate.

Hearts were perfused as described in Table 3-1. CoA esters were extracted from hearts using 6% perchloric acid and seperated by HPLC as as described in the "**CHAPTER II**". Triangles, DCA-treated hearts. Squares , control hearts. (r value = 0.88)

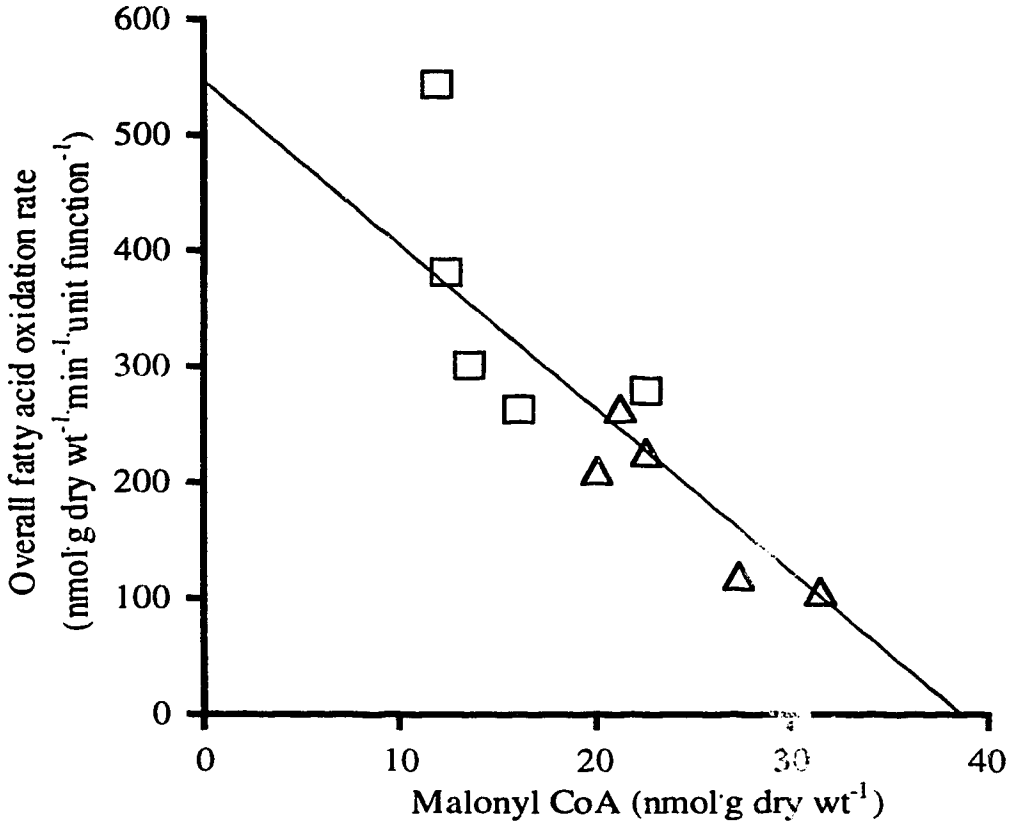
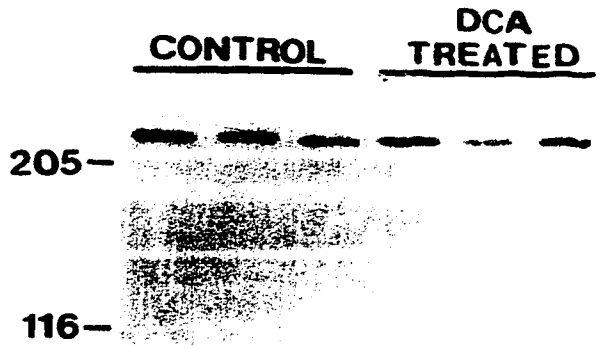



**Figure 3-2.** Correlation between malonyl-CoA levels and total palmitate oxidation rates.

Palmitate oxidation rates shown are the sum of exogenous and endogenous palmitate oxidation in isolated hearts perfused in the presence or absence of dichloroacetate. Hearts were perfused as described in Table 3-1. 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. (r value = -0.83)

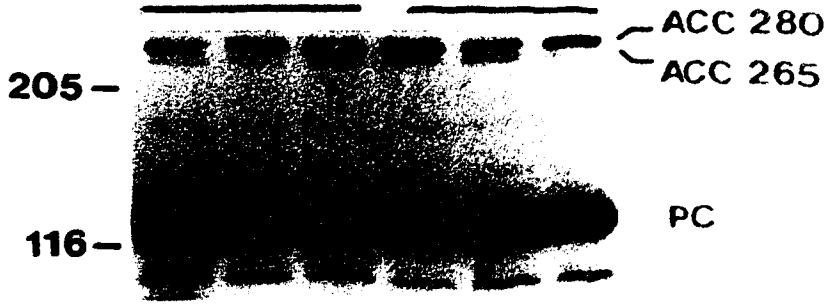
**Figure 3-3.** Immunoblot and Streptavidin-peroxidase analysis of acetyl CoA carboxylase in tissue extracts from isolated perfused hearts.

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-peroxidase (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 "**CHAPTER II**". SDS-PAGE was performed followed by transfer to nitrocellulose membrane. Immunoblotting was then performed as described in "**CHAPTER II**".




ACC 265 Ab


STREPTAVIDIN



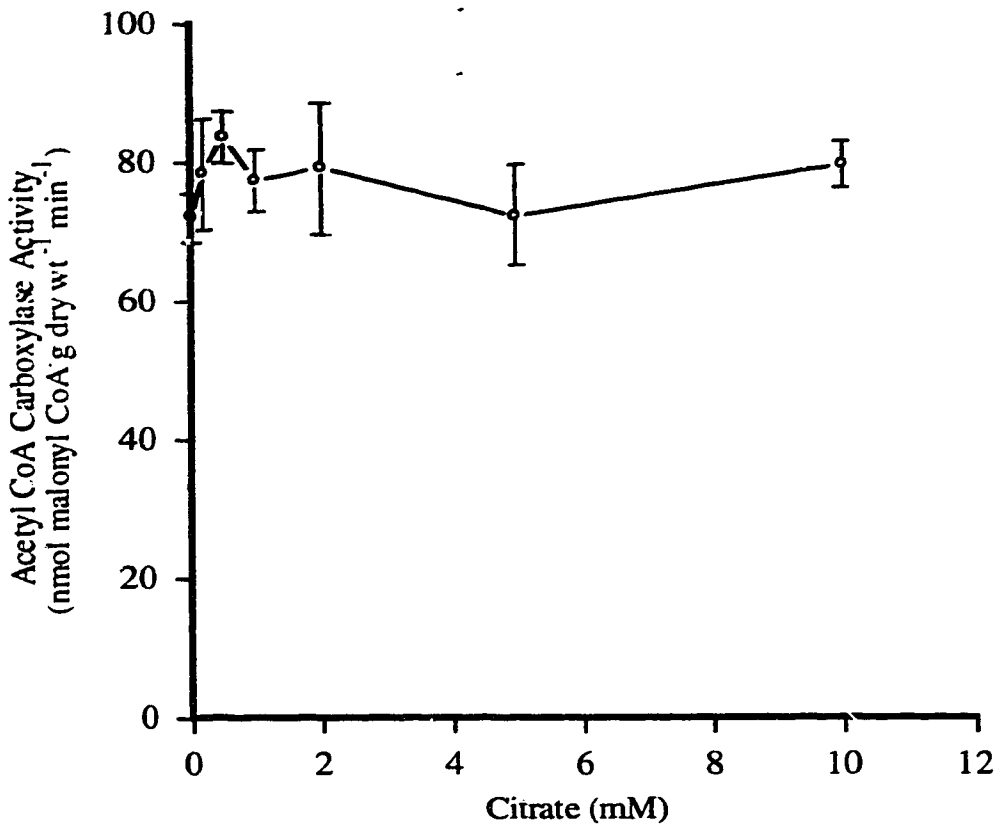



**Figure 3-4.** Citrate dependence of acetyl CoA carboxylase activity isolated from control hearts.

Data represent the mean ± standard error of the mean of six hearts. 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 tissue, as described in **"CHAPTER II"**.

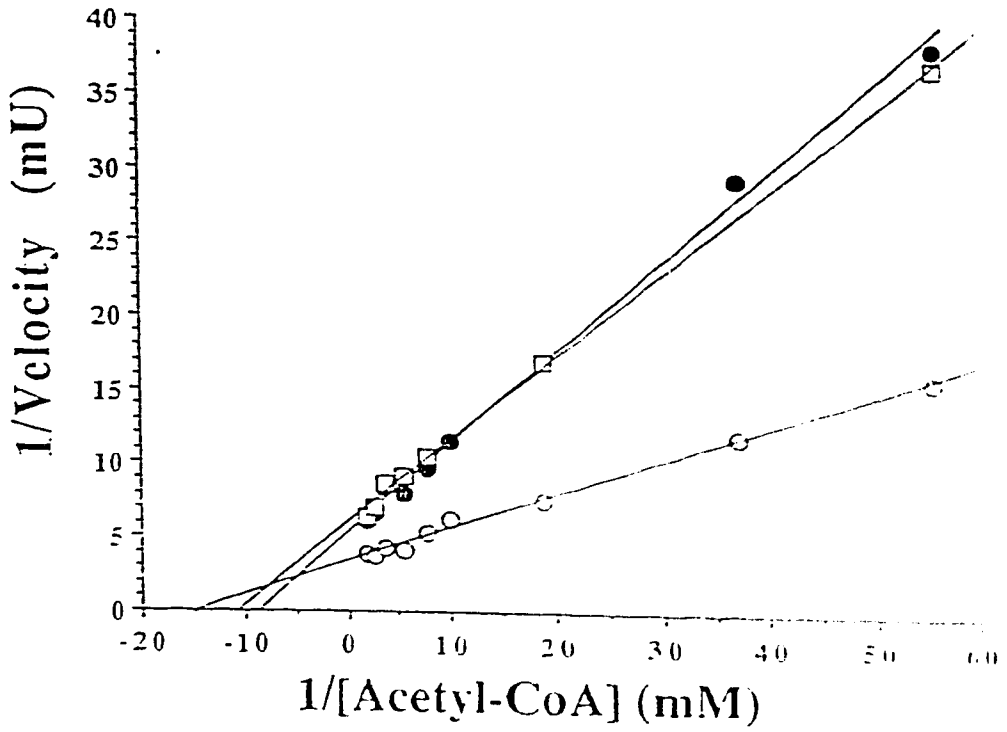
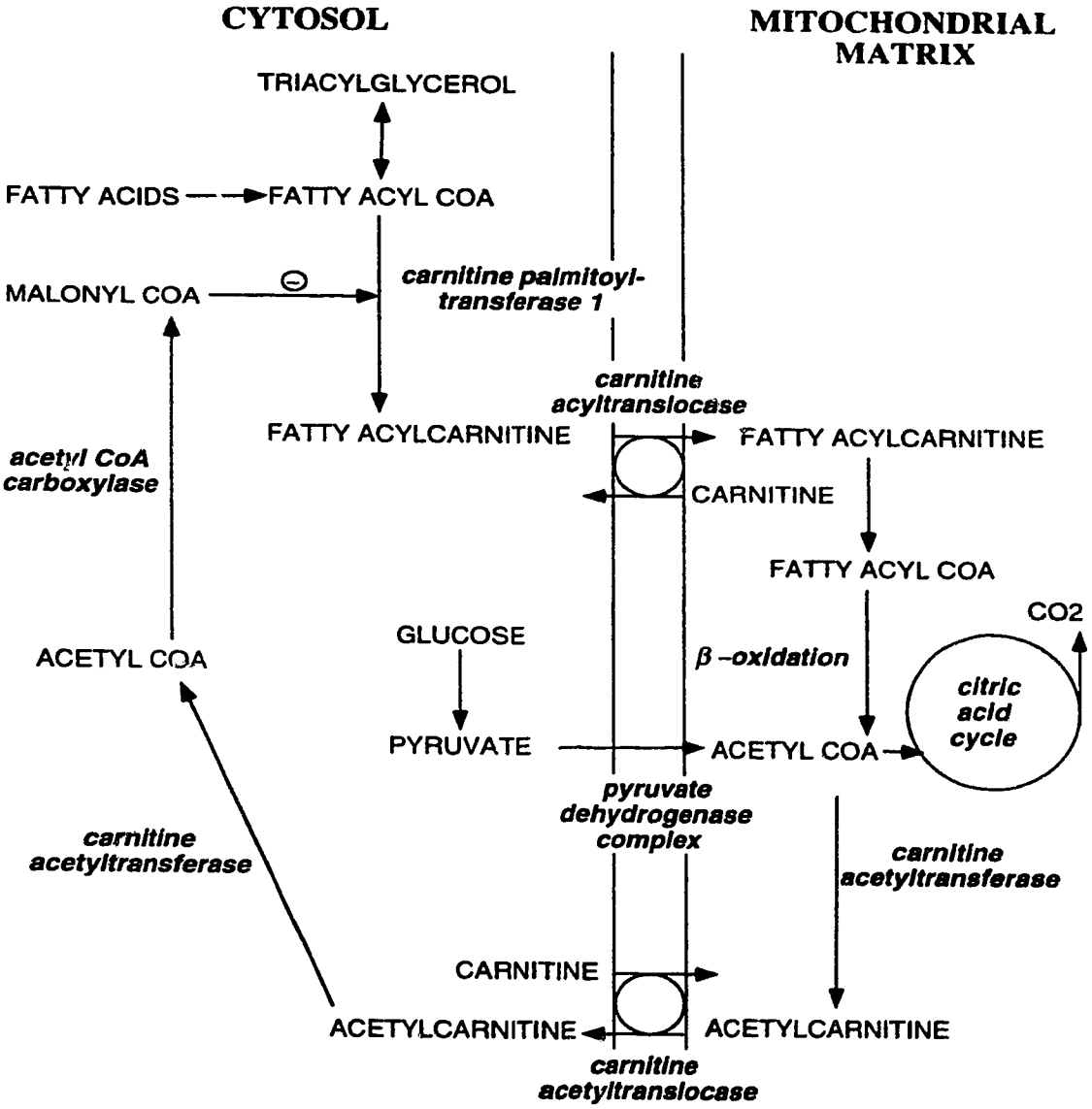



**Figure 3-5.** Acetyl-CoA kinetics of ACC-265 and ACC-280 isoenzymes.

ACC was isolated and purified in parallel from rat white adipose tissue (WAT, open circles), heart (closed circles) and skeletal muscle (SM, quadriceps and soleus, squares). The graph depicts the Lineweaver-Burke transormation of activity measured at variable acetyl-CoA concentrations (10-500 μM). These data obtained from a single set of parallel isolates are representative of three such simultaneous isolations.

**Figure 3-6** Proposed relationship between pyruvate dehydrogenase, acetyl-CoA carboxylase, and oxidative metabolism of fatty acids and glucose in the heart..

Increasing pyruvate dehydrogenase complex activity will increase the supply of acetyl-CoA for carnitine acetyltransferase and the short chain carnitine carrier system. As a result, cytosolic acetyl-CoA levels increase, resulting in an increase in acetyl CoA carboxylase activity. Increased malonyl-CoA production will then inhibit carnitine palmitoyltransferase 1 activity, resulting in a decrease in fatty acid oxidation.


CYTOSOL
MITOCHONDRIAL MATRIX
TRIACYLGLYCEROL
FATTY ACIDS
FATTY ACYL COA
MALONYL COA
carnitine palmitoyl-transferase 1
carnitine acyltranslocase
FATTY ACYLCARNITINE
FATTY ACYLCARNITINE
CARNITINE
FATTY ACYL COA
acetyl CoA carboxylase
GLUCOSE
β -oxidation
CO2
ACETYL COA
citric acid cycle
PYRUVATE
ACETYL COA
pyruvate dehydrogenase complex
carnitine acetyltransferase
carnitine acetyltransferase
CARNITINE
ACETYLCARNITINE
ACETYLCARNITINE
carnitine acetyltranslocase

# CHAPTER IV

## Differential tissue regulation of acetyl CoA carboxylase following acute streptozotocin-induced diabetes[7]

### *A) Introduction*

Diabetes is a prominent disease within North American society. Generally regarded as a disease involving abnormalities in glucose metabolism, diabetics also exhibit a number of disorders associated with alterations in lipid metabolism. This can include high circulating levels of very low density lipoproteins (VLDL) and triacylglycerols (Howard, 1987; Reaven,1982; Abrams *et al.*, 1982), as well as an increase in circulating free fatty acids (Reaven *et al.*, 1988). These abnormalities in lipid metabolism are in large part due to an overproduction of lipids by the key lipogenic tissues (i.e. liver and adipose tissue). The abnormalities in lipid metabolism also have important consequences on fatty acid utilization by muscle tissue. For instance, hearts from uncontrolled diabetics can become almost entirely reliant upon fatty acid oxidation as a source of ATP production, primarily at the expense of fatty acid oxidation (Garland *et al.*, 1962; Wall and Lopaschuk, 1989).

Acetyl coenzyme A carboxylase (ACC) catalyses the carboxylation of acetyl CoA to form malonyl CoA and is regarded as the rate-limiting enzyme in the *de novo* synthesis of fatty acids. In addition to being a substrate for fatty acid synthase, malonyl CoA is also a potent inhibitor of carnitine palmitoyltransferase

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[7] A version of this chapter has been submitted for publication. Gamble, J., Witters, L.A., and Lopaschuk, G.D. (1995) *FEBS Lett.*

1 (CPT 1) (McGarry *et al.*, 1978; Chung *et al.*, 1992; Cook, 1984), an enzyme which regulates the mitochondrial uptake of fatty acids in the form of acyl CoA esters. This fact has led to the hypothesis that acetyl CoA carboxylase may be a potentially important regulator of fatty acid oxidation. Accordingly, an isozyme of ACC has been found in tissues which have a low capacity for fatty acid synthesis (i.e., heart, brown adipose tissue and skeletal muscle), providing further support for this hypothesis (Bianchi, *et al.*, 1990). We (**CHAPTER III**) and others (Awan and Saggerson, 1993) have since provided direct evidence that ACC is an important regulator of myocardial fatty acid oxidation via the production of malonyl CoA.

As is the case with all carboxylases, the ACC protein has a covalently linked biotin group. This allows one to use a streptavidin probe with Western blots to determine the isozyme distribution and relative protein levels. For ACC activity measurements, an HPLC protocol was used for direct determination of malonyl CoA levels produced by ACC.

*B) Methods*

*i) model*

The acute streptozotocin-induced model of diabetes was used in this study as described in **CHAPTER II**.

*ii) specific protocol*

Forty-eight hours following injection of streptozotocin, animals were sacrificed and the tissues were extracted as outlined in **CHAPTER II**. The crude cytosolic fraction was prepared for analysis of the ACC content and activity. The wet transfer method was used for transferring protein samples onto nitrocellulose. ACC activity was determined by preparing a stock solution for incubation at 37° C and removing aliquots at 1 minute intervals over the 4 minute incubation period for later determination of malonyl CoA levels by HPLC. For citrate kinetics, incubations were performed for 4 minutes in the presence of seven different citrate concentrations varying from 0 to 10 mM and malonyl CoA levels determined using HPLC. Eadie-Hofstee plots were generated for determination of kinetic parameters. As is the case with most carboxylases, the ACC protein has a covalently linked biotin group thereby allowing one to use a streptavidin probe with Western blots to determine the isozyme distribution and relative protein levels. Northern blotting was also performed by isolating total RNA from heart tissue using the guanidine monothiocyanate method and fractionating the RNA on 1% agarose gels. Gels were stained with ethidium bromide and photographed under UV light to ensure equal loading of RNA in each sample (based on equal intensities of 18 and 28S RNA). RNA was transferred from the gels onto the solid nitrocellulose support over an 18 hour period using the capillary transfer method. Membranes were probed with a cDNA insert generated against liver ACC-280 that was $^{32}$P-labelled using the random primer method. Hybridization was carried out overnight at 42° C followed by washings with 2 x SSPE, 0.1% SDS initially at room temperature and again at 55° C. The membranes were then exposed to film for 48 hours @ -70° C.

## C) *Results*

### i) *Animal Characteristics*

In order to induce diabetes, animals were injected, via the tail vein, with streptozotocin (110 mg/kg), a procedure that we have previously found enduces a ketotic diabetic state after 48 hours (Saddik and Lopaschuk, 1994). Table 4-1 displays the influence of the diabetic state on the various animal characteristics. While body weight remained unchanged in the control animals over the 48 hour period, the diabetic animals lost approximately 23 g of body weight over this time. This loss of body weight in diabetic animals was accompanied by dramatic increases in both the plasma glucose (2.4 fold) and free fatty acids (2.5 fold). Urine analysis (Ketostix) at the time of sacrifice indicated that the diabetic animals were also severely ketotic and hyperglycaemic.

### ii) *Effect of acute diabetes on ACC protein expression*

It has been recently demonstrated that ACC exists as 2 distinct isoenzymes with varying tissue distribution (Bianchi *et al.*, 1990). Western blots were performed in this study to determine ACC enzyme distribution in the various tissues and the influence of diabetes on protein expression. Using streptavidin as a probe to specifically recognize biotin-containing enzymes, we are able to determine the expression of both ACC isozymes simultaneously in addition to the other carboxylases present in the fractions. As shown in Figure 4-1, heart and liver tissue express both the 280 kDa and 265 kDa isoenzymes. Densitometry scanning of these blots reveals that while the 280 kDa predominates in heart tissue,

both isoenzymes are present in relatively equal quantity in liver (Figure 4-3). These two tissues also differ in the expression of ACC following the onset of diabetes. While in the liver the expression of both isoenzymes are dramatically reduced in response to diabetes (21% and 38% for ACC-265 and ACC-280, respectively), there is no significant change in expression of either ACC isoenzyme in the diabetic heart.

In contrast to liver and heart, white adipose and skeletal muscle contain only a single ACC isoenzyme (Figure 4-2). White adipose tissue exclusively expresses ACC-265, which was completely absent in extracts from the diabetic animals. In contrast, skeletal muscle exclusively expresses the higher molecular weight isoenzyme (ACC-280), which was unaffected by the diabetic state (Figure 4-2). The streptavidin which was used as a probe for these blots allowed for the identification of a number of other carboxylases also present in our isolated fractions as a result of the high affinity of avidin for the biotin groups inherent to all carboxylases. As can be seen in Figures 4-1 and 4-2, both the pyruvate and propionyl CoA carboxylases are present in all four tissue types and, unlike ACC, appear to remain expressed at relatively constant levels under both control and diabetic conditions. These two carboxylases therefore served as a form of internal control assuring adequate transfer of proteins from the gels to the membranes.

### *iii) Effect of acute diabetes on acetyl CoA carboxylase enzyme activity*

In addition to ACC protein levels, enzyme activity was also investigated in the control and diabetic tissues by directly measuring the production of malonyl CoA, the product of the enzymatic reaction. Variable citrate concentrations were

also used in a separate series of assays for determination of affinity constants in each of the four tissues from the control and diabetic animals (Table 4-2). Despite the lack of change in heart ACC protein in diabetes there was a 42% decrease in ACC activity in hearts from diabetic animals. In liver, the loss of ACC protein seen in the Western blots was accompanied by a significant decrease in ACC activity, with less than 50% activity remaining in liver from diabetic rats relative to controls. A similar pattern was also seen in white adipose tissue isolated from diabetic animals where the disappearance of the ACC protein on the Western blots was paralleled by a dramatic reduction in enzyme activity in the diabetic state to only 13 % of the activity seen in control tissue. In contrast to these changes, skeletal muscle ACC isolated from control and diabetic rats exhibited virtually identical enzyme activity and was apparently resistant to the diabetic state in terms of both enzyme expression and functional activity.

*iv) Northern blot*

In order to determine whether the alterations in ACC following diabetes was the result of changes at the level transcription, as has been found previously in adipose tissue (Pape *et al.*, 1988), Northern blotting was performed on various tissues isolated from control and diabetic rats. Equal amounts of RNA were loaded in each lane as determined by ethidium bromide staining of the gels. As shown in Figure 4-4, an mRNA species of the expected size of approximately 10 kb was detected in the heart tissue from both control and diabetic animals but no apparent differences were found between the control and diabetic groups. Unfortunately, this analysis was restricted to only heart tissue as, for an unknown

reason, bands could never be detected when probed with a cDNA insert of ACC-265.

*D) Summary*

In this study it was demonstrated that the vast majority of changes in ACC induced by the diabetic state occurred in those tissues where the production of malonyl CoA is important in lipogenesis. Tissues which are primarily oxidative in nature revealed only minimal changes ACC expression following the transition to the diabetic state, although a significant decrease in ACC activity was observed in hearts from diabetic rats. The underlying explanation for this differential regulation of ACC among tissues and the relative insensitivity of ACC-280 to down-regulation in the diabetic state requires further investigation.

**Table 4-1.** Animal characteristics of control and acute streptozotocin-induced diabetic rats.

| Condition | Body weight (g) | | Plasma glucose (mM) | Plasma free fatty acids (mM) |
|---|---|---|---|---|
| | pre | post | | |
| control | 361 ± 9 | 366 ± 10 | 6.28 ± 0.3 | 0.43 ± 0.1 |
| diabetic | 345 ± 4 | 321 ± 3* | 14.78 ± 1.3* | 1.07 ± 0.2* |

* significantly different from corresponding control

Values are the mean ± standard error of the mean of six control and six diabetic animals.

**Table 4-2.** Enzyme activity and citrate kinetics in ACC enzyme isolates.

Data represent the mean +/- standard error of the mean for measurements taken with tissue from six control and six diabetic animals. ACC activity was measured in cytosolic fractions isolated from frozen tissue as described in "**CHAPTER II**". Malonyl CoA levels were measured using HPLC and activity is expressed as the amount of malonyl CoA produced·minute$^{-1}$·mg protein$^{-1}$. Citrate kinetics were determined as described in "**CHAPTER II**" using variable citrate concentrations ranging from 0-10 mM. Values were determined using an Eadie-Hofstee transformation from which the $K_m$ was determined.

| Condition | ACC activity (pmol·min$^{-1}$·mg protein$^{-1}$) | $K_m$ Citrate ( mM) |
|---|---|---|
| *Heart* | | |
| control | 162.8 ± 2.1 | 6.98 |
| diabetic | 94.9 ± 7.0* | 5.38 |
| *Liver* | | |
| control | 70.7 ± 5.8 | nd |
| diabetic | 31.0 ± 5.8* | nd |
| *White adipose* | | |
| control | 363.2 ± 133.2 | 1.85 |
| diabetic | 48.2 ± 15.0* | nd |
| *Skeletal muscle* | | |
| control | 108.6 ± 16.8 | 4.37 |
| diabetic | 109.8 ± 28.2 | 3.36 |

* significantly different from corresponding control
nd; not detectable

**Figure 4-1.** Western blot analyses of the carboxylase distribution in tissues from control and diabetic animals expressing both ACC isoenzymes.

Cytosolic fractions were isolated from the various tissues and subjected to SDS-PAGE (3%-5%) as described in "**CHAPTER II**". Following transfer to nitrocelluluose, the blots were probed with peroxidase-labeled streptavidin in order to visualize the biotin-containing carboxylases.

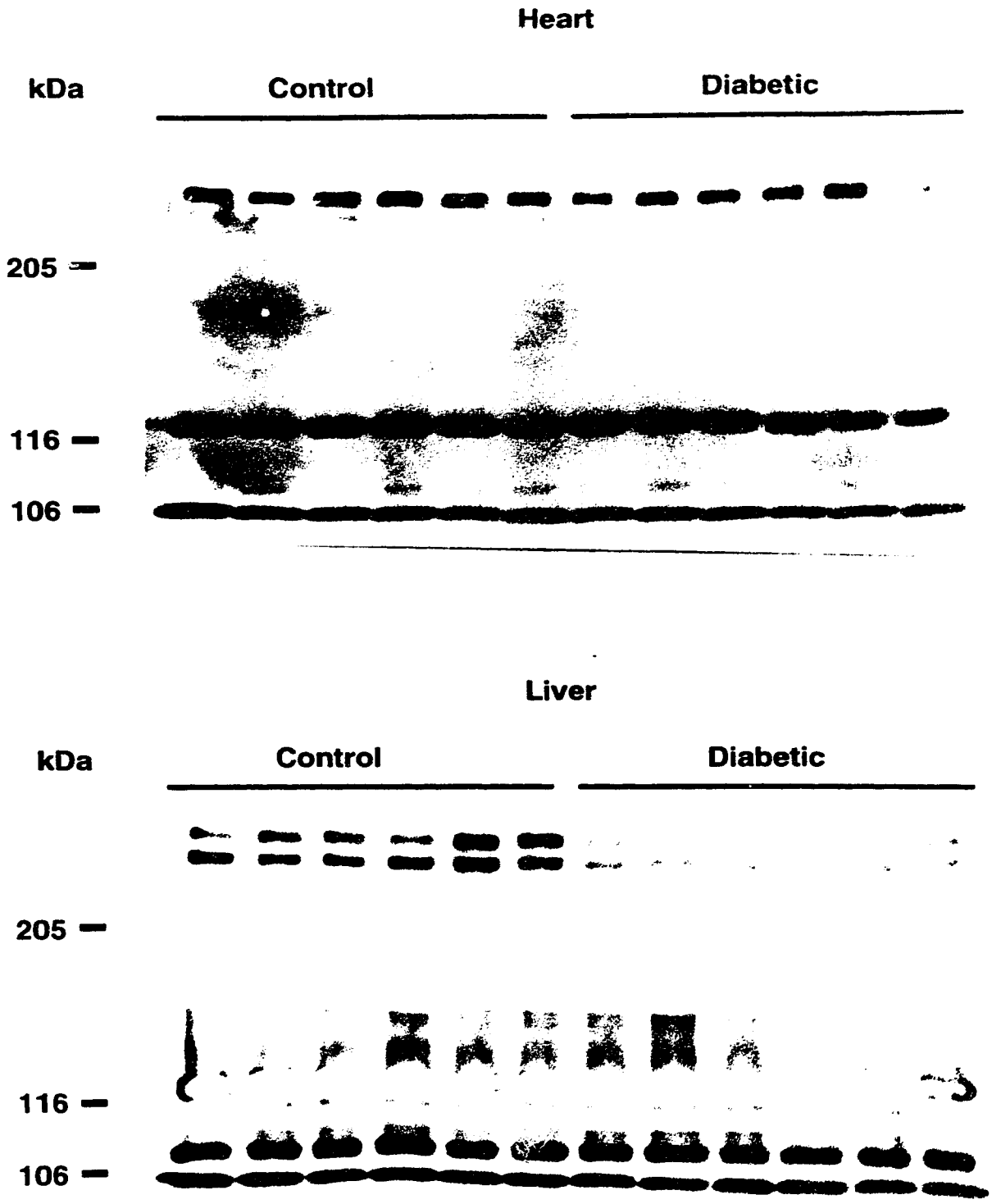
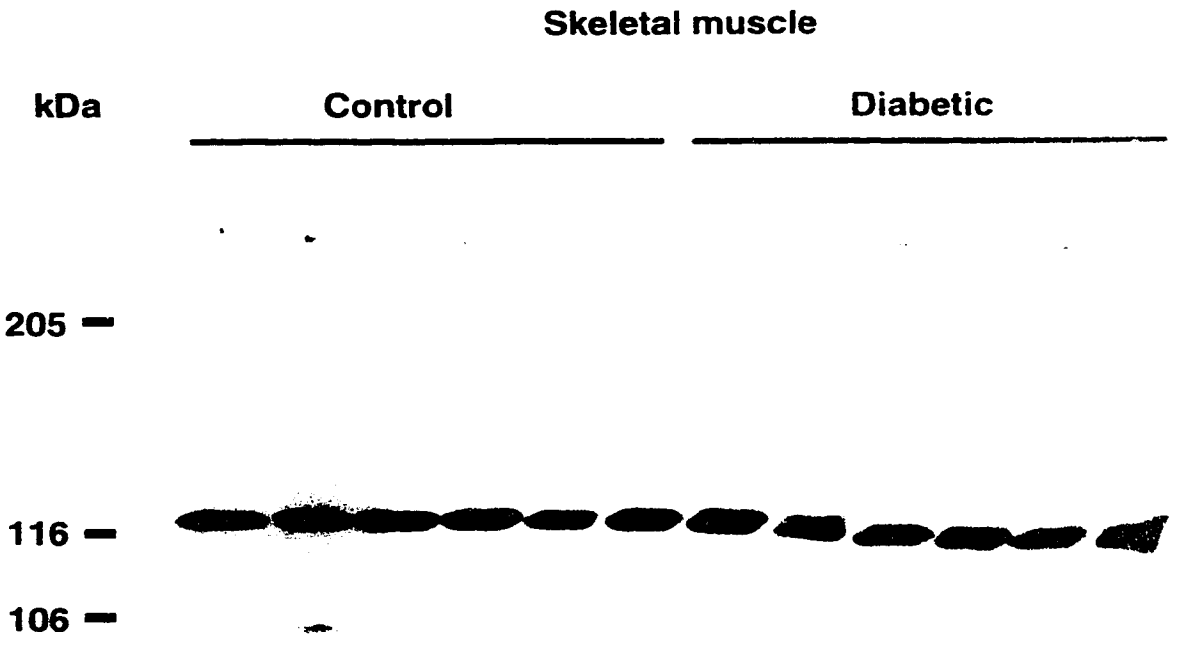

Heart
kDa
Control
Diabetic
205
116
106
Liver
kDa
Control
Diabetic
205
116
106

**Figure 4-2.** Western blot analyses of the carboxylase distribution in tissues from control and diabetic animals expressing a single ACC isoenzyme.

Cytosolic fractions were isolated from the various tissues and subjected to SDS-PAGE (3%-5%) as described in "**CHAPTER II**". Following transfer to nitrocelluluose, the blots were probed with peroxidase-labeled streptavidin in order to visualize the biotin-containing carboxylases.


Skeletal muscle
kDa
Control
Diabetic
205
116
106

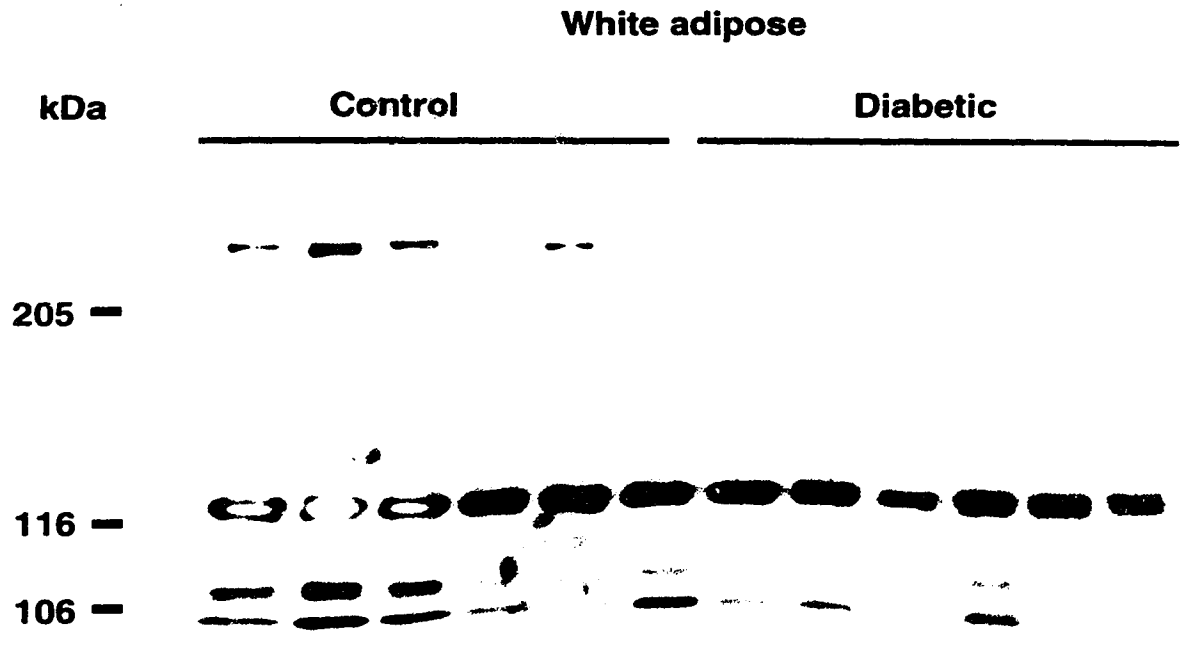
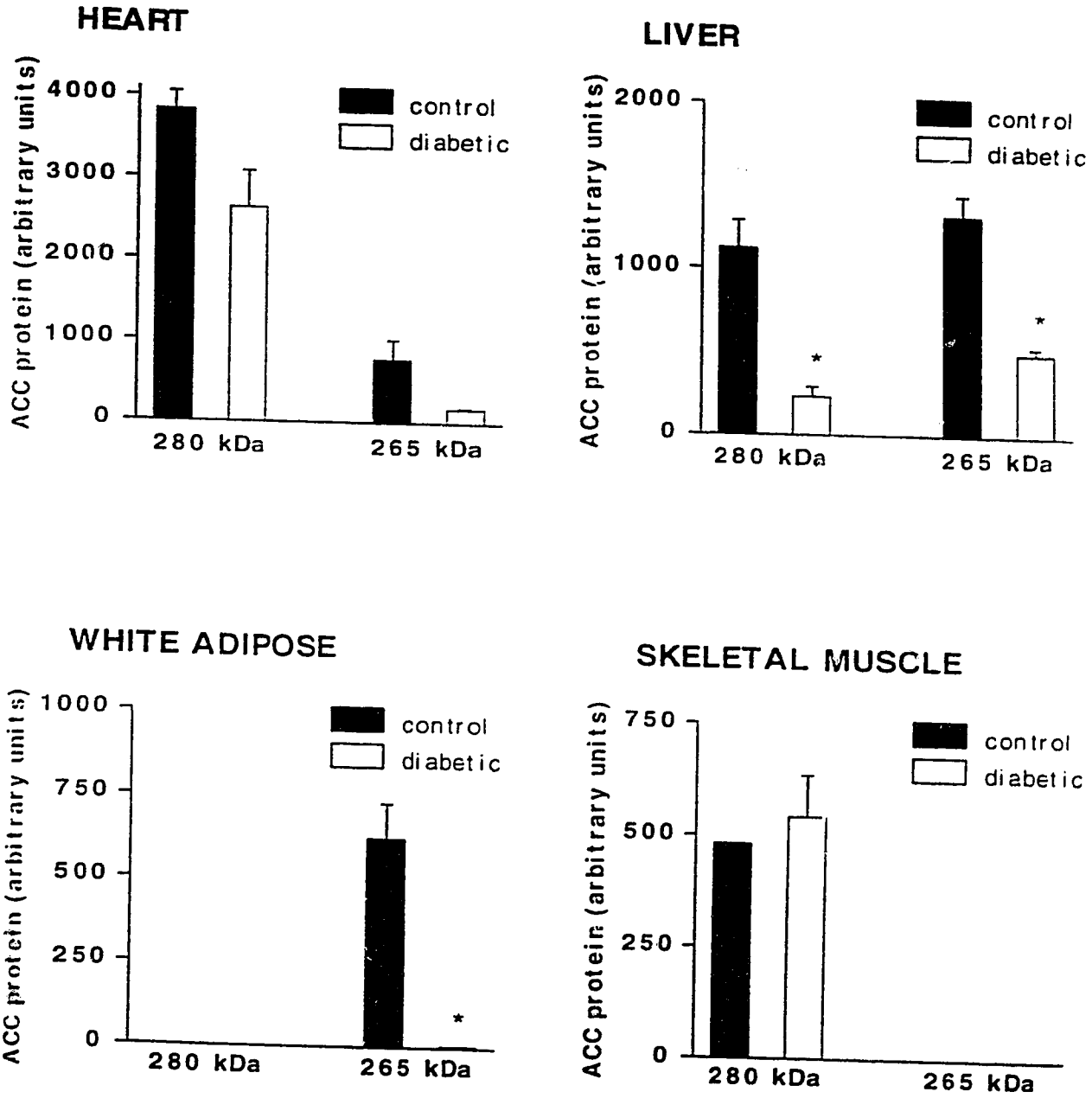

White adipose
kDa
Control
Diabetic
205
116
106

**Figure 4-3.** Relative ACC protein levels and isoenzyme distribution in tissues isolated from control and diabetic animals.

Following Western blot analyses described in Figures 4-1 and 4-2, the lanes on the x-ray film were subjected to scanning densitometry in order to determine the relative changes in ACC protein levels following the transition to the diabetic state. Each value was calculated from the results of scanning 4 lanes of each tissue type in both the control and diabetic state and expressed as a relative absorbance with respect to the control state.


HEART
ACC protein (arbitrary units)
4000
3000
2000
1000
0
control
diabetic
280 kDa
265 kDa
LIVER
ACC protein (arbitrary units)
2000
1000
0
control
diabetic
*
*
280 kDa
265 kDa
WHITE ADIPOSE
ACC protein (arbitrary units)
1000
750
500
250
0
control
diabetic
*
280 kDa
265 kDa
SKELETAL MUSCLE
ACC protein (arbitrary units)
750
500
250
0
control
diabetic
280 kDa
265 kDa

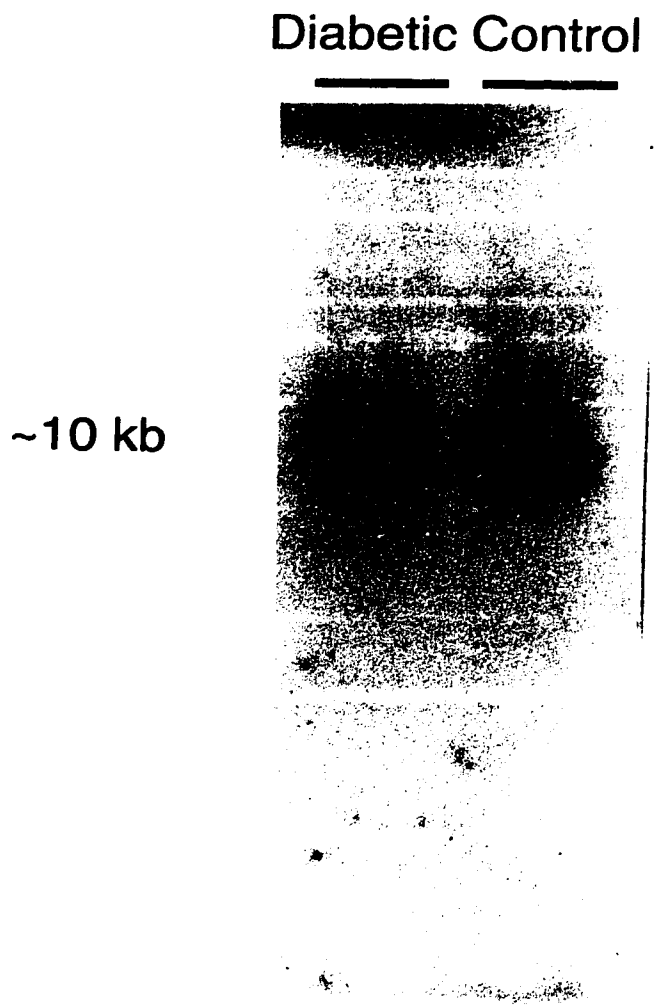



**Figure 4-4.** Northern blot of heart ACC mRNA from control and diabetic rats.

Total RNA was prepared from frozen ventricular tissue of control and streptozotocin-induced diabetic rats (20 µg load) as described in **CHAPTER II**. A $^{32}$P-labeled cDNA probe for ACC-280 was used for the detection of the mRNA of interest. Exposure time of the autoradiograph was 48 hours.

# CHAPTER V

## Regulation of myocardial metabolism in insulin resistance: the involvement of acetyl CoA carboxylase and 5' AMP-activated protein kinase[8]

### *A) Introduction*

Under normal conditions, approximately 70% of the total energy production comes from the oxidation of fatty acids while the remaining 30% is derived from glucose (Lopaschuk and Saddik, 1992). However, in diabetes, myocardial glucose use is markedly impaired and fatty acids can account for as much as 99% of the total myocardial energy production (Randle, 1986).

Acetyl CoA carboxylase (ACC) plays an important role in regulating both hepatic fatty acid biosynthesis (Kim *et al.*, 1989) and myocardial fatty acid oxidation (**CHAPTER III**; Awan and Saggerson, 1993) via its production of malonyl CoA. In the liver, malonyl CoA serves as a donor of carbon units to fatty acid synthase for elongation of the acyl chain, whereas in heart, malonyl CoA is a very potent inhibitor of carnitine palmitoyltransferase 1, an enzyme important in the uptake of fatty acids into the mitochondria (Chung *et al.*, 1992; Cook 1984; Murthy and Pande, 1987) where they are subsequently oxidized. Previous work has shown that in the heart, ACC plays an important role in a feedback loop that maintains a balance between glucose and fatty acid oxidation (**CHAPTER III**).

ACC is subject to both long and short term regulation. Long term regulation involves changes in enzyme levels (Bianchi *et al.*, 1990; Witters, 1986), whereas an important short term regulatory mechanism for ACC activity is

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[8] A version of this chapter has been submitted for publication. Gamble, J., Russell, J.C., and Lopaschuk, G.D. (1995) *Biochim. Biophys Acta.*

covalent phosphorylation (Pape *et al.*, 1988). Recently, a unique protein kinase which is activated by AMP was recognized to have the ability to regulate fatty acid and sterol biosynthesis via phosphorylation of both ACC and HMG-CoA reductase, respectively (Sato *et al.*, 1993; Hardie, 1992; Davies *et al.*, 1990). This phosphorylation results in a profound inactivation of both enzymes. The 5'-AMP-activated protein kinase (AMPK) has been extensively characterized in both mammalian and non-mammalian cells and tissues where it shows a strong homology with the SNF1 protein kinase of *S. cerevisiae* (Woods *et al.*, 1994; Carling *et al.*, 1994; Mitchellhill *et al.*, 1994). AMPK has been termed a metabolite sensing protein kinase as it is activated under a number of situations of cell stress, including glucose depletion, ATP depletion or heat shock (Witters, 1991). In hepatocytes, AMPK has also been shown to be inhibited by insulin (Witters and Kemp, 1992), suggesting that under circumstances of insulinopenia or insulin resistance AMPK may be activated thereby potentially altering cellular substrate metabolism.

The development of cardiomyopathies is extremely prevalent among the diabetic population (Kannel and McGee, 1979; Garcia *et al.*, 1974; Gwilt *et al.*, 1985). Alterations which are intrinsic to the diabetic myocardium itself also contribute to the increased incidence and severity of myocardial infarctions and post-infarct complications seen in the diabetic. For instance, the increased reliance of hearts from diabetic animals on fatty acids as an energy substrate may be an important factor contributing to the development of cardiomyopathies and the severity of ischemic injury in the diabetic (Liedtke *et al.*, 1984;1988; Lopaschuk *et al.*, 1988). The majority of experimental studies involving abnormalities in heart metabolism in diabetes have focussed on animal models which are insulin deficient (ie, chemically induced diabetes and the spontaneously diabetic "BB"

Wistar rats) (Dhalla *et al.*, 1985; Garland and Randle, 1964; Lopaschuk and Tsang, 1987; Heyliger *et al.*, 1986). These models being representative of the insulin-dependent type I diabetes. Relatively few studies on diabetic metabolic abnormalities, however, have been conducted on animals in an insulin resistant state, which is more closely related to type II, or non insulin-dependent diabetes.

The JCR:LA corpulent rat provides a unique rat model for the study of insulin resistance (ie. type II diabetes). These animals if homozygous for the corpulent gene (*cp*) exhibit a syndrome characterized by obesity, hypertriglyceridemia, and hyperinsulinemia with impaired glucose tolerance (Russell *et al.*, 1990; Dolphin *et al.*, 1990). Homozygous (*cp/cp*) males of the JCR:LA strain spontaneously develop atherosclerotic and ischemic myocardial lesions whereas females and heterozygotes do not. The dramatic hyperinsulinemia (20 fold increase versus controls), insulin resistance and hyperlipidemia in these animals have profound effects on cellular energy metabolism (Lopaschuk and Russell, 1991).

In this study myocardial substrate metabolism and the activities of two key enzymes involved in the regulation both carbodydrate and fatty acid metabolism, namely ACC and AMPK, were studied in the insulin resistant JCR:LA corpulent rat model.

*B) Methods*

*i) model*

The animal model used in this study was the JCR:LA male rat. Lean control animals were JCR:LA rats heterozygous for the corpulent *cp* gene. Insulin-resistant animals were JCR:LA rats homozygous for the corpulent *cp* gene. The JCR:LA strain was developed as described in **CHAPTER II.**

*ii) specific protocol*

Hearts were extracted from halothane-anesthetized animals and perfused as working hearts for 40 minutes under aerobic conditions. The perfusion solution consisted of the standard Krebs-Henseleit solution containing 11 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 1.75 mM $Ca^{2+}$ and 2000 μU/ml insulin. Glucose and palmitate were trace-labeled with [U-$^{14}$C] glucose and [9,10-$^{3}$H] palmitate, respectively. Steady-state oxidative rates were calculated by quantitative collection of $^{14}CO_2$ and $^{3}H_2O$ as described in **CHAPTER II.** Buffer samples were taken at 10 minute intervals over the course of the perfusion as were recordings of mechanical function.

## *C) Results*

### *i) Animal characteristics*

Depicted in Table 5-1 are the body weights, heart weights, heart weight to body weight ratios, serum glucose levels and serum fatty acid levels in the lean control and corpulent JCR:LA insulin-resistant rats. Body weights were significantly greater in animals homozygous for the corpulent gene versus lean controls. In addition, corpulent animals exhibited greater heart weights than did lean animals. This relative increase in body weight however, exceeded that of the increase in heart mass, thereby resulting in a significantly higher heart weight to body weight ratio in the lean control animals as compared to their insulin-resistant counterparts. At the time of sacrifice, blood samples were taken from the carotid artery for determination of serum gluose and fatty acid levels in the control and insulin-resistant animals. In accordance with previous studies (Lopaschuk and Russell, 1991; Dolphin *et al.*, 1988; 1987) no significant difference was found between glucose levels of control or insulin-resistant rats. While we did not measure insulin levels in the present study, it has previously been shown that serum insulin levels in insulin-resistant rats can range fron 400-1000 μU/ml, which is markedly elevated compared to the lean control animals which exhibit levels of 15-20 μU/ml.

### *ii) Heart function in insulin-resistant rats*

Mechanical function was monitored continuously over the 40 minute perfusion protocol in spontaneously beating hearts from both the control and

insulin-resistant JCR:LA rats. In order to maintain function throughout the protocol in these hearts, it was necessary to include 2000 μU/ml insulin and 1.75 mM $Ca^{2+}$ in the perfusate as determined previously (Lopaschuk and Russell, 1991). All hearts were subject to a constant preload of 11.5 mm Hg and an afterload of 80 mm Hg. As shown in Table 5-2, heart rate was significantly lower in the insulin-resistant rats versus controls whereas no differences were evident in peak systolic pressure or developed pressure. This reduction in heart rate however, was sufficient to cause a significant reduction in the rate pressure product (HR x PSP) in the insulin-resistant hearts. Both coronary and aortic flows were also continuously monitored which allowed for the determination of cardiac work, the product of cardiac output and peak systolic pressure. As shown in Table 5-2, there was a 34% decrease in cardiac work in the insulin-resistant rats versus their lean controls. While the direct cause of the depressed heart function in the hearts from insulin resistant animals is not readily apparent, one cannot discount a potential alteration in functioning of the coronary endothelium. An impaired coronary flow may contribute to the development of the myocardial lesions in this model which are believed to be ischemic in origin.

### *iii) Glucose and palmitate oxidation in JCR:LA rat hearts*

Glucose and palmitate oxidation were determined in the spontaneously beating isolated working rat hearts via quantitative collection of $^{14}CO_2$ and $^{3}H_2O$, respectively. As shown in figure 5-1, the generation of the $^{14}CO_2$ and $^{3}H_2O$ was linear between 10 min and 40 min of the perfusion protocol. During this period, steady-state oxidative rates were determined. Steady-state rates of glucose and palmitate oxidation are shown in Table 5-3. In order to account for determined

differences in heart weight and mechanical function, both rates are presented as values normalized for these parameters. Glucose oxidation rates, normalized per gram dry weight of tissue, were significantly reduced in the insulin-resistant rat hearts versus those of lean controls. Despite the impaired mechanical function in the insulin resistant hearts, glucose oxidation rates remained significantly lower than rates in control hearts when normalized for cardiac work. Palmitate oxidation rates are also presented in Table 5-3. In contrast to glucose oxidation, no significant differences were found in absolute rates of palmitate oxidation between the control and insulin-resistant hearts when values were normalized for dry tissue weight. While fatty acid oxidation rates were normalized for cardiac work in the insulin-resistant hearts these differences in rates did not reach statistical significance.

Using the steady-state rates for glucose and palmitate oxidation shown in Table 5-3, steady-state rates of ATP production from these exogenous substrates were calculated and are presented in Figure 5-2. In the hearts from lean control animals, there was approximately an equal contribution of both glucose and fatty acid oxidation to overall ATP production. On the other hand, in hearts from insulin-resistant animals the contribution of glucose oxidation to ATP production fell markedly to only 30% while the contribution of fatty acid oxidation to ATP production now rose to 70%.

*iv) Acetyl CoA carboxylase (ACC) in hearts from insulin-resistant rats*

We (**CHAPTER II**) and others (Awan and Saggerson, 1993) have shown that ACC is an important regulator of fatty acid and glucose metabolism in the heart. In control hearts, ACC is present as two distinct isoenzymes of differing molecular weight which can be distinguished by their migration during SDS-PAGE. A Western blot using extracts from the control and insulin resistant JCR:LA rat hearts is presented in Figure 5-3. These blots were probed with streptavidin (which recognizes biotin moieties in enzymes) in order to identify both ACC isoenzymes simultaneously in addition to the other carboxylases which may be present in the extracts. Similar to hearts of the Sprague-Dawley strain (CHAPTER III), hearts from both the control and insulin resistant JCR:LA animals contain the 280 kDa and the 265 kDa ACC isoenzymes, with the 280 kDa isoenzyme predominating. In the control and insulin-resistant hearts, the content of which appears to be invariant suggesting that insulin-resistance does not alter the pattern of cardiac ACC expression.

*v) ACC and AMPK activity in hearts from control and insulin-resistant rats*

In order to explore whether the alterations in substrate metabolism between control and insulin-resistant hearts may be due to changes in ACC activity as opposed to changes in expression, PEG fractions were prepared from the frozen tissue and ACC activity was measured using the "$CO_2$ fixation" technique (Bianchi *et al.*, 1990; Thampy, 1989). Because AMPK can phosphorylate and inhibit ACC

activity in the liver, the present study also set out to determine the if myocardial ACC is also under phosphorylation control. In addition, citrate is thought to activate ACC either through polymerization of the enzyme or through stabilization of the dimer configuration necessary for ACC activity (Kim *et al.*, 1989). Indeed, it has been proposed that the phosphorylated form of ACC is more dependent upon citrate for its activity (Jamil and Masden, 1987a,b). The results of the ACC assays are shown in Figure 5-4. For all hearts, the presence of 10 mM citrate resulted in enhanced ACC activity. In perfused hearts from lean control animals ACC activity was approximately 2.5 fold higher than activity measured in hearts from the insulin-resistant animals. The removal of NaF and NaPPi from the isolation procedure (which allows for dephosphorylation of ACC) also resulted in an activation of ACC and this was more pronounced in the hearts from insulin resistant animals suggesting that the enzyme may be in a more phosphorylated state.

AMPK activity was also measured in the extracts as AMPK is a potent regulator of ACC activity via its ability to phosphorylate and inhibit ACC. This assay involves the incorporation of $^{32}P$ into a synthetic peptide (SAMS) which corresponds to the amino acid sequence on ACC which is phosphorylated by AMPK (Davies *et al.*, 1989). As presented in Table 5-4, in hearts from insulin-resistant JCR:LA animals the AMPK activity was markedly elevated as compared to activity seen in control hearts. These results are consistent with the studies primarily conducted in liver and adipose tissue (Hardie, 1992; Davies *et al.* 1989) and suggest that like these tissues, AMPK is also an important regulator of ACC activity in the heart.

Levels of CoA esters were also determined in both control and insulin-resistant hearts frozen at the end of perfusion. Malonyl CoA levels were found to

be significantly reduced in the insulin-resistant hearts (6.33 ± 0.2 versus 5.42 ± 0.1 nmol·g dry $wt^{-1}$ in control and insulin-resistant hearts, respectively, $p < 0.05$) as were levels of acetyl CoA (16.16 ± 2.0 versus 9.20 ± 0.6 nmol·g dry $wt^{-1}$ in control and insulin resistant hearts, respectively, $p < 0.05$). Unfortunately, one cannot determine the subcellular distribution of the various CoA esters therefore it cannot be concluded that these values represent levels which are accessible to the cytosolic enzymes. Compartmentalization of malonyl CoA must exist within the heart given that these levels are sufficient to cause a complete inhibition of CPT I activity and thereby fatty acid oxidation. Taken together, these results indicate that both the substrate (acetyl CoA) and the product (malonyl CoA) of ACC are significantly lower in the hearts from insulin-resistant animals which exhibited the higher proportion of ATP coming from fatty acid oxidation (Figure 5-2).

### *D) Summary*

This model of insulin resistance provides a unique tool to examine the role of ACC and AMPK in regulating fatty acid oxidation in a pathological state characterized by an altered metabolic status. Despite a greater reliance of insulin-resistant hearts on fatty acid oxidation as a source of ATP, no alterations in ACC protein levels were detected. However, ACC activity was decreased suggesting that the decrease was due to allosteric regulation or covalent phosphorylation. Insulin resistant animals exhibited an increase in AMPK activity, which accompanied the concomitant decrease in ACC activity. This study represents one of the first demonstrations of AMPK activity in heart and its correlation with myocardial ACC activity. The results obtained in this series of experiments

provide further support for the contention that like the liver, AMPK, acting via ACC, is an important mechanism by which the heart can regulate fatty acid and carbohydrate metabolism.

**Table 5-1.** Animal characteristics of the control and insulin resistant JCR:LA rats

| | *Lean control* | *Insulin resistant* |
|---|---|---|
| Heart wt (g) | 1.11 ± 0.05 | 1.34 ± 0.04* |
| Body wt (g) | 310 ± 4 | 465 ± 9* |
| Heart wt:body wt ratio (x1000) | 3.6 ± 2 | 2.9 ± 1* |
| Plasma free fatty acids (mM) | 0.34 ± 0.02 | 0.40 ± 0.02* |
| Plasma glucose (mM) | 13.4 ± 0.7 | 12.7 ± 1.0 |

*, significantly different from lean control.

Values are the mean ± standard error of the mean of 8 lean control and 5 insulin resistant animals per group. Serum samples were obtained from the animals at the time of sacrifice.

**Table 5-2.** Mechanical function in isolated working spontaneously beating control and insulin-resistant JCR:LA rat hearts

| | *Lean control* | *Insulin resistant* |
|---|---|---|
| Heart Rate (beats·min$^{-1}$) | 299 ± 4 | 248 ± 18* |
| Peak Systolic Pressure (mm Hg) | 107 ± 5 | 105 ± 4 |
| HR x PSP (beats·min$^{-1}$·mmHg$^{-3}$) | 31 ± 1 | 26 ± 2* |
| Cardiac Output (ml·min$^{-1}$) | 32 ± 6 | 21 ± 3* |
| Cardiac Work (ml·mm Hg$^{-1}$·min$^{-1}$· 10$^{-1}$) | 38 ± 8 | 25± 3* |

*, significantly different from lean control.

Values are the mean ± standard error of the mean of 8 lean control and 5 insulin resistant hearts in each group. Hearts were perfused as described in "**CHAPTER II**".

**Table 5-3.** Steady-state oxidative rates in hearts from control and insulin-resistant JCR:LA rats.

| | *Lean control* | *Insulin resistant* |
|---|---|---|
| **Glucose Oxidation** | | |
| *Absolute rates* ($nmol \cdot g\ dry^{-1} \cdot min^{-1}$) | 1453 ± 252 | 574 ± 146* |
| *Rates normalized for cardiac work* ($nmol \cdot mmHg \cdot ml^{-1}$) | 10.0 ± 1.1 | 7.5 ± 1.9* |
| **Palmitate Oxidation** | | |
| *Absolute rates* ($nmol \cdot g\ dry^{-1} \cdot min^{-1}$) | 379 ± 35 | 313 ± 80 |
| *Rates normalized for cardiac work* ($nmol \cdot mmHg \cdot ml^{-1}$) | 3.2 ±0.7 | 4.9 ± 0.5 |

*, significantly different from lean control hearts.

Values are the mean ± standard error of the mean of 8 lean control and 5 insulin resistant hearts in each group. Steady-state oxidative rates were determined by quantitative collection of $^{14}CO_2$ and $^{3}H_2O$ for glucose and palmitate respectively as outlined in "**CHAPTER II**". Rates are expressed normalized for either dry heart weight or for mechanical work performed by the hearts.

**Table 5-4.** AMPK activity in control and insulin resistant rat hearts

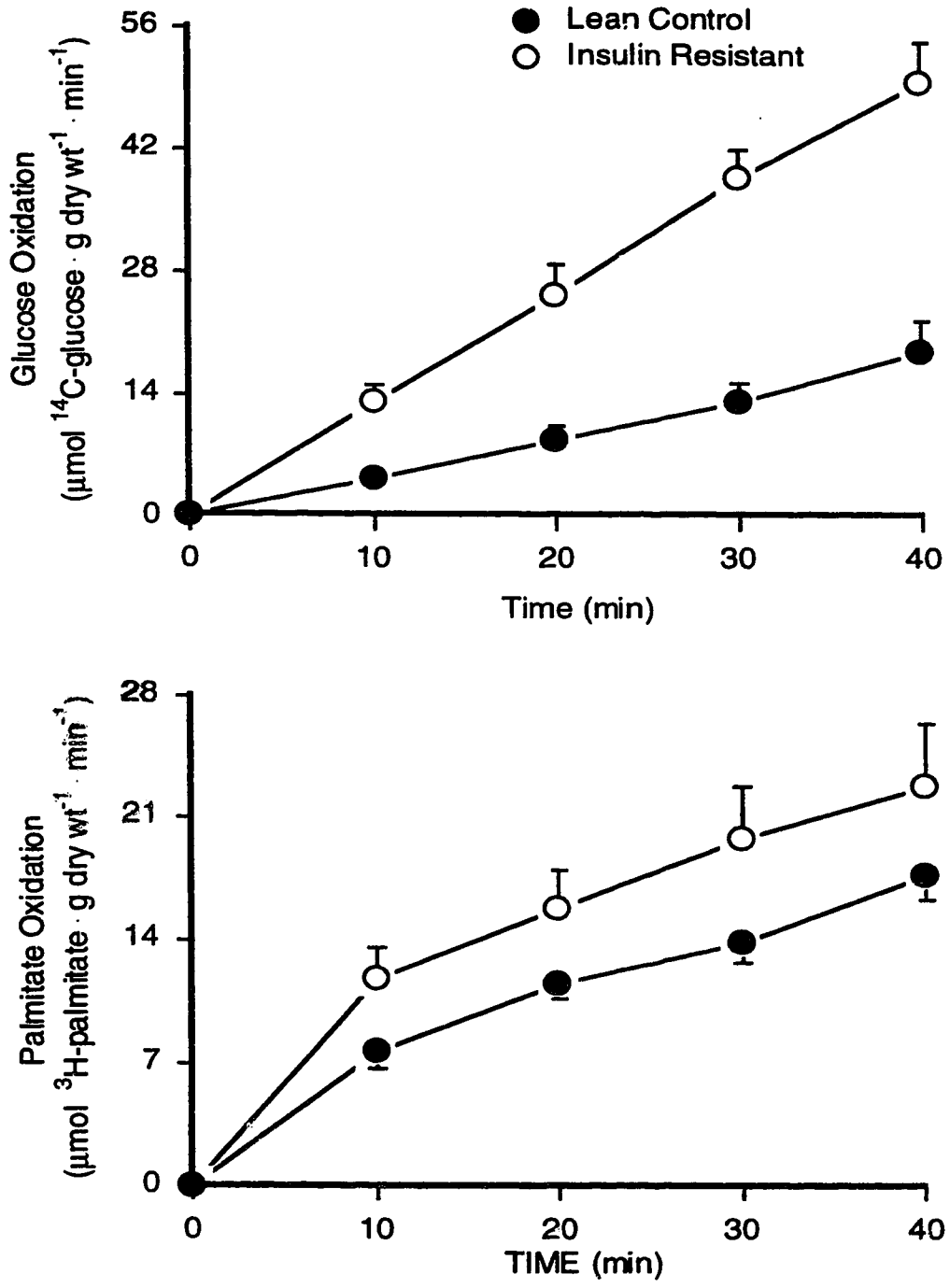
| | AMPK activity ($pmol \cdot min^{-1} \cdot mg\ protein^{-1}$) |
|---|---|
| *Lean control* | 272 ± 35 |
| *Insulin resistant* | 499 ± 52* |

*, significantly different from lean control.

Values are the mean ± standard error of the mean of 6 lean control and 5 insulin resistant hearts in each group. Fractions containing AMPK were prepared from frozen heart tissue of control and insulin-resistant JCR:LA rats using a 6% polyethylene glycol procedure outlined in "**CHAPTER II**". AMPK activty was measured using the incorporation of $^{32}P$ into the synthetic SAMS peptide as outlined in "**CHAPTER II**".

**Figure 5-1.** Cumulative glucose oxidation (*upper panel*), and palmitate oxidation (*lower panel*) in hearts from control and insulin resistant JCR:LA rats.

Values are the mean ± standard error of the mean of 8 lean control and 5 insulin resistant rat hearts. Cumulative glucose oxidation and palmitate oxidation rates were determined as described in "**CHAPTER II**".


Lean Control
Insulin Resistant
Glucose Oxidation
(µmol $^{14}$C-glucose · g dry wt$^{-1}$ · min$^{-1}$)
56
42
28
14
0
0
10
20
30
40
Time (min)
Palmitate Oxidation
(µmol $^{3}$H-palmitate · g dry wt$^{-1}$ · min$^{-1}$)
28
21
14
7
0
0
10
20
30
40
TIME (min)

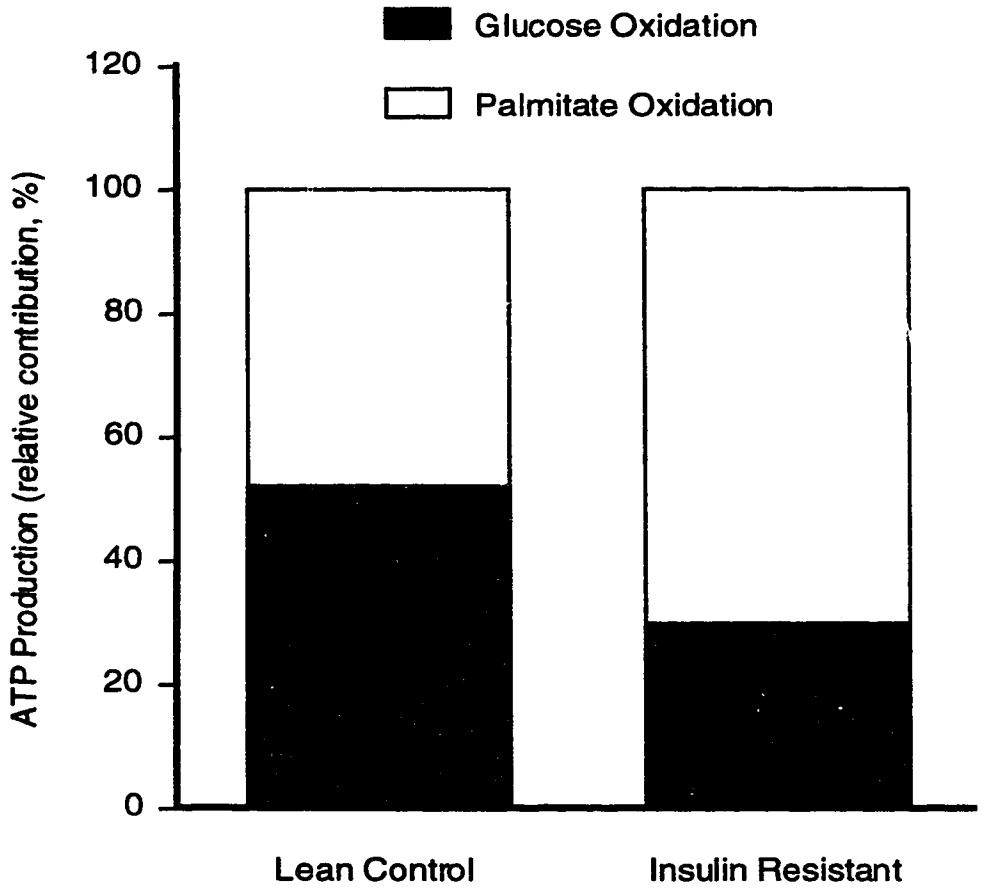



**Figure 5-2.** The relative contribution of glucose and palmitate oxidation to overall ATP production in control and insulin-resistant hearts.

Relative contribution of glucose oxidation and palmitate oxidation were calculated from the steady state oxidative rates shown in Table 5-3, using a value of 38 moles of ATP produced per mole of glucose oxidized and 129 moles of ATP produced per mole of palmitate oxidized as described in "**CHAPTER II**".

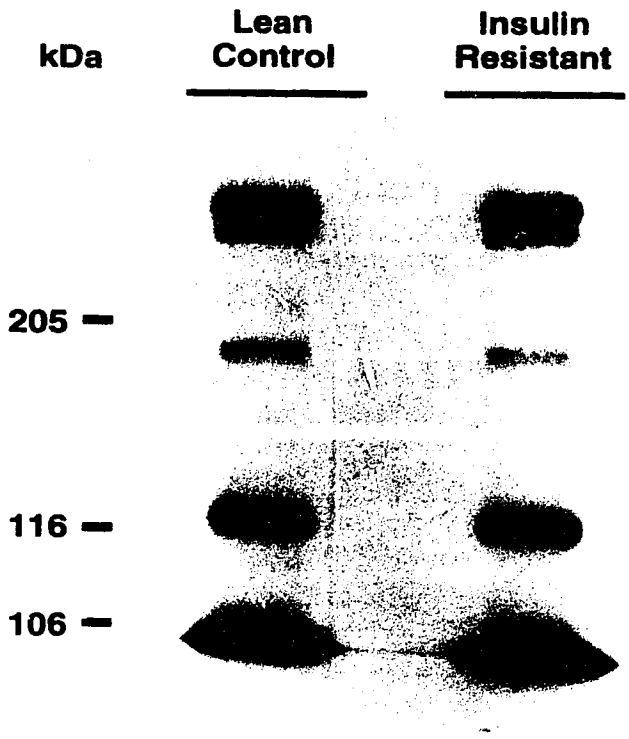



**Figure 5-3.** Western blot of ACC isoenzymes in heart tissue from JCR:LA rats.

Fractions containing ACC were isolated from both control and insulin-resistant rat heart tissue using a polyethylene glycol extraction procedure as outlined in "**CHAPTER II**". Protein samples were then fractionated using SDS-PAGE and transferred to nitrocellulose followed by incubation with streptavidin and chemiluminescent visualization onto x-ray film.

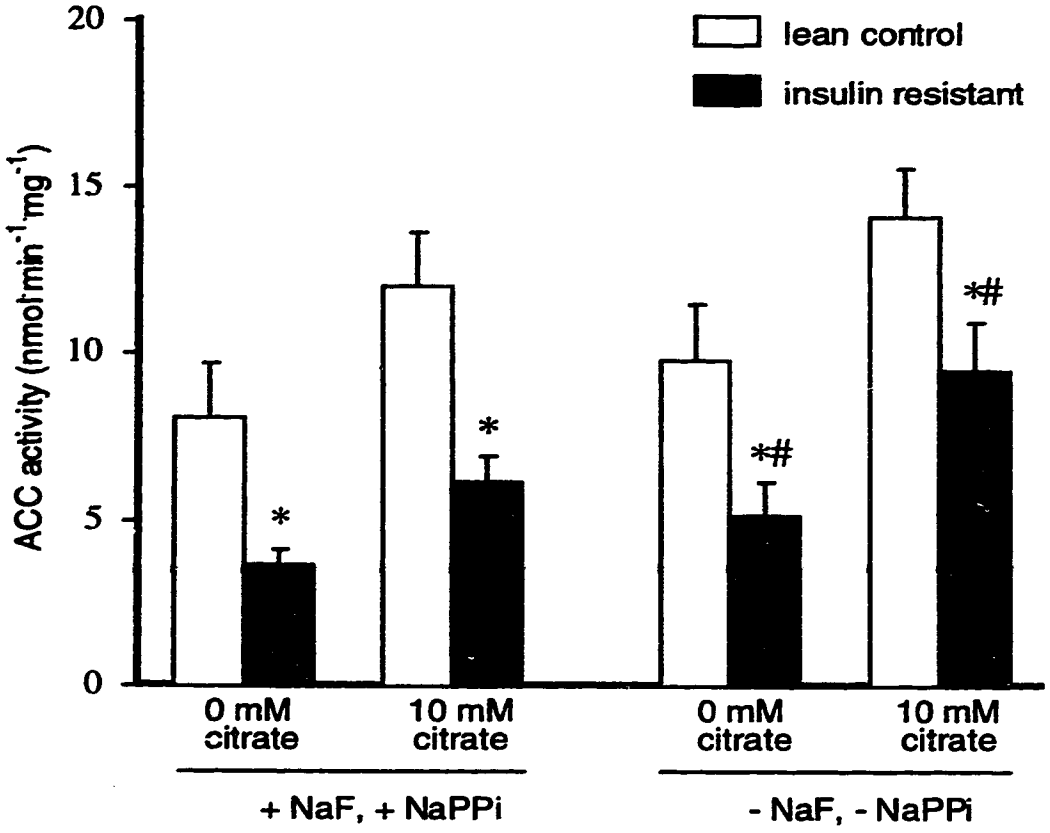



**Figure 5-4.** ACC activity in PEG extracts isolated from control and insulin-resistant rat hearts.

Values are the mean ± standard error of the mean of 7 control and 5 insulin resistant hearts in each group. ACC activity was measured in PEG extracts from frozen tissue as described in "**CHAPTER II**". In order to dephosphorylate ACC, heart tissue was homogenized in the presence of NaF and NaPPi and resuspended in their presence or absence as indicated on the abscissa. ACC activity was measured at 0 and 10 mM citrate in all of the conditions.

*, significantly different from lean control

#, significantly different from corresponding activity measured in the presence of NaF, NaPPi

# CHAPTER VI

## Hormonal regulation of glucose and fatty acid metabolism in the heart[9]

### *A) Introduction*

The extensive amount of contractile work performed by the heart results in an enormous demand for energy production. This demand is primarily met by the oxidation of carbohydrates and fatty acids. Under aerobic conditions, fatty acid oxidation accounts for the majority (~60%) of ATP produced in the heart. The remainder is derived, to a large extent, by carbohydrate oxidation primarily in the form of glucose (Neely and Morgan, 1974; Newsholme *et al.*, 1962; Saddik and Lopaschuk, 1991). These proportions are variable and are dependent on the concentration of glucose and fatty acids delivered to the myocyte (Saddik and Lopaschuk, 1991).

Alterations in cytosolic malonyl CoA levels is an important regulator of myocardial fatty acid oxidation by virtue of its ability to inhibit cardiac carnitine palmitoyltransferase 1 (CPT1) at very low concentrations ($IC_{50}$ ~ 30 nM)(Cook, 1984; Kudo *et al.*, 1995; McGarry *et al.*, 1978). Inhibition of CPT1 prevents fatty acids from gaining access to the mitochondrial matrix thus preventing their passage through the β-oxidation spiral. Malonyl CoA is the product of acetyl CoA carboxylase (ACC), the activity of which plays an important role in the feedback loop between carbohydrate and fatty acid metabolism (**CHAPTER III**; Awan and Saggerson, 1993; Kudo *et al.*, 1995).

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[9] A version of this chapter has been submitted for publication. Gamble, J., and Lopaschuk, G.D. (1995) *J. Biol. Chem.*

ACC exhibits differential tissue expression of at least two distinct isoenzymes (Bianchi *et al.*, 1991). Heart tissue expresses predominantly the 280 kDa isoenzyme, with the 265 kDa isoenzyme expressed to a lesser extent (Thampy, 1989; **CHAPTER III**). This is in contrast to the liver, which expresses the 265 kDa isoenzyme to a much greater extent than the higher molecular weight ACC isoenzyme (Bianchi *et al.*, 1991). Based on this distribution, it has been hypothesized that the 265 kDa isoenzyme may be important regulator of fatty acid biosynthesis in liver and adipose tissue whereas the 280 kDa isoenzyme may govern the capacity for fatty acid oxidation in tissues such as heart and skeletal muscle.

A number of studies have addressed the response of ACC in liver and adipose tissue to a variety of hormonal states (Holland *et al.*, 1984; Sim and Hardie, 1988; Swenson and Porter, 1985; Witters, 1981; Bianchi *et al.*, 1992). This hormonal regulation of ACC activity in these lipogenic tissues is thought to be manifest through a cycle of phosphorylation-dephosphorylation. The degree of phosphorylation is thought to be reflected by the dependence of enzyme activity on the allosteric activator citrate (Jamil and Masden 1987a;1987b).

While the hormonal control of ACC in liver and adipose tissue has been studied extensively in relation to fatty acid biosynthesis less is known regarding the hormonal control of tissue such as skeletal muscle and heart which express the 280 kDa isoenzyme of ACC (Saha *et al.*, 1995; Duan and Winder, 1993). In the liver, insulin has been shown to activate ACC (Mabrouk *et al.*, 1990) through a dephosphorylation mechanism involving the inhibition of 5' AMP-activated protein kinase (AMPK) (Witters and Kemp, 1992). The counterregulatory hormone glucagon, on the other hand, inhibits ACC activity (Kim, 1983; Kim *et al.*, 1989; McGarry and Foster, 1980) by activation of cAMP-dependent protein

kinase which phosphorylates the 265 kDa isoenzyme of ACC at Se -79 (Davies *et al.*, 1990). While it appears that a similar mechanism may be operant in skeletal muscle (Duan and Winder, 1993; Saha *et al.*, 1995), the question as to whether or not these regulatory mechanisms apply to hormonal control of oxidative metabolism in the heart remains obscure. Recently, Awan and Saggerson (1993) have demonstrated that incubation of cardiac myocytes with insulin could acutely increase levels of malonyl CoA and reduce fatty acid oxidation. In turn, epinephrine caused a decrease in malonyl CoA levels associated with an increase in fatty acid oxidation. It should be pointed out however, that their measurements of malonyl CoA levels were derived from perfused hearts whereas their fatty acid oxidation values were determined in isolated cardiac myocytes. As mentioned previously, the interpretation of fatty acid oxidation rates must be done with caution given the limitation of the low work demand imposed in the isolated cardiac myocyte model.

In the present study, we have directly examined the effects of both insulin and glucagon on glucose and palmitate oxidation in the isolated working rat heart model. In addition, we have also attempted to gain inference as to a potential mechanism underlying these effects by determining the impact of these hormones on enzyme activity and phosphorylation status of myocardial ACC and AMPK.

## *B) Methods*

### *i) model*

The animal model used was the male Sprague-Dawley rat weighing between 250-300 grams. Animals were maintained as outlined in **CHAPTER II.**

*ii) specific protocol*

Hearts were extracted from pentobarbital-anaesthetized rats and perfused as working hearts for 40 minutes under aerobic conditions. The perfusion solution consisted of a standard Krebs-Henseleit solution containing 11 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 2.5 mM $Ca^{2+}$ and the presence of either 100 μU/ml insulin (control and glucagon groups) or 1000 μU/ml insulin (insulin group). Glucagon-treated hearts received glucagon (0.05 μM) 5 minutes into the perfusion protocol and a recording of mechanical function was taken prior to the addition. Glucose and palmitate were trace-labeled with [U-$^{14}$C] glucose and [9,10-$^{3}$H] palmitate, respectively. Steady-state oxidative rates were calculated by quantitative collection of $^{14}CO_2$ and $^{3}H_2O$ as described in **CHAPTER II**. Buffer samples were taken at 10 minute intervals over the course of the perfusion as were recordings of mechanical function.

*C) Results*

*i) Effects of insulin and glucagon on heart function.*

Heart function was monitored continuously over the course of the perfusions in the isolated working rat hearts. Spontaneously beating hearts were subjected to a constant left atrial preload of 11.5 mm Hg and an aortic afterload of 80 mm Hg. Table 6-1 shows the influence of hormonal stimulation with either insulin or glucagon on the various parameters of mechanical function. Insulin added at a concentration of 1000 μU/ml had no effect on any of the indicies of heart function. The addition of 0.05 μM glucagon resulted in a significant increase

in both heart rate and rate-pressure product. In all groups, heart function remained stable over the course of the 40 minute perfusion protocol.

*ii) Effects of insulin and glucagon on glucose and palmitate oxidation in isolated working rat hearts.*

Steady-state rates of both glucose and palmitate oxidation were determined in the isolated working hearts perfused in the presence of insulin or glucagon. Cumulative glucose and palmitate oxidation rates were linear between 10 and 40 minutes of the perfusions under all conditions studies (Figures 6-1 and 6-2). Based on the accumulation of $^{14}CO_2$ and $^{3}H_2O$, steady-state rates of oxidation can be derived for glucose and palmitate, respectively. Figure 6-1 also shows the steady-state rates of glucose oxidation under the three conditions studied. While increasing insulin had no effect on glucose oxidation rates, the addition of glucagon resulted in a 59% increase in glucose oxidation rates relative to control. This is similar to the effect that we have found previously using the positive inotrope epinephrine (Collins-Nakai *et al.*, 1994). In contrast to the increase in glucose oxidation rates, glucagon did not change steady-state palmitate oxidation rates as compared to controls when hearts were administered with glucagon . Increasing insulin however, did result in a significant decrease in palmitate oxidation rates relative to control and glucagon perfused hearts when normalized for dry weight of the heart (Figure 6-2).

*iii) Effects of insulin and glucagon on myocardial acetyl CoA carboxylase activity*

In order to characterize the hormonal influences on heart ACC in this study, ACC activity was measured in PEG extracts from hearts perfused in the presence of insulin and/or glucagon. To obtain information with regards to the phosphorylation state of ACC under these conditions, extracts were prepared in the presence and absence of both NaF and NaPPi and ACC activity measured (Table 6-2). In agreement with studies on ACC from liver and adipose tissue, insulin resulted in a 1.9 fold activation of myocardial ACC activity in PEG extracts isolated under conditions which preserve the phosphorylated state of ACC whereas glucagon reduced ACC activity to 50% of control levels (Table 6-2). When PEG fractions were extracted in the absence of NaF, NaPPi, an increase in ACC activity was seen in all hearts. This is thought to be due to a dephosphorylation and activation of ACC during isolation. The largest percent increase in ACC activity was seen in the glucagon-perfused hearts (82.1%) which is suggestive of a greater phosphorylated state. In conjunction with this, the smallest increase in ACC activity from enzyme isolated in the absence of the phosphatase inhibitors was in hearts perfused with insulin. This effect of an enhanced increase in ACC when PEG extracts from glucagon-treated hearts are prepared in the absence of NaF and NaPPi was maintained regardless of whether ACC activity was measured in the presence or absence of 10 mM citrate (data not shown). These results are consistent with results found in hepatocytes where insulin is thought to activate ACC through dephosphorylation via an inhibition of a 5'-AMP activated protein kinase (Witters and Kemp, 1992) and glucagon is thought to phosphorylate and

inactivate ACC via phosphorylation by the 5'AMP-activated protein kinase (Hardie, 1989, Sim and Hardie, 1988).

*iv) Acetyl CoA carboxylase isoenzyme distribution in the heart*

Acetyl CoA carboxylase is known to exist as at least 2 isoenzymes with differing tissue distribution (Bianchi *et al.*, 1990). As we (**CHAPTER III**) and others (Thampy, 1989) have shown previously, heart tissue expresses both the high and low molecular weight isoenzymes of ACC. In order to determine whether any of the differences in enzyme activity could be attributed to changes in the protein expression in response to the exposure of the hearts to insulin and glucagon, Western blotting was performed on the PEG extracts as shown in Figure 6-5.

As expected, when the blots are probed with streptavidin, which recognizes the biotin moieties in various carboxylases, a number of bands are recognized in our fractions. The two lower bands represent pyruvate carboxylase and proprionyl CoA carboxylase (approximately 120 and 107 kDa, respectively). The upper two bands correspond to the 265 and 280 kDa isoenzymes of ACC. Although the 280 kDa isoenzyme appears to be the predominant isoenzyme expressed in heart tissue, there is no apparent change in the relative amounts of either the 280 or 265 kDa isoenzymes in response to perfusion in the presence of insulin and glucagon.

*v) Co A ester levels in isolated working hearts perfused with insulin and glucagon*

The product of the ACC catalyzed reaction is malonyl CoA. To determine whether changes in ACC activity would be reflected in changes in overall tissue levels of malonyl CoA levels, CoA esters were isolated from frozen ventricular tissue and separated and quantified using HPLC. Perfusing hearts in the presence of 1000 μU/ml insulin or 0.05 μM glucagon resulted in no significant differences in tissue levels of malonyl CoA (42.1 ± 8 nmol·g dry $wt^{-1}$ in controls versus 37.9 ± 7 and 45.7 ±9 nmol·g dry $wt^{-1}$ in insulin and glucagon groups, respectively). It should be pointed out however, that compartmentalization of malonyl CoA probably exists (**CHAPTER II**; McGarry, 1995) and the predictive value of total tissue levels of malonyl CoA as a predictor of malonyl CoA available to inhibit CPT I remains uncertain.

*vi) 5'AMP-activated protein kinase activity*

AMPK is capable of inhibiting ACC activity in liver and adipose tissue by causing phosphorylation of the enzyme (Sims and Hardie 1988). AMPK itself can be activated by an upstream kinase kinase. The activation of hepatic ACC by insulin is apparently manifested through an inhibition of AMPK (Witters and Kemp, 1992). In order to determine whether the alterations in ACC activity in this study could be explained by changes in AMPK activity we measured AMPK activity in our hearts as shown in Figure 6-3. When hearts were perfused in the presence of 1000 μU/ml insulin, the increase in ACC activity (Table 6-2) was accompanied by an inhibition of AMPK of approximately 40%. Inhibition of

AMPK in the insulin-treated hearts persisted when AMPK was assayed in the presence of saturating amounts of AMP. This is consistent with a decreased phopshorylation of AMPK. Glucagon treatment, on the other hand, did not result in any significant change in AMPK activity compared to control hearts. AMPK activity in all groups of hearts could be dramatically reduced by pretreatment with PP2A (data not shown) suggesting that cardiac AMPK activity is indeed under phosphorylation control.

A correlation between ACC and AMPK activity in control and insulin-treated hearts is shown in Figure 6-4. Glucagon-treated hearts were omitted from the figure because based on Figure 4-3, glucagon appears to affect cardiac ACC activity independent of actions via AMPK. As shown in Figure 6-4, a negative correlation exists between ACC and AMPK activity. Hearts treated with a high dose of insulin exhibit higher ACC activity and this is associated with lower AMPK activity as compared to controls.

*D) Summary*

A number of conclusions can be drawn with respect to the hormonal regulation of myocardial metabolism. Perfusion of hearts with glucagon stimulates glucose oxidation with no effect on palmitate oxidation. Insulin, on the other hand, suppresses fatty acid oxidation with a concomitant decrease in AMPK activity and increase in ACC activity. In accordance with these results, using an indirect measure of ACC phosphorylation, it was found that the ability of insulin to stimulate ACC activity is associated with a dephosphorylation of the enzyme, most likely the result of a decrease in AMPK activity. Therefore, this study has

demonstrated that like the liver, insulin has the ability to alter myocardial fatty acid oxidation via an ACC and AMPK mediated mechanism.

**Table 6-1.** Effects of insulin and glucagon on mechanical function in isolated working rat hearts

| *Condition:* | Heart rate (beats·min$^{-1}$) | Peak systolic pressure (mm Hg) | Cardiac output (ml·min$^{-1}$) | Heart rate x PSP (x $10^{-3}$) |
|---|---|---|---|---|
| Control | 298 ± 11 | 120 ± 3 | 65 ± 4 | 36 ± 1 |
| Insulin | 288 ± 6 | 130 ± 4 | 71 ± 3 | 35 ± 1 |
| Glucagon | | | | |
| pre | 295 ± 12 | 127 ± 6 | 70 ± 6 | 37 ± 2 |
| post | 338 ± 14*#$ | 128 ± 5 | 75 ± 8 | 43 ± 2*#$ |

*, significantly different from control
#, significantly different from insulin
$, significantly different from pre glucagon values

Values represent the means ± standard error of the mean of 6-8 hearts in each group. Spontaneously beating hearts were perfused as described in "**CHAPTER II**". Insulin was present throughout the 40 minute perfusion protocol at either 100 μU/ml (control) or 1000 μU/ml (insulin). Glucagon (0.05 μM), when present was added 5 minutes into the perfusion and heart function was recorded just prior to the addition (pre) and at 10 minute intervals thereafter (post). PSP, peak systolic pressure.

**Table 6-2.** Acetyl CoA carboxylase activity in isolated working rat hearts treated with insulin and glucagon.

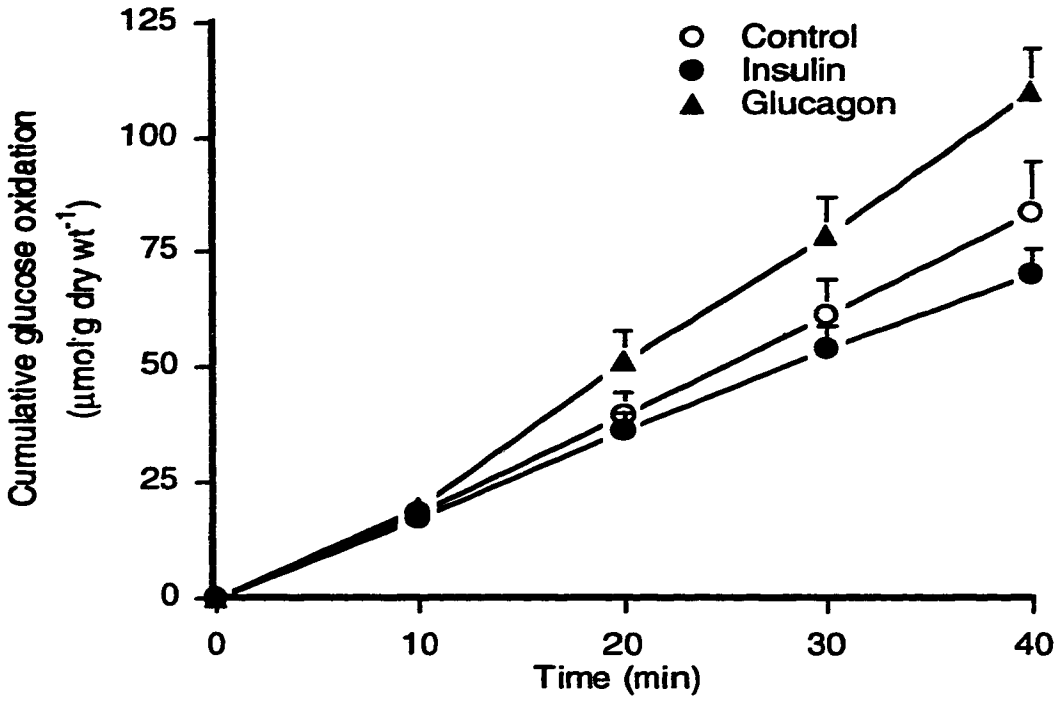
| | Acetyl CoA carboxylase activity (nmol·min$^{-1}$·mg protein$^{-1}$) | | |
|---|---|---|---|
| *Condition:* | *isolated with NaF, NaPPi* | *isolated without NaF, NaPPi* | *% change* |
| Control | 4.92 ± 0.8 | 6.29 ± 1.2 | 27.8 |
| Insulin | 9.49 ± 1.6* | 10.38 ± 0.7* | 9.3 |
| Glucagon | 2.47 ± 0.4*# | 4.50 ± 0.5# | 82.1 |

*, significantly different from control
#, significantly different from insulin

Values are the mean ± standard error of the mean of 6 hearts in each group. Hearts were perfused as described in "**CHAPTER II**". ACC activity was determined in 6% PEG precipitates (isolated in the presence or absence of NaF, NaPPi) from frozen, pulverized ventricular tissue and assayed in the absence of added citrate as described in "**CHAPTER II**".

**Figure 6-1.** Glucose oxidation rates in hearts treated with insulin or glucagon.

Data are the mean ± standard error of the mean of 6 control, 7 insulin and 6 glucagon hearts in each group. Glucose oxidation rates were determined during the 40 minute perfusion protocol as described in "**CHAPTER II**". *Upper panel:* time-course of the cumulative glucose oxidation over the 40 minute aerobic perfusion protocol for control, insulin and glucagon-treated hearts. *Lower panel:* Steady-state glucose oxidation rates between 10 and 40 min of perfusion.


Cumulative glucose oxidation
(µmol·g dry wt⁻¹)
125
100
75
50
25
0
0
10
20
30
40
Time (min)
Control
Insulin
Glucagon

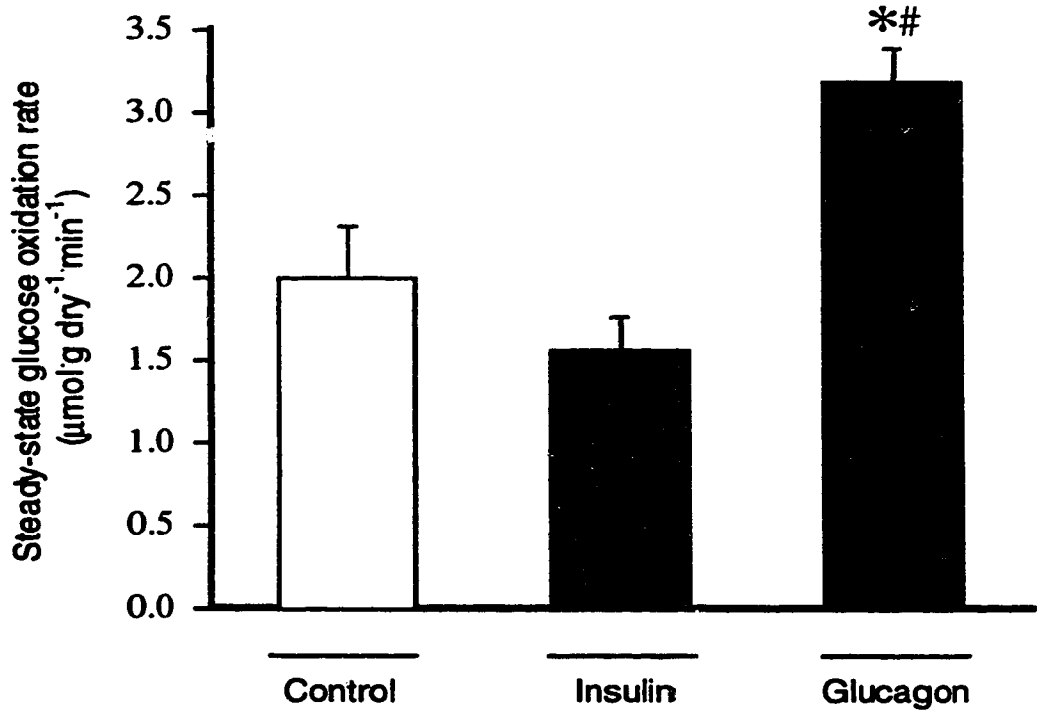
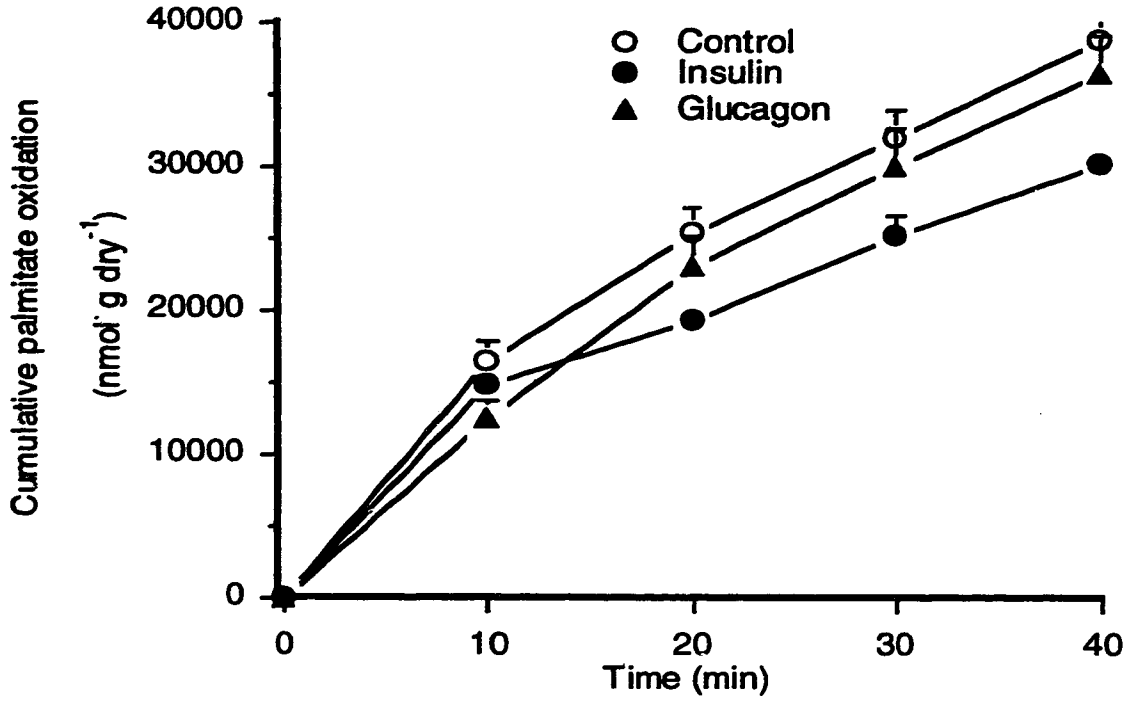
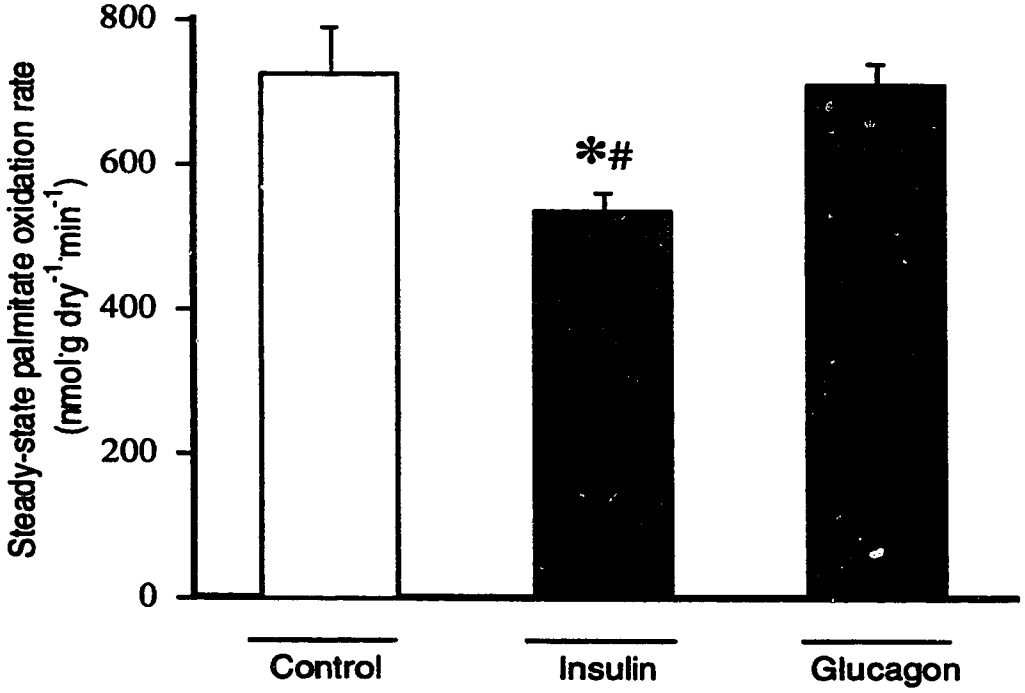

Steady-state glucose oxidation rate
(µmol·g dry⁻¹·min⁻¹)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*#
Control
Insulin
Glucagon

**Figure 6-2.** Steady-state palmitate oxidation rates in hearts treated with insulin or glucagon.

Data are the mean ± standard error of the mean of at least 7 hearts in each group. Palmitate oxidation rates were determined during the 40 minute perfusion protocol as described in "**CHAPTER II**". *Upper panel:* time-course of the cumulative palmitate oxidation over the 40 minute aerobic perfusion protocol for control, insulin and glucagon-treated hearts. *Lower panel:* Steady-state palmitate oxidation rates between 10 and 40 min of perfusion.


Cumulative palmitate oxidation
(nmol·g dry⁻¹)
40000
30000
20000
10000
0
Control
Insulin
Glucagon
0
10
20
30
40
Time (min)


Steady-state palmitate oxidation rate
(nmol·g dry⁻¹·min⁻¹)
800
600
400
200
0
*#
Control
Insulin
Glucagon

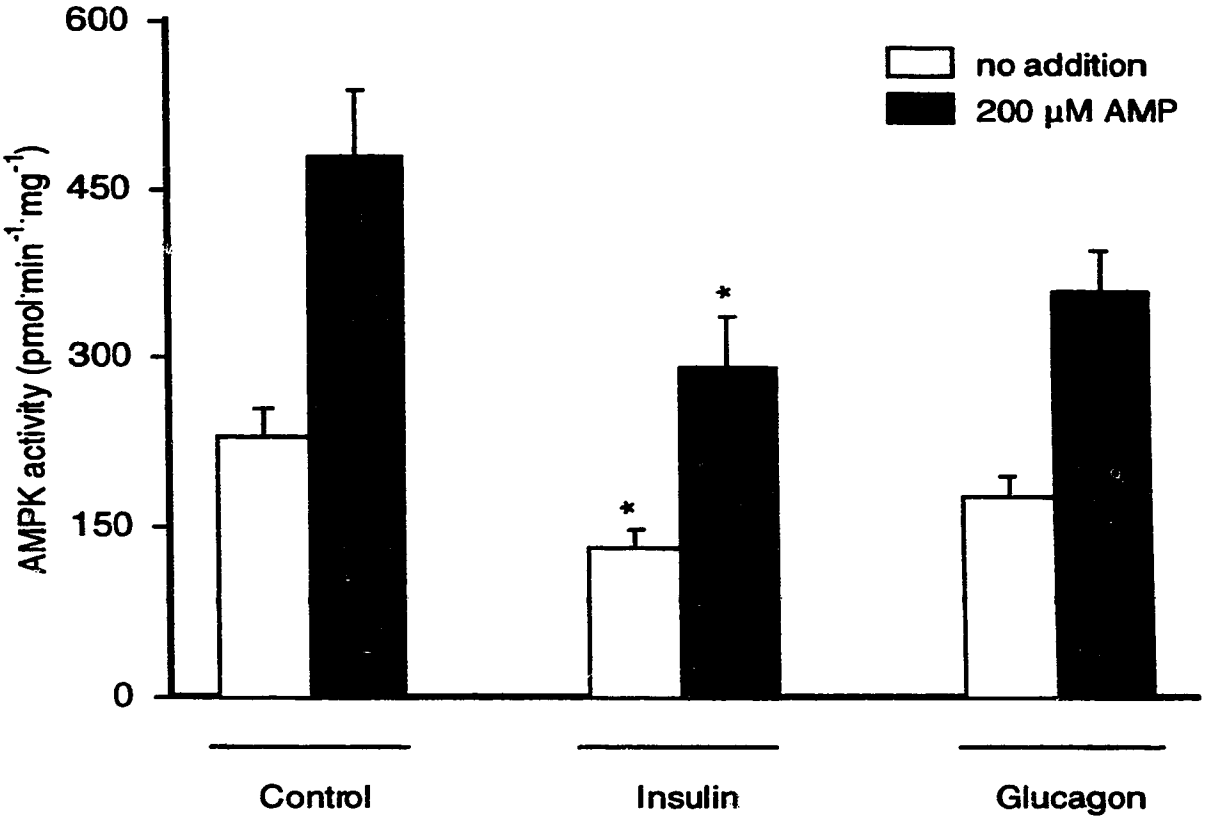



**Figure 6-3.** AMPK activity in hearts treated with insulin or glucagon.

Values represent the means ± standard error of the mean of 5 control, 4 insulin and 5 glucagon hearts in each group. Hearts were perfused as described in **"CHAPTER II"**. AMPK was measured in 6% polyethylene glycol (PEG) precipitation from heart tissues frozen at the end of perfusion. 5'-AMP activated protein kinase activity was measured by monitoring the incorporation of $^{32}P$ into the SAMS peptide. Assays were conducted in the absence of added AMP or in the presence of 200 µM AMP.

*, significantly different from control

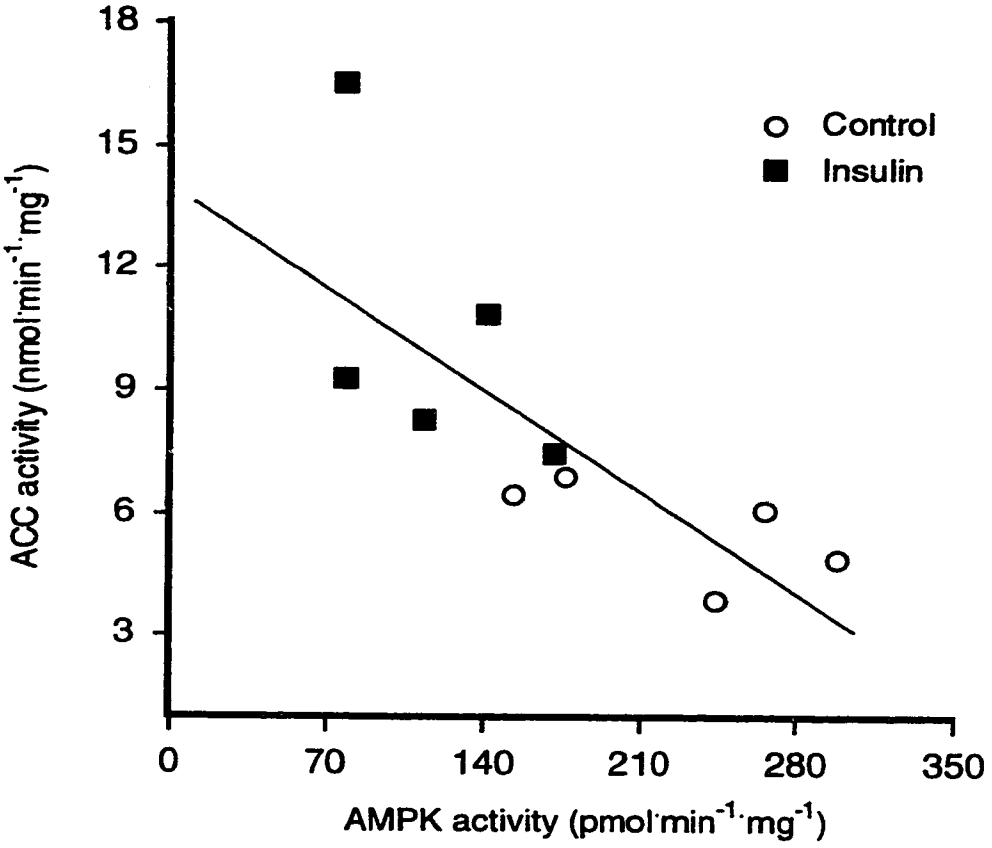



**Figure 6-4.** Correlation between myocardial ACC and AMPK activity.

Plotted are the values for ACC and AMPK activity from control and insulin-treated hearts determined as described in **CHAPTER II.** Hearts were frozen at the end of the perfusion protocol and enzyme activity was measured in 6% PEG extracts. (r value = -0.76)

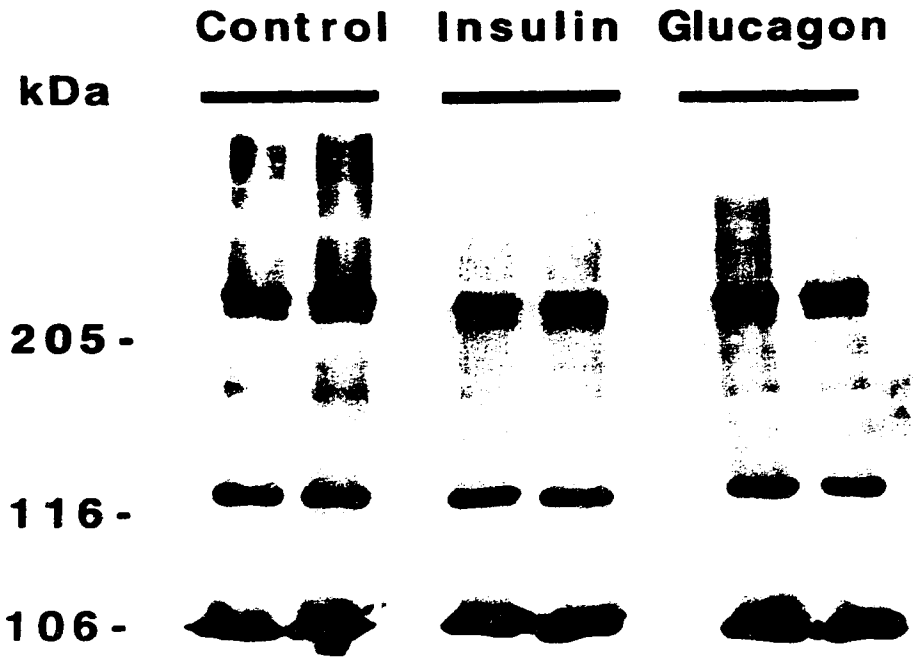
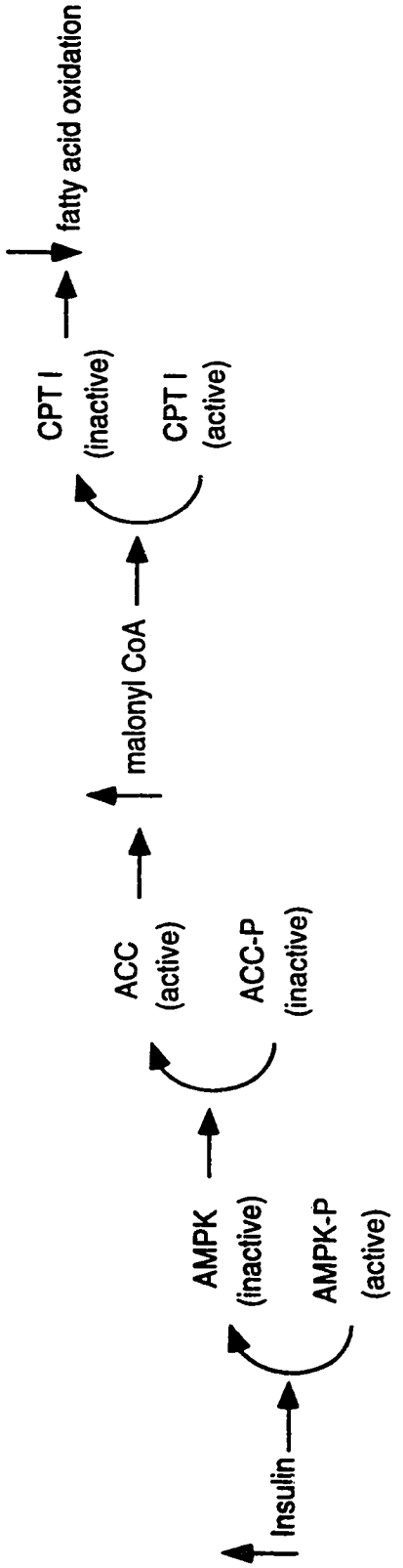



**Figure 6-5.** Western blot analysis of heart ACC.

ACC protein content was measured in polyethylene glycol fractions obtained from hearts frozen at the end of the 40 min perfusion period as described in "**CHAPTER II**". Forty-five μg of total protein was loaded in each lane. SDS-PAGE was carried out on the 3-5% acrylamide gels and the bands were subsequently transferred to nitrocellulose membrane. Membranes were probed with perxidase-conjugated streptavidin and bands were visualized using chemiluminescence and exposure to x-ray film.

**Figure 6-6.** Hypothetical scheme by which insulin decreases myocardial fatty cid oxidation.

A rise in myocardial insulin levels results in a dephosphorylation and inactivation of the 5'AMP-activated protein kinase (AMPK). The reduction in the kinase activity causes an activation of acetyl CoA carboxylase (ACC) via dephosphorylation. This increased ACC activity gives rise to enhanced levels of malonyl CoA, the product of the ACC reaction.. Malonyl CoA is a potent inhibitor of carnitine palmitoyltransferase I, the enzyme considered to be rate-limiting for fatty acid oxidation. Malonyl CoA therefore causes an inhibition of CPT I activity thereby reducing the uptake of fatty acids into the mitochondria, the site for fatty acid ß-oxidation. As a result, myocardial fatty acid oxidation rates decline in response to the reduced uptake of fatty acids into the mitochondria.


Insulin
AMPK
(inactive)
AMPK-P
(active)
ACC
(active)
ACC-P
(inactive)
malonyl CoA
CPT I
(inactive)
CPT I
(active)
fatty acid oxidation

# CHAPTER VII

# DISCUSSION

Historically, the regulation of myocardial fatty acid oxidation in the heart by malonyl CoA has been inferred base primarily on the ability of malonyl CoA to inhibit myocardial CPT I (McGarry *et al.*, 1983). The origin of malonyl CoA in heart tissue remained obscure until Thampy (1989) and Bianchi *et al.*, (1990) identified isoenzymes of ACC in heart tissue. However, at the time of initiation of this thesis research, a direct relationship between ACC, malonyl CoA and myocardial fatty acid oxidation had not yet been made. Accordingly, this was addressed in this thesis by directly measuring fatty acid oxidation in the isolated working rat heart perfused in the presence of physiological levels of fatty acids followed by subsequent biochemical analysis of ACC activity and expression in these tissues.

## *A) Acetyl CoA carboxylase regulation of fatty acid oxidation in the normal heart*

To initially address the potential regulatory role of ACC in myocardial fatty acid metabolism we used isolated working rat hearts in which overall fatty acid oxidation rates (i.e., endogenous and exogenous) were directly measured. If hearts were perfused with 1.2 mM palmitate, 93% of ATP production originates from exogenous and endogenous fatty acid oxidation. Addition to the perfusate of the PDC agonist DCA stimulated glucose oxidation and decreased the contribution of palmitate oxidation to 65% of myocardial ATP production. This

decrease in fatty acid oxidation was associated with an increase in tissue levels of malonyl CoA. 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 the production of acetyl CoA by PDC may supply ACC with substrate thereby stimulating its activity. For this to hold true, one would expect that depressing glucose oxidation should result in a diminished ACC activity. Indeed, when PDC activity was decreased by removing glucose from the perfusate malonyl CoA levels dropped to virtually undetectable levels (Table 3-5). This was accompanied by a subsequent increase in myocardial fatty acid oxidation rates.

Despite the increase in malonyl CoA levels in DCA-treated hearts, no difference was found between ACC activity measured in extracts prepared from frozen cardiac tissue of DCA-treated and control hearts. Historically, the $CO_2$ fixation has been employed as a measure of ACC activity. This involves using [$^{14}$C]bicarbonate and measuring its incorporation into malonyl CoA. However, the strepavidin-probed Western blot shown in Figure 3-3c demonstrates the high concentration of other carboxylases in the heart relative to ACC. Although these carboxylases were not positively identified, based on their estimated molecular weight they are assumed to represent pyruvate carboxylase (120 kDa) and propionyl CoA carboxylase (106 kDa). We therefore developed an ACC assay based on measuring the end product of the ACC reaction, malonyl CoA . This assay provides a direct 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 $CO_2$ fixation assay were observed.

Western blotting also revealed the presence of both the 265 kDa and the 280 kDa isoenzymes of ACC in the heart. The reason why the heart expresses both isoenzymes is not readily apparent. Whether or not the heart contains the

ACC-265 for the purpose fatty acid synthesis or elongation is not understood. While there may be fatty acid elongation in the heart to provide long chain fatty acids targeted for membranes the heart does not appear to display fatty acid synthase activity (Awan and Saggerson, 1993) although fatty acid synthase mRNA is expressed in heart (Semenkovich *et al.*, 1995).

The lack of difference in ACC activity between control and DCA-treated hearts is unlikely to be due to non-specific phosphorylation/dephosphorylation during the preparation of the cytosolic fraction, since isolation conditions employed were specifically designed to preserve the phosphorylation state of the enzyme (Thampy and Wakil, 1988). Furthermore, cardiac ACC activity did not show any significant citrate dependency, which occurs when the 265 kDa isoform of ACC is inhibited by phosphorylation (Thampy and Wakil, 1988). The lack of effect of DCA on ACC activity *in vitro* also speaks against a covalent modification of ACC as the mechanism behind the increase in malonyl CoA levels in these hearts.

ACC-265 has been shown to be regulated over two different time frames. A rapid regulation (minutes) involves changes in covalent phosphorylation, allosteric regulation and polymerization (Bianchi *et al.*, 1990; Witters, 1986). A long term (hours-days) regulation involves changes in enzyme mass (Pape *et al.*, 1988) together with changes in enzyme activity caused by the aforementioned rapid regulation (Jamil and Masden, 1987a; Thampy and Wakil, 1988). While liver ACC was reported to be dependent on citrate for activation (Thampy and Wakil, 1985; Lane *et al.*, 1979), Thampy and Wakil (1988) found that it is the phosphorylated form of the enzyme that is citrate dependent. In other words, when livers were quickly frozen upon excision, a more active, citrate-independent ACC with lower phosphate content resulted. Davies *et al.*, (1992) recently

reported that a highly 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 ischemia/hypoxia during removal procedure) and with an increased activity of 5'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 or DCA-treated hearts (Figure 3-4). The data in the present study suggests that the increase in malonyl CoA as a result of DCA treatment was caused by an increase in the amount of acetyl CoA available to the enzyme. In other words, heart ACC activity can be driven by the supply of substrate. As depicted in Figure 3-1, 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 (in which ACC-280 predominates) exhibited lower affinity for acetyl CoA ($Km$ = 117 μM) compared to in white adipose tissue ($Km$ = 67 μM) in which ACC-265 predominates. Cytosolic levels of total CoA in the heart are in the range of 15-50 μM (Idell-Wenger *et al.*, 1978). Although cytosolic content of acetyl CoA cannot be accurately determined, it is obviously lower than overall CoA levels. Although one can not determine the subcellular distribution of CoA esters, one can conclude that the availability of cytosolic acetyl CoA to ACC may be an important factor modulating cardiac ACC activity. In contrast, cytosolic CoA levels in the liver (Williamson and Corkey, 1979) are much greater than in the heart, and the affinity of ACC-265 for acetyl CoA is higher (Bianchi *et al.*, 1990) suggesting that acetyl CoA supply is probably less important as a regulator of hepatic ACC activity.

Although most of the acetyl CoA in the heart is intra-mitochondrial, a mechanism exists in the heart for the transport of acetyl groups out of the mitochondria via a carnitine acetyltransferase (CAT), and carnitine acetyltranslocase (Lysiak *et al.*, 1986;1988; Pearson and Tubbs, 1967). As shown in Figure 3-6, 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, an increase in intra-mitochondrial acetyl CoA production due to 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 intra-mitochondrial acetyl CoA levels in our perfused hearts (Idell-Wenger *et al.*, 1978). 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 CoA thiolase (Schulz, 1991;1994). This, however, does not explain the increase in malonyl CoA levels we observed in DCA-treated hearts. Furthermore, while an increase in intra-mitochondrial acetyl CoA can decrease fatty acid ß-oxidation, this does not decrease mitochondrial fatty acid uptake (Schulz, 1994). Further support for the export of acetyl CoA to cytosol resulting in ACC activation

comes from recent work from this laboratory using L-carnitine-supplemented hearts. Increasing intracellular carnitine levels was shown to increase glucose oxidation in fatty acid perfused hearts (Broderick *et al.*, 1992), 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 cannot be explained by an inhibition of thiolase (i.e intramitochondrial acetyl CoA levels decrease under these conditions). We hypothesize that the 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 activity and malonyl CoA production, resulting in a decrease in fatty acid oxidation due to a decreased mitochondrial fatty acid uptake because of CPT I inhibition.(Figure 3-6). To date, however, we have not determined the effects of carnitine supplementation on myocardial malonyl CoA levels. However, in a recent study, we did observe that increasing myocardial carnitine levels with propionyl carnitine treatment causes a dramatic rise in myocardial malonyl CoA levels (Schonekess *et al.*, in press).

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 shunt fatty acids in the form of acyl-CoA towards complex lipid synthesis.

Cardiac levels of malonyl CoA reported in this study are comparable to previously reported values of 4-5 nmol·g wet$^{-1}$ (16-20 nmol·g dry $^{-1}$) (Singh *et al.*, 1984; McGarry *et al.*, 1983). Although the increase in malonyl CoA levels in DCA-treated hearts was significant (compared to untreated perfused hearts), this

increase was only around 40%. Cardiac CPT 1 is highly sensitive to inhibition by malonyl CoA. It is possible therefore, that even with a slight change in malonyl CoA concentration in the cardiac myocyte, a dramatic change in fatty acid oxidation could be expected. In addition, these data suggest that most of the malonyl CoA present in the heart is inaccessible to CPT I. The malonyl CoA content measured in the hearts used in this study is similar to that previously reported (Singh *et al.*, 1984; McGarry *et al.*, 1983). Conversion of these values to a concentration would result in cytosolic levels of at least 5 μM. However, as McGarry *et al.*, (1983) have previously shown heart CPT 1 is extremely sensitive to malonyl CoA inhibition (the $IC_{50}$ is between 50 and 100 nM). As a result, if all of the malonyl CoA present in the heart were accessible to CPT 1, fatty acid oxidation would always be completely inhibited. This of course is not the case, suggesting that most of the malonyl CoA produced in the heart is inaccessible to CPT 1. Alternatively, as suggested by McMillin *et al.*, 1994, this lack of ability to completely supress myocardial fatty acid oxidation despite high levels of malonyl CoA may also be explained by the interaction of malonyl CoA with a binding site distinct from the inhibitory site. Malonyl CoA acting at this site may alter the interaction of malonyl CoA at the inhibitory site thereby allowing for CPT I activity in the midst of the high malonyl CoA levels in the cell.

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 (in addition to its natural substrate propionyl-CoA) (Hulsmann, 1966). Scholte *et al.*, (1986) 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 (1989) has shown that ACC is the enzyme involved in the synthesis of malonyl CoA in the

heart. We (Kudo *et al.*, unpublished data) have also shown that cardiac ACC is also localized to the cytosolic compartment. Therefore, the increase in cardiac malonyl CoA levels seen in DCA-treated hearts in this study can be explained by an increase in ACC activity resulting from an increase in cytosolic acetyl CoA concentrations.

In summary, in normal hearts we have demonstrated a direct link between malonyl CoA production by ACC and a decrease in myocardial fatty acid oxidation rates. Both ACC-280 and ACC-265 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.

### *B) Differential tissue regulation of acetyl CoA carboxylase in acute diabetes*

Having shown that ACC is an important regulator of myocardial fatty acid oxidation (**CHAPTER II**) in addition to its well established role in lipogenesis, we were interested in determining what influence diabetes has on the ACC expression and activity in a variety of both lipogenic and non-lipogenic tissues. To date, information regarding the influence of diabetes on tissues which primarily oxidize rather than synthesize fatty acids is non-existent. Therefore, tissue ACC expression and activity was measured in heart, liver, white adipose tissue and skeletal muscle from streptozotocin-induced insulin-deficient diabetic rats.

Diabetics can have marked abnormalities in both fatty acid biosynthesis by lipogenic tissues and fatty acid utilization by tissues which oxidize fatty acids. It has long been established that ACC has an important role in lipid biosynthesis, since it is the first committed step in fatty acid biosynthesis (Kim *et al.*, 1989). It has also been recently established that ACC has an important role in regulating fatty acid oxidation (**CHAPTER III**; Awan and Saggerson, 1993). The product of ACC, malonyl CoA, is a potent inhibitor of carnitine palmitoyltransferase 1, which is the rate limiting enzyme for entry of fatty acids into the mitochondria. The results of **CHAPTER IV** demonstrate that ACC expression and activity in tissues associated with lipogenesis are affected to a much greater extent than tissues in which fatty acid oxidation is more prominent. This may be indicative of a mechanism of ACC regulation that is either tissue or isoenzyme-specific. Indeed, brain ACC, which expresses exclusively the 265 kDa isoenzyme of ACC, exhibits a decrease in enzyme activity and expression during the first 4 weeks

post-birth. In contrast, there were no changes in cardiac, skeletal muscle and liver ACC over the same course of development (Brooke-Spencer *et al.*, 1993, Lopaschuk *et al.*, 1994b).

This differential regulation of ACC may be a reflection of the regulatory mechanisms associated with the two isoenzymes of ACC. The 265 kDa isoenzyme of ACC has been shown to be regulated over two different time frames. Long term regulation is associated with changes in enzyme mass, due to mechanisms of enzyme synthesis and degradation, in addition to the changes in enzyme activity. A rapid regulation (minutes) can also occur, which involves changes in covalent phosphorylation mediated by various hormones, allosteric regulation and protein polymerization (see Kim *et al.*, 1989 for review). Allosteric regulation can involve fatty acyl CoA inhibition of enzyme activity, or citrate stimulation of activity (see Hardie, 1989 for review). Polymerization can also influence enzyme activity, and it is believed that the enzyme requires at least a dimer configuration for proper interaction of the catalytic subunits (Kim *et al.*, 1989). Covalent phosphorylation of the enzyme is another important mechanism regulating ACC activity, with a number of different kinase acting at one or more of the seven putative phosphorylation sites on the enzyme (Kim *et al.*, 1989). Complicating this scenario, however, is the recent discovery of isozymic forms of ACC which are expressed in different tissues throughout the body (Bianchi *et al.*, 1990). While the characterizations listed above have resulted almost exclusively from study of the 265 kDa isozyme of ACC, there is only minimal information on the regulation of the more recently discovered 280 kDa ACC isozyme (**CHAPTER III**; Winz *et al.*, 1994; Thampy, 1989).

The cloning of a cDNA for the 265 kDa ACC (Bai *et al.*, 1986) has allowed for the study of gene activity in a variety of physiological states, including

diabetes. However, despite the importance of ACC in lipid metabolism, the effects of diabetes on ACC expression and activity has not been extensively studied. The influence of both nutritional status and diabetes on ACC mRNA has been previously described in epididymal fat (Pape *et al.*, 1988). The induction of diabetes virtually eliminated the high level of mRNA normally expressed in this tissue, a condition which could be reversed within 6 hours following injection of of these animals. While insulin returned the mRNA levels comparable to those of the non-diabetic animals, an induction of gene activity, albeit to a lesser degree, was also observed in control animals following insulin treatment. As we demonstrate in **CHAPTER IV**, the loss of mRNA in white adipose tissue found by others (Pape *et al.*, 1988) is accompanied by a dramatic decrease in ACC protein expression and activity.

My inability, after numerous attempts, to detect ACC-265 mRNA in tissues from control and diabetic rats also warrants further attention. The probe that was used was originally obtained from Dr. Ki-Han Kim, and had been used routinely for the detection of ACC-265 mRNA in liver and adipose tissue. The RNA was isolated from all tissues simultaneously as were the labelling of the cDNA probes. In addition, when the plasmids containing the cDNA were subjected to a restriction digest they did yield a fragment of the expected size. Even more frustrating with this inability to detect ACC-265 mRNA is that a positive identification of the band thought to represent ACC-280 mRNA (Figure 4-2) cannot be done. The initial intention was to strip and reprobe the same membranes with the ACC-265 probe to re-inforce the identity of each mRNA band. Unfortunately, this was not possible so at the present moment one can only infer that the detected mRNA band corresponds to ACC-280.

Interestingly, skeletal muscle which expressed solely ACC-280 was not altered by the diabetic state in terms of either expression or activity. The underlying reason for this lack of change is not readily apparent. Whether this reflects a different pattern of regulation between the two isoenzymes is not known. Relatively little information exists with respect to the regulation of the 280 kDa isoenzyme of ACC. We have previously reported that heart ACC may be primarily regulated by substrate supply (acetyl CoA)(**CHAPTER III**). However, Brownsey's group (Winz *et al.*, 1994) has recently suggested that phosphorylation of hepatic ACC by the catalytic subunit of cyclic AMP-dependent protein kinase occurs much more rapidly in ACC-280 versus ACC-265. We have also recently shown that ACC appears to be phosphorylated and inhibited in the reperfused ischemic heart, which leads to a drop in malonyl CoA levels and an acceleration of fatty acid oxidation (Kudo *et al.*, 1995). Our laboratory has also shown indirectly (Kudo *et al.*, 1996 in press) and directly Kudo *et al.*, unpublished data) that phosphorylation of ACC inhibits enzyme activity. Further work in this area is needed in order to draw any definitive conclusions as to the role of phosphorylation in the regulation of ACC-280.

The origin of the two ACC isoenzymes is also an issue that requires clarification. Biochemical and immunologic evidence would suggest that these are two unique enzymes (Bianchi *et al.*, 1990, Winz *et al.*, 1994; Bianchi *et al.*, 1992). It is highly unlikely that ACC-265 is simply a proteolytic fragment of the 280 kDa form. However, multiple species of ACC-265 are known to exist as a result of differential splicing of a single primary transcript under the control of two distinct promoters (Luo and Kim, 1990). Whether or not ACC-280 is a product of this gene is not definitively known. Taken together, this data suggests

that the origin of, and the regulatory control of the two ACC isoenzymes are quite different.

In the present study it was found that heart ACC activity was altered by the diabetic state with no change in protein content, suggesting that allosteric control may be involved in this response. One possibility is the involvement of a recently characterized cardiac 5' AMP-activated protein kinase (AMPK) (Kudo *et al.*, 1995). This kinase is known to be an important regulator of both lipid and sterol synthesis in liver via its phosphorylation of both acetyl CoA carboxylase and 3-hydroxy 3-methyl glutaryl CoA reductase, respectively (Hardie, 1992). AMPK has been termed a "metabolite -sensing protein kinase" due to its activation during situations of cell stress such as ATP and glucose depletion (Witters *et al.*, 1991). Interestingly, the activation of hepatic ACC by insulin has been shown to be mediated by an inhibition of AMPK (Witters and Kemp, 1992). It has also been demonstrated that in hearts from insulin-resistant animals there is an increase in AMPK activity associated with both a depressed ACC activity and an accelerated fatty acid oxidation (**CHAPTER V**). It is possible that the decrease in ACC activity observed in acutely diabetic rat hearts and liver (Table 4-2) occurs secondary to an increase in AMPK activity, which could occur secondary to a decrease in circulating insulin levels. This hypothesis remains to be tested. It is also not known at this point whether or not AMPK may be involved in the tissue specific regulation of ACC, in a manner similar to the zonal expression of ACC activity in the liver (Witters *et al.*, 1994).

The results of the present study demonstrate that the acetyl CoA carboxylase isoenzymes found in various tissues are differentially regulated in acute streptozotocin-induced diabetes. ACC-280 which, based on tissue distribution, is assumed to be involved primarily in regulating fatty acid oxidation

appears somewhat resistant to the effects of diabetes in terms of protein expression in both heart and skeletal muscle. The only effect of diabetes was a decrease in cardiac ACC activity. In contrast, the 265 kDa ACC isoenzyme expression and activity are markedly diminished in the diabetic state. Whether this is the result of genetic control mechanisms or allosteric regulation, respectively, of the protein remains to be determined. These changes in ACC may reflect the physiological response to the high levels of circulating fatty acids in the diabetic whereby a depressed lipogenic capacity is accompanied by an up-regulation of fatty acid oxidation capacity.

While this model of streptozotocin-induced diabetes is more representative of a type I insulin-deficient diabetes, models also exist for the more common non-insulin-dependent, type II diabetes. Unlike the insulin deficient state, the transition to overt type II diabetes is accompanied by the onset of insulin-resistance. Therefore, we were interested in examining what alterations in myocardial metabolism and ACC occur in a genetically-induced model of insulin-resistance known to exhibit forms of cardiomyopathy.

### *C) Acetyl CoA carboxylase and 5' AMP-activated protein kinase regulation of myocardial fatty acid metabolism in insulin resistance*

Non-insulin dependent (type II) diabetes mellitus in humans is often characterized by obesity, hyperlipidemias, insulin resistance and a predisposition to the development of coronary heart disease. Traditionally, the chronically-induced rat has been used as a model to study the influence of diabetes on the heart. Unfortunately, this situation does not mimic the situation seen in the majority of humans with diabetes who are prone to the development of atherosclerosis. In addition, the subcellular defects which have been discovered in diabetic hearts are almost certainly a result of alterations in lipid profiles and the appearance of vascular lesions. The insulin-resistant JCR:LA corpulent rat provides a valuable model to study the influence of diabetes on the heart as it exhibits a number of the metabolic alterations seen in the human disease.

When homozygous for the corpulent gene (*cp*) male rats of the JCR:LA strain spontaneously develop myocardial lesions which are ischemic in origin and correlate strongly with the hyperinsulinemia experienced by these animals. This hyperinsulinemia is just one of a number of profound metabolic derrangements which are also characteristic of these animals. Results from euglycemic insulin clamp experiments have suggested that these corpulent animals are extremely hyperinsulinemic, a condition which may be secondary to a marked peripheral insulin resistance (Russell *et al.*, 1994). Despite the fact that these animals have normal fasting glucose levels, they are also extremely glucose intolerant (Russell *et al.*, 1987).

In addition to the abnormalities in carbohydrate metabolism, corpulent JCR:LA animals exhibit an altered lipid metabolic profile. Hypersecretion of very

low density lipoproteins (VLDL) in conjunction with hypertriglyceridemia (Russell *et al.*, 1991) and an exaggerated level of *de novo* fatty acid synthesis (Shillabeer *et al.*, 1992) all contribute to the pathogenesis of obesity and atherosclerosis in these animals. Interestingly, interventions which lower plasma lipid and insulin levels have been found to prevent the onset of the ischemic myocardial lesions (Dolphin *et al.*, 1988; Russell *et al.*, 1987).

Normalization of insulin levels is also able to reverse a number of the cardiomyopathic changes which arise in the insulin-deficient models of diabetes. However, attempts to reduce the high levels of circulating inslulin in the corpulent rats has proven to be futile in that the they ultimately become overtly diabetic and glucosuric if levels fall below 100 μU/ml (Russell and Miyazaki, unpublished observations). While one might expect that insulin deficiency and insulin resistance may have similar effects on cellular metabolism, namely a reduction in insulin-stimulated glucose metabolism, this issue has not clearly been addressed. Indeed in **CHAPTER IV**, we show that a marked 64% reduction in glucose oxidation occurs in the corpulent rat hearts versus controls (Table 4-3), a result which is consistent with those we have found in insulin-deficient models of diabetes such as chemically-induced and the genetic model of the BB Wor rat (Wall and Lopaschuk, 1989; Broderick *et al.*, 1992b). Myocardial fatty acid oxidation, on the other hand, appears to be a metabolic parameter which remains uncompromised in both the insulin-resistant JCR:LA rat (Table 4-3) and both chemically-induced and genetic models of insulin-deficient diabetes (Lopaschuk and Tsang, 1987; Wall and Lopaschuk, 1989).

In normal Sprague-Dawley rats, the oxidation of fatty acids, accounts for approximately 50-75% percent of the total myocardial ATP production. While absolute rates of palmitate oxidation remained unaltered between the lean and

corpulent hearts, the diminution of glucose oxidation in the corpulent hearts (Table 4-3) resulted in a significant increase in the contribution of fatty acids to 70% of ATP production while that in the lean counterparts remained similar to values seen in Sprague-Dawley strains ie. ~50% (Table 3).

A number of sites may be responsible for this alteration in the balance of fatty acid and glucose metabolism in the JCR:LA corpulent rat heart. We have recently shown that ACC is an enzyme that plays a key role in regulating energy substrate balance in the heart (Saddik *et al.*, 1993). ACC has traditionally been studied for its role in fatty acid biosynthesis in the liver (Kim *et al.*, 1989) but its role in other tissues such as skeletal muscle and particularly heart, which are oxidative in nature, is gaining interest as of late (**CHAPTER III**; Awan and Saggerson, 1993; Winz *et al.*, 1994). This interest has been peaked by studies which have shown that malonyl CoA, which has been known for many years to be present in heart, was derived from ACC expressed in the heart (Thampy, 1989). Until recently however, the role of malonyl CoA in regulating fatty acid oxidation was only inferred based on its appearance in tissues which were primarily involved in the oxidation of fatty acids and the knowledge that malonyl CoA was a potent inhibitor of carnitine palmitoyltransferase 1 (CPT 1) (McGarry *et al.*, 1989), an enzyme important for the uptake of fatty acids into the mitochondria where they are subsequent oxidized. In addition, ACC was discovered to exist as two distinct isoenzymes which differ in their tissue distribution (Bianchi *et al.*, 1990). This has led to speculation that the larger of the two isoenzymes (280 kDa), which predominates in heart, skeletal muscle and liver, may regulate fatty acid oxidation whereas the 265 kDa isoenzyme which predominates in liver, white adipose tissue and lactating mammary gland may function primarily in fatty acid biosynthesis (Bianchi *et al.*, 1990). As the Western blot in Figure 5-1 shows, the

JCR:LA rat heart is similar to the Sprague-Dawley strain in that expresses both the predominant 280 kDa and to a lesser extent the 265 kDa ACC isoenzyme. The relative amounts of either protein appear to remain unchanged between the lean and insulin-resistant rat hearts.

We have shown that ACC activity is important in modulating the balance of myocardial glucose and fatty acid oxidation in the isolated perfused rat heart and have proposed a feedback loop involving the interaction of three key enzymes, namely CPT 1, carnitine acetyl translocase (CAT) and ACC (**CHAPTER III**). The classic study of Randle *et al.*, (1963) has suggested a similar regulation in liver whereby fatty acids can regulate carbohydrate metabolism. Our model on the other hand, describes how alterations in glucose metabolism are able to manifest changes in fatty acid oxidation. When glucose oxidation is stimulated by DCA there was a parallel reduction in palmitate oxidation that was associated with both an increase in ACC activity and an increase in myocardial malonyl CoA levels. Furthermore, if glucose was removed from the perfusion medium, there was a depression in ACC activity and malonyl CoA levels were reduced very low levels and an increase in palmitate oxidation ensued (Saddik *et al.*, 1993). Together, these results suggested a central role for ACC in counterbalancing alterations in energy substrate utilization in the heart.

This regulatory pathway also appears to be active in the insulin-resistant JCR:LA rat heart. Hearts from corpulent animals derived the majority of ATP from the oxidation of fatty acids and exhibited lower ACC activity than the lean controls. Theoretically, the reduction in glucose oxidation in the hearts from corpulent JCR:LA rats should also reduce the intramitochondrial acetyl CoA concentration and provide less substrate for CAT since it has been shown that acetyl CoA from glucose may be preferrentially used as a substrate for CAT , as

opposed to acetyl CoA derived from ß-oxidation (Lysiak, *et al.*, 1986). This, in turn, should shuttle less acetyl CoA back into the cytosol and reduce its availability as a substrate for ACC and cause a concommitent reduction in malonyl CoA levels. These series of events may underly the mechanism for the increased fatty acid oxidation in the diabetic heart. In addition to the absolute levels of malonyl CoA, one must also consider the sensitivity of CPT 1 to inhibition by malonyl CoA as a potential mechanism for altering fatty acid oxidation.

In the liver, the $IC_{50}$ of CPT 1 for malonyl CoA increases dramatically in diabetes (Saggerson and Carpenter, 1981; Cook *et al.*, 1984). As a result, higher concentrations of malonyl CoA are required to inhibit fatty acid oxidation and the ability of malonyl CoA to regulate hepatic fatty acid metabolism becomes less important. In contrast, heart CPT 1 is much more sensitive to inhibition by malonyl CoA than in the liver (Chung *et al.*, 1992) and diabetes does not alter the sensitivity of heart CPT 1 to inhibition by malonyl CoA (Cook and Lappi, 1992). Together, these studies would suggest that in the heart, the absolute production of malonyl CoA by ACC may be the key factor in the regulation of mitochondrial fatty acid uptake.

A great deal of attention has been focussed on the regulation of ACC itself which may involve mechanisms for both rapid (minutes) and long term (hours/days) control. Long term regulation involves genetic manipulation in terms of tissue-specific activity of a number of promoters active on the ACC gene and changes in transcriptional rate (Kim *et al.*, 1989). Rapid regulation of ACC can occur due to both covalent phosphorylation by a number of different kinases and allosteric regulation (Kim *et al.*, 1989). Citrate has classically been regarded as a feed forward activator of ACC. Insulin is also known to activate liver ACC via its ability to inhibit the activity of the 5'-AMP-activated protein kinase (AMPK)

(Witters and Kemp, 1992). The results of the present study are entirely consistent with this hypothesis. In both insulin-deficient and insulin-resistant diabetes one would expect that the low intracellular insulin concentrations would allow for a more active AMPK, a reduction in ACC activity and an increase in fatty acid oxidation.

Little is presently known regarding AMPK in the heart. In the liver, AMPK is thought to act as a "metabolic sensor" in that it functions to conserve energy by inhibiting anabolic processes when cellular ATP fall and AMP levels rise. Enzymes known to be acted upon by AMPK include 3-hydroxy-3methyl glutaryl CoA reductase (Sato *et al.,* 1993), hormone-sensitive lipase (Hardie, 1992) and ACC (Hardie, 1992; 1989). Although little is known about the regulation of AMPK itself, hepatic AMPK is activated by AMP and is activated in situations of cell stress, such as substrate depletion (Witters *et al.,* 1991). Activity of AMPK in the heart exceeds that in the liver and Northern blotting has revealed a higher level of AMPK mRNA in rat heart versus liver (Gao *et al.*, 1995).

Based on these observations, both ACC and AMPK in the heart appear to be important regulators of energy substrate metabolism. Previous studies have shown that fatty acid inhibition of myocardial glucose use is an important factor contributing to both the onset of diabetic cardiomyopathies and the increased sensitivity of diabetics to ischemic injury (Broderick *et al.,* 1993; Lopaschuk *et al.,* 1988;1992). Furthermore, pharmacological agents which either stimulate myocardial glucose oxidation (Nicholl *et al.,* 1991) or inhibit fatty acid oxidation (Pieper *et al.,* 1984) can acutely improve function in hearts obtained from diabetic rats. Thus novel pharmacological agents designed to inhibit AMPK and/or activate ACC may provide a means of favourably altering the metabolic status of the diabetic heart and ultimately improve its function.

In summary, results from **CHAPTER V** demostrate that insulin-resistant animals of the JCR:LA strain, which express the corpulent gene (*cp/cp*), exhibit depressed heart function and a shift in energy metabolism toward a predominance of fatty acid as the major source of ATP. This divergence away from glucose metabolism in these hearts is associated with a decrease in ACC activity and an increase in AMPK activity. This activation of AMPK and subsequent inactivation of ACC probably results in a decreased inhibition of CPT I by malonyl CoA, thereby permitting an acceleration of fatty acid oxidation.

### *D) Hormonal regulation of myocardial fatty acid and glucose oxidation*

Having determined that ACC and AMPK play an important role in the regulation of myocardial fatty acid oxidation in models of both insulin-deficiency and insulin-resistance we were interested in examining directly whether hormonal control of heart ACC and AMPK alters fatty acid oxidation. This is particularly relevant given the increasing number of studies involving lipogenic tissues where the hormonal control of fatty acid biosynthesis is thought to be manifested through the interaction of AMPK and ACC. To date, a similar regulatory mechanism had not been examined in non-lipogenic tissue such as heart.

Within the cardiac muscle cell, the integrated regulation of contractile activity and the pathways associated with energy substrate metabolism remains, to a large extent, undefined. The influence of various hormones on these parameters is even less clearly understood. In **CHAPTER IV**, we examined the influence of insulin and glucagon on energy substrate utilization and on the enzymes thought to be important in regulating these metabolic pathways, namely ACC and AMPK.

In a previous study, (Collins-Nakai *et al.*, 1994) we have found that following inotropic stimulation of isolated working rat hearts with epinephrine, the increase in ATP required to meet the increased contractile function was derived almost exclusively from an up-regulation of glucose metabolism. This increase was proposed to result from the stimulation of the pyruvate dehydrogenase complex secondary to the increase in intramitochondrial $Ca^{++}$ levels (Denton *et al.*, 1978; McCormack *et al.*, 1990; Moreno-Sanchez, R., and Hansford, R.G, 1988). The results from this study are entirely consistent with the notion that inotropes that act via cAMP-dependent protein kinase (in this case

glucagon), selectively stimulate myocardial glucose metabolism and that this may occur via a $Ca^{++}$-dependent stimulation of PDC.

The lack of effect of glucagon on palmitate oxidation is in agreement with our previous studies with epinephrine and with the work of Kriesberg (1966). However, our results contrast the work by Gousios and Felts (1963) and a more recent study of Awan and Saggerson (1993). One factor that may have influenced the results in the latter study is that the authors utilized an isolated myocyte preparation which, athough beating, is not performing external work. The pathways of oxidative metabolism are driven by the increased demand for ATP resulting from the high levels of mechanical performance in the working heart preparations. In support of this, the palmitate oxidation rates reported by Awan and Saggerson (1993) are a mere one-sixtieth of those typically found in the working heart model. Thus, the window by which palmitate oxidation could potentially be increased by glucagon is much lower in working heart models as compared to an isolated cardiac myocyte preparation.

Hearts treated with a high dose of insulin (1000 μU/ml) did result in a significant reduction in steady-state rates of palmitate oxidation (Figure 6-2 and Table 6-2). This finding is entirely consistent with an earlier study that suggested that insulin may decrease myocardial palmitate oxidation while having no effect on free fatty acid extraction (Gousios and Felts, 1963). Our present findings of a reduced palmitate oxidation in response to insulin and the effects of insulin on ACC and AMPK activity provides the basis for the model proposed in Figure 6-4 and described below.

We have previously proposed an integral role for ACC in regulating the balance of between myocardial carbohydrate and fatty acid metabolism (**CHAPTER III**). Using dichloroacetate (DCA), and agent which causes a net

activation of the pyruvate dehydrogenase complex, we found a stimulation of myocardial glucose oxidation at the expense of fatty acid oxidation. This was accompanied by an increase in the levels of malonyl CoA which were inversely correlated to rates of fatty acid oxidation and positively correlated to acetyl CoA levels. Based on these previous results (**CHAPTER III**) we propose that a rise in intra-mitochondrial acetyl CoA levels activates carnitine acetyltranslocase which shuttles acetyl groups out of the mitochondria into the cytosol. Once re-esterified to CoA, the acetyl units act as a substrate for ACC which produces malonyl CoA and inhibits fatty acid oxidation at the level of CPT 1. Based on the results of **CHAPTER VI**, we propose that insulin does not decrease fatty acid oxidation by increasing this shuttle pathway, but rather by directly activating ACC activity.

The mammalian ACC enzyme itself is a large, complex multifunctional enzyme which catalyzes the carboxylation of acetyl CoA to form malonyl CoA (Kim *et al.*, 1989). Extensive characterization has been carried out on the 265 kDa isoenzyme at the level of both the protein and the gene. Regulation of this enzyme is complex and involves a number of both short and long term control mechanisms. Under certain physiological conditions, ACC can undergo rapid reversible phosphorylation resulting in an inhibition of enzyme activity Lane *et al.*, 1974; Kim *et al.*, 1983). However, this relationship between phosphorylation state and ACC activity is somewhat controversial with a number of reports indicating a direct relationship (Tipper and Witters, 1983; Munday *et al.*, 1988; Carling *et al.*, 1988) whereas others could not demonstrate such an effect (Lane *et al.*, 1973; Beaty and Lane, 1983).

The ACC-265 protein contains a number of sites which can be phosphorylated by at least seven different kinases. One of the most important kinases involved in the phosphorylation-induced inhibition of ACC activity is the

5' AMP-activated protein kinase (AMPK) which phosphorylates hepatic ACC on ser-79 (Hardie, 1989). AMPK, as the name implies, is activated by AMP either through direct allosteric activation (Carling *et al.*, 1989) or via a phosphorylation of an upstream kinase kinase (Moore *et al.*, 1991). This is supported by the results of **CHAPTER VI** which showed that regardless of the perfusion condition, heart AMPK activity was markedly reduced *in vitro* following either isolation under conditions which favour dephosphorylation of the enzyme or after incubation with protein phosphatase 2A. AMPK is a multi-substrate kinase that responds to alterations in the energy status of the cell and thus has been termed a "stress kinase". Indeed in rat hepatocytes, the induction of cellular stress by either substrate depletion (Moore *et al.*, 1991; Louis and Witters, 1992; Witters *et al.*, 1991) or heat shock (Corton *et al.*, 1994) results in an activation of AMPK which serves to switch off ATP consuming biosynthetic pathways, such as fatty acid and cholesterol synthesis, in an attempt to preserve ATP for the more essential processes such as maintenance of ion homeostasis.

Phosphorylation of hepatic ACC by AMPK is accompanied by marked decreases in the ACC-265 enzyme activity. Until recently, it was not known whether this same phenomenon occurred in the heart. Recent studies have suggested that the heart does express a significant amount of mRNA encoding AMPK (Gao *et al.*, 1995; Verhoeven *et al.*, 1995; Aguan *et al.*, 1994). In addition, we have recently shown that myocardial ACC activity is inversely correlated with both AMPK activity and levels of fatty acid oxidation in hearts subjected to ischemia followed by reperfusion (Kudo *et al.*, 1995) providing strong evidence for a role of AMPK in regulating myocardial fatty acid oxidation via phosphorylation and thus inhibition of ACC. Indeed, in **CHAPTER VI** an inverse correlation was found between myocardial ACC and AMPK activity in

control hearts and hearts treated with a high dose of insulin. On the other hand, hearts treated with glucagon did not reveal an altered AMPK activity although ACC activity was depressed. This would suggest that the effects of glucagon on heart ACC activity are brought about independently of changes in AMPK and may be due to direct phosphorylation of ACC by the cAMP-dependent protein kinase.

One factor that complicates interpretations concerning the regulation of ACC is the presence of at least 2 isoenzymes that vary in terms of their tissue distribution (Bianchi *et al.*, 1991). The heart contains a 265 kDa isoenzyme in addition to a 280 kDa isoenzyme which is predominantly expressed (**CHAPTER II**). Unfortunately, unlike the 265 kDa isoenzyme of ACC, the 280 kDa ACC has not been extensively characterized at either the protein or gene level. Trumble *et al.*, (1995) have recently performed a kinetic characterization of affinity-purified ACC from rat skeletal muscle which is expressed exclusively as a 272 kDa protein. Kinetic constants were found to differ from that of adipose tissue ACC which expresses the 265 kDa isoenzyme of ACC. In addition, based on recent structural evidence of both the ACC-280 protein (Winz *et al.*, 1994) and potential molecular cloning (Abu-Elheiga *et al.*, 1995), it does appear that the 280 kDa isoenzyme of ACC is most likely the product of a distinct gene . While it is not known whether the phosphorylation sites on ACC-265 are also present in ACC-280, it has been suggested that the ACC-280 in liver is more readily phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Winz *et al.*, 1994). In this study we did not attempt to correlate these changes in phosphorylation state to alterations in ACC-280 enzyme activity. However, our laboratory has also shown indirectly (Kudo *et al.*, 1996 in press) and directly

Kudo *et al.*, unpublished data) that phosphorylation of ACC inhibits enzyme activity

In **CHAPTER VI**, we found that glucagon treatment did result in an inhibition of myocardial ACC activity measured *in vitro* and that this was apprently due to an increase in ACC phosphorylation. This effect was not accompanied by similar changes in AMPK activity suggesting that increased phosphorylation of ACC is due to a kinase other than AMPK, most likely protein kinase A. Insulin, on the other hand, caused an activation of ACC that was associated with an apparent dephosphorylation of the enzyme due to an inhibition of AMPK activity. The reduced citrate dependence of ACC activity in hearts treated with insulin (Table 6-2) is also consistent with this theory given that earlier work has suggested that citrate dependence may be indicative of phosphorylation state (Jamil and Masden, 1987a,b). As suggested in the review by Kim (1989) citrate is thought to form a complex which maintains a configuration of the ACC dimer that is compatible with enzyme activity in the phosphorylated state. In addition, the greatest increase in citrate dependent ACC activity measured *in vitro* was in the hearts treated with glucagon (Table 6-2). Whether or not this ability of insulin to alter ACC activity and phosphorylation state is mediated by a direct inhibition of the AMPK itself or the kinase kinase or through a direct activation of a phosphatase remains to be determined. In either case, this activation of ACC with a concommitant decrease in the activity of the AMPK is similar to what is observed in studies on isolated hepatocytes (Witters and Kemp, 1992). These increase in ACC activity was not however, translated into changes in tissue malonyl CoA levels despite the fact that insulin did suppress fatty acid oxidation. However, as mentioned previously, one can not

conclude that measurements of tissue malonyl CoA levels are not necessarily predictive of those levels which are accessible to CPT I.

In summary, it was demonstrated that in the heart, glucagon causes a marked stimulation of glucose oxidation that is likely due to stimulation of the pyruvate dehydrogenase complex secondary to an increase in intramitochondrial $Ca^{2+}$. While glucagon was able to diminish ACC activity presumably through phosphorylation, this was not paralleled by an increase in the activity of AMPK. In contrast, perfusing hearts in the presence of insulin results in a supression of fatty acid oxidation that is associated with an increase in ACC activity and a reduced AMPK activity. Therefore, as depicted in Figure 6-4, this mechanism involving an integrated regulation of ACC and AMPK represents a method by which insulin may regulate myocardial fatty acid oxidation. This scheme may have implications in the pathology of diabetes where excessive myocardial fatty acid oxidation contributes to the poor heart function seen both prior to and following infarction.

## *E) Conclusion*

At the time research towards this thesis was initiated no information existed with respect to the role of ACC in non-lipogenic tissue apart from the demonstration of an isoenzyme of ACC expressed in heart. The work presented in this thesis has demonstrated that:

1) ACC is an important regulator of myocardial fatty acid oxidation rates via the production of malonyl CoA.

2) the various isoenzymes of ACC may be differentially regulated in lipogenic versus non-lipogenic tissues in disease states such as insulin-deficient diabetes.

3) further evidence supporting the ability of ACC to regulate myocardial fatty acid oxidation rates was also found in a genetic model of insulin-resistance.

4) like the liver, cardiac AMPK is capable of regulating myocardial ACC activity and therefore rates of fatty acid oxidation.

5) it was demonstrated that the ability of hormones, particularly insulin, to regulate myocardial metabolism may be via a pathway involving AMPK and ACC.

Therefore, with the resurgence of studies aimed at a metabolic approach to the study of heart function in health and disease, the work presented in this thesis has provided the groundwork necessary for the development of pharmacological agents targeting metabolic pathways in non-lipogenic tissue such as heart and skeletal muscle. This may have important implications in disease states where an over-reliance of tissues on fatty acid oxidation is detrimental.

# CHAPTER VIII

# FUTURE DIRECTIONS

## A) *5'AMP-activated protein kinase regulation of ACC*

While an extensive amount of work has been done regarding the regulation of ACC-265 by AMPK, much less is known with respect to AMPK regulation of ACC-280. The studies presented in this thesis have provided intial impetus for research in this area. However, a number of questions still remain to be answered in terms of the interaction of ACC-280 and AMPK. Initially, it must be determined whether the sites phosphorylated by AMPK on ACC-280 are the same as those on ACC-265. Unfortunately, there is very limited information on the peptide sequence of ACC-280 (Winz *et al.*, 1994) and the cDNA has proved extremely difficult to clone. Therefore, future studies need to be conducted to determine the peptide sequence of ACC-280 in order to reveal the potential phosphorylation sites.

These issues also have implications from a technical standpoint. The current method used to measure AMPK activity in heart tissue is based on the ability of the enzyme to phosphorylate the SAMS peptide. The SAMS peptide was designed to mimic the peptide sequence phosphorylated by AMPK on liver ACC-265. Therefore, the question remains as to the absolute AMPK activity that could be obtained if a peptide was used that corresponded to the heart or skeletal muscle ACC-280 sequence. Using an *in vitro* incubation of purified AMPK and semi-purified ACC, one could

determine the phosphorylation sites using a sequence analysis of the gel slices following electrophoretic separation of the ACC-265 and ACC-280 bands. The tissue most amenable to these studies would be skeletal muscle tissue (gastrocnemius) as it expresses only the ACC-280 isoenzyme (**CHAPTER IV**).

*B) Pharmacological manipulation of ACC/AMPK and metabolism*

Given the detrimental role of fatty acids in the development of various cardiovascular pathologies, the ability to pharmacologically manipulate fatty acid metabolism is of great interest. A few studies relevant to the work of this thesis have recently been published in this regard Arbeeny *et al.*, (1992) using a hamster model to study VLDL metabolism, found that admintistration of the ACC inhibitor, tetradodecyloxy furoic acid (TOFA), reduced fatty acid and triglyceride synthesis and decreased VLDL secretion by 90%. Numerous attempts were made over the course of research toward this thesis to examine the effect of TOFA on myocardial fatty acid oxidation. Unfortunately, a number of problems were encountered such as impure TOFA, solubility problems. and variable results in addition to the fact that the working heart model is not practical for determining dose-response relationships for pharmacological studies. This is clearly an area however, that needs to be explored further.

In another study, Sullivan *et al.*, (1994) have used the cell permeable activator of the 5' AMP-activated protein kinase, AICAR to examine the regulation of lipogenesis in rat adipocytes. These authors

demonstrated that stimulation of AMPK by AICAR is associated with a marked reduction in lipogenesis through an increased phosphorylation of ACC. Interestingly, preincubation of the adipocytes with AICAR also reduced the response of the adipocytes to the lipolytic actions of isoprenaline. As described by Hardie. (1989) AMPK is able to inhibit the action of cAMP on hormone-sensitive lipase by phosphorylating the site adjacent to the serine phosphorylated by cAMP-dependent protein kinase. A similar lipid lowering effect of AICAR has also been demonstrated in isolated rat hepatocytes where a simultaneous AMPK-dependent inhibition of both ACC and HMG-CoA reductase supresses fatty acid and cholesterol biosynthesis in a dose-dependent manner (Henin *et al.*, 1995). Clearly, similar studies should be performed in the intact heart where a direct link between AMPK and fatty acid oxidation could be explored.

A potential benefit of inhibition of fatty acid synthesis also applies to the JCR:LA corpulent rat model which was used in this thesis (**CHAPTER V**). When these insulin-resistant animals which exhibit profound hyperlipidemia and atherosclerotic lesions were treated with MEDICA 16, an ACC and ATP-citrate lyase inhibitor, a reduction in hepatic VLDL triglyceride production was found in conjunction with an activation of VLDL catabolism (Russell *et al.*, 1991). While the current evidence suggests that the hyperinsulinemia and hyperlipidemia in these animals contributes to the vascular and myocardial lesions, future studies with agents such as MEDICA 16 may provide insight into the use of hypolipidemic drugs in the treatment of insulin resistance.

The aspect that has never been looked at is the effects of these pharmacological manipulation of ACC and AMPK control of fatty acid

metabolism in non-lipogenic tissues. This is particularly important in the heart and skeletal muscle which primarily oxidize fatty acids. While inhibiting ACC in the liver may diminish fatty acid synthesis and be beneficial in states such as obesity, or hyperlipidemias, this may have grave consequences in muscle. Reducing ACC activity, and thereby malonyl CoA levels in muscle, favours an up-regulation of fatty acid oxidation and may render the animal in a state of insulin resistance. Similarly, in heart, an enhanced fatty acid oxidation is detrimental to the recovery of function when hearts are subjected to ischemia followed by reperfusion. A similar arguement also holds true for targeting AMPK. While it would be desireable to inhibit AMPK in heart to reduce fatty acid oxidation, this may result in a stimulation of fatty acid and cholesterol synthesis in liver and result in a hypertriglyceridemic, hypercholesterolemic animal.

One factor that may be taken advantage of is the differential expression of the ACC isoenzymes among tissues (**CHAPTER IV**). If it were possible to target only ACC-265 for pharmacological interventions designed to prevent hyperlipidemias and only ACC-280 when attempting to optimize myocardial metabolism the above noted problems could be avoided. Once again, these concerns highlight the need for further characterization of ACC-280 both structurally and functionally in order to fully address the therapeutic potential of manipulating this pathway.

*C) Differential regulation of ACC isoenzymes in pathologic states*

From the combined results presented in **CHAPTERS IV** and **V**, the activity of heart ACC is depressed in the insulin-deficient or insulin

resistant states. The insulin-deficient state caused marked depression in the expression and activity of ACC in liver and adipose tissue. Whether insulin-resistance causes a similar effect in the tissues studied in **CHAPTER IV** remains to be determined. This was originally to be a focus of this thesis but the tissues were not available at the appropriate time. Moreover, the influence pharmacological treatment with agents such as MEDICA to reduce the lipid abnormalities and improve heart function in the JCR:LA rat is another avenue yet to be studied. As discussed above, selective action on the lipogenic and oxidative pathways would be the desired feature in this corpulent, hyperlipidemic model.

*D) Role of AMPK in the regulation of myocardial glycogen metabolism*

The majority of work presented in this thesis has focussed on the regulation of myocardial fatty acid metabolism by ACC and AMPK. Interestingly, AMPK may also function in the regulation of pathways outside lipid metabolism. AMPK exhibits strong structural and functional homology to the SNF1 protein kinase family in yeast (Mitchelhill *et al.*, 1994). This SNF1 kinase in yeast is thought to function as a derepressor of genes associated with the metabolism of non-glucose sugars (i.e., invertase) (Carling *et al.*, 1994). Thus, a role for AMPK in mammalian carbohydrate metabolism may exist. Indeed, Carling and Hardie, (1989) have shown that AMPK does phosphorylate and inactivate glycogen synthase *in vitro*. The issue as to whether this mechanism may control glycogen synthesis in a physiological setting has not been determined. Using a labeling protocol similar to that described in **CHAPTER III**, it is possible to study glycogen turnover in the working heart and in turn, the effects of

pharmacological interventions such as AICAR which may activate AMPK and suppress glycogen synthesis.

E) *Determination of cellular CoA ester concentration*

One of the factors that has complicated our studies is the inability to measure the levels of CoA esters in various subcellular compartments. As a result, our studies were restricted to presenting values as total cellular CoA ester levels. As a reult we could not determine the accessibility of the various CoA esters to the enzymes associated with metabolic pathways. This difficulty is primarily due to the low concentrations of CoA esters in the cytosol versus mitochondria and the inability to accurately separate fractions containing the esters, due in part, to their amphiphilic nature (i.e., some cytosolic long chain acyl CoA can bind to and sediment with mitochondria following homogenization and centrifugation). In addition, attempts to isolate mitochondria and cytoplasmic fractions from fresh hearts would mean that malonyl CoA and other esters would be actively metabolized during the isolation rendering subsequent measures invalid. Unfortunately, at present there are no methods known by which one can distinguish the levels of CoA esters among the cytosol, mitochondria or any other subcellular compartment in the intact heart although such a technique would prove to be extremely useful in metabolic studies. Therefore, the development of a methodology to accurately determine the levels of CoA esters within the subcellular compartments should be the focus of future work.

# CHAPTER IX

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# CHAPTER X

## Curriculum Vitae


## JAMES GAMBLE

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|---|---|
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**EDUCATION**

| | |
|---|---|
| Sept. '91 to present | Ph.D.<br>University of Alberta<br>Department of Pharmacology<br>Thesis: Acetyl CoA carboxylase regulation of cellular energy metabolism |
| Sept. '88 to Sept. '90 | M.Sc.<br>University of Windsor<br>Department of Kinesiology<br>Thesis: The force-interval relationship in rat cardiac muscle; evidence for length modulation of force recovery |
| Sept. '84 to June '88 | B.Sc. (Hons)<br>York University<br>Department of Physical Education |

## AWARDS

**Research Studentship** - Muttart Diabetes Research and Training Center (July 1992 to July 1993)

**Mary Louise Imry Graduate Award** - University of Alberta (June 1993)

**Research Traineeship** - Heart and Stroke Foundation of Canada (July 1993 to present)

**Finalist - Student Presentation Award** - Canadian Cardiovascular Society Meeting, Vancouver, British Columbia (October 1993).

**J. Gordon Kaplan Graduate Award** - University of Alberta (July 1994)

**Research Studentship** - Alberta Heritage Foundation for Medical Research (January1994 to present)

**Research Fellowship** - Heart and Stroke Foundation of Canada (January 1995)
Endocrine/Metabolism Division
Dartmouth Medical School, Hanover N.H. (USA)
Supervisor: Dr. Lee A. Witters

## RESEARCH EXPERIENCE

| | |
|---|---|
| Sept. '91 to present | Graduate student<br>Supervisor: Dr. G.D. Lopaschuk |
| Sept. '90 to Sept. '91 | Research Assistant, Department of Biological Sciences, University of Windsor<br>Supervisor: Dr. P.B. Taylor |
| Sept. '88 to Sept. '90 | Graduate student<br>Supervisor: Dr. K.A. Kenno |

## TEACHING EXPERIENCE

| Date: | Course: | Activity: |
|---|---|---|
| 05/94 | Cardiovascular Pharmacology | lecture (1 hour) |
| 08/93 | Biochemical Techniques | lab instructor (6 hours) |
| 09/92 | Pharmacological Techniques | lab instructor (3 hours) |
| 10/92 | Pharmacological Techniques | lab instructor (3 hours) |
| 09/91<br>09/92 | Computing Science<br>Bank of Montreal staff | instructor (52 hours) |
| 01/91 | Cardiovascular Physiology | lecture (1 hour) |
| 01/90 | Exercise Physiology | lab instructor (52 hours) |
| 09/89 | Human Anatomy | lab instructor (52 hours) |
| 01/89 | Exercise Physiology | lab instructor (52 hours) |
| 09/88 | Human Anatomy | lab instructor (52 hours) |

## ORAL PRESENTATIONS

1. Glycolysis and glucose oxidation during reperfusion of ischemic hearts from diabetic rats. *Department of Pharmacology Seminar*, University of Alberta, March 1992.

2. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. Student presentation award competition. *Canadian Cardiovascular Society Meeting*, Vancouver Canada, October 1993.

3. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *American Heart Association Meeting*, Atlanta, U.S.A., November 1993.

4. Acetyl CoA carboxylase regulates myocardial fatty acid oxidation. *Department of Pharmacology Seminar*, University of Alberta, February, 1993.

5. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *Lipid and Lipoprotein Research Group Annual Retreat*, May, 1993

6. Differential tissue regulation of acetyl CoA carboxylase in diabetes. *Department of Pharmacology Seminar*, University of Alberta, January, 1994.

7. Differential tissue expression of acetyl CoA carboxylase in diabetes. *Lipid and Lipoprotein Research Group Annual Retreat*, May, 1994

8. Acetyl CoA carboxylase regulation of myocardial fatty acid oxidation in insulin resistance. *Department of Pharmacology Seminar*, University of Alberta, March, 1995.

9. Acetyl CoA carboxylase regulation of fatty acid oxidation in the JCR:LA corpulent rat heart. *Lipid and Lipoprotein Research Group Annual Retreat*, May, 1995.

## PUBLICATIONS

1. **Gamble, J.**, Taylor, P.B., and Kenno, K.A. Myocardial stretch alters twitch characteristics and calcium loading of the sarcoplasmic reticulum in rat ventricular muscle. *Cardiovasc. Res.* 26:865-870, 1992.

2. Lopaschuk, G.D., Broderick, T., and **Gamble, J.** Pharmacological control of fatty acid and glucose metabolism in the heart (invited review). *Cardiologia* 37: 29-33, 1992.

3. **Gamble, J.**, and Lopaschuk, G.D. Glycolysis and glucose oxidation during reperfusion of ischemic hearts from diabetic rats. *Biochim. Biophys. Acta* 1225: 191-199, 1994.

4. Saddik, M., **Gamble, J.**, Witters, L.A., and Lopaschuk, G.D. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *J. Biol. Chem.* 268: 25836-25845, 1993).

5. Lopaschuk, G.D., Belke, D.D., **Gamble, J.**, Itoi, T., and Schonekess, B.O. Regulation of fatty acid oxidation in the heart. (invited review, *Biochim. Biophys. Acta* 1213:263-276, 1994).

6. Lopaschuk, G.D., and **Gamble, J.** Acetyl-CoA carboxylase: a novel regulator of fatty acid oxidation in the heart. (1993 Merck-Frost Award to GDL, invited review, *Can. J. Physiol. Pharmacol.* 72:1101-1109).

7. Broderick, T.L., Panagakis, G., DiDomenico, D., **Gamble, J.**, Lopaschuk, G.D., Shug, A.L., and Paulson, D.J. L-carnitine effects on cardiac function and myocardial substrate utilization in carnitine-deficient hearts. (*Cardiovasc. Res.* in press)

8. **Gamble, J.**, Makinde, O., Russell, J., and Lopaschuk, G.D. Regulation of myocardial metabolism in insulin resistance: the role of acetyl CoA carboxylase and AMP-activated protein kinase. (submitted).

9. **Gamble, J.**, Witters L.A., and Lopaschuk, G.D. Differential tissue regulation of acetyl CoA carboxylase in diabetes. (submitted).

10. Makinde, A.O., **Gamble, J.**, Witters, L.A., and Lopaschuk, G.D. Hormonal regulation of 5'AMP activated protein kinase in the newborn rabbit heart. (manuscript in preparation)

11. **Gamble, J.**, , and Lopaschuk, G.D. Hormonal regulation of energy substrate metabolism in the heart. (submitted).

## ABSTRACTS

1. **Gamble, J.**, Kenno, K.A., Taylor, P.B., and Helbing, R. The effect of resting muscle length on the force-interval relationship in rat ventricular muscle. *J. Mol. Cell. Cardiol.* 22(suppl. I) S32 (1990).

2. **Gamble, J.**, Kenno, K.A., and Taylor, P. Diastolic length alters myocardial SR calcium loading. *J. Mol. Cell. Cardiol.* 23(suppl. III) S77 (1991).

3. **Gamble, J.**, and Lopaschuk, G.D. Glucose oxidation and glycolysis in fatty acid perfused diabetic rat hearts during global ischemia-reperfusion. *J. Mol. Cell. Cardiol.* 24(suppl. III) S22 (1992).

4. **Gamble, J.**, Saddik, M., and Lopaschuk, G.D. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *J. Mol. Cell. Cardiol.* 25(suppl. I) S66 (1993).

5. **Gamble, J.**, Saddik, M., and Lopaschuk, G.D. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *Can. J. Cardiol.* 9(suppl. E) 73E (1993).

6. **Gamble, J.**, Saddik, M., Witters, L.A., and Lopaschuk, G.D. Acetyl CoA carboxylase regulation of fatty acid oxidation in the heart. *Circulation.* 88(4) pt 2. (1993).

7. **Gamble, J.**, Colins-Nakai, R., Barr, R., Humen, D., and Lopaschuk, G.D. Ranolazine stimulates glucose oxidation in isolated working rat hearts. *Can J. Physiol. Pharmacol.* 72(suppl. 1) P1.4.9 (1994).

8. **Gamble, J.**, Witters L.A., and Lopaschuk, G.D. Differential tissue expression of acetyl CoA carboxylase in diabetes. *Can. J. Cardiol.* 10(suppl. C) 7 (1994).

9. **Gamble, J.**, Makinde, O., Russell, J., and Lopaschuk, G.D. Heart AMP-activated protein kinase activity in insulin-resistant rats. *Western Perinatal Research Meeting* (1995).

10. Makinde, O., **Gamble, J.**, and Lopaschuk, G.D. Insulin inhibits 5'AMP-activated protein kinase in the newborn heart. *Western Perinatal Research Meeting* (1995).

11. **Gamble, J.**, Makinde, O., Russell, J., and Lopaschuk, G.D. Heart AMP-activated protein kinase activity in insulin-resistant rats. *J. Mol. Cell. Cardiol. 27 (5)* (1995).

12. Makinde, O., **Gamble, J.**, and Lopaschuk, G.D. Insulin inhibits 5'AMP-activated protein kinase in the newborn heart. *J. Mol. Cell. Cardiol. 27 (5)* (1995).

13. **Gamble, J.**, Witters L.A., and Lopaschuk, G.D. Regulation of Acetyl CoA carboxylase (ACC) in diabetes. *J. Mol. Cell. Cardiol.* (1995).