Aspects of fatty acid handling in the diabetic rat heart

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ASPECTS OF FATTY ACID HANDLING IN THE DIABETIC RAT HEART

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ASPECTS OF FATTY ACID HANDLING IN THE DIABETIC RAT HEART

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
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Chapter 1

General Introduction

Background and aims of the study

Fatty acid transport and metabolism

In order to supply the organs in the body with sufficient amounts of blood, the heart is in a continuous need of energy. Although the heart can be considered as an omnivorous organ, circulating long-chain fatty acids (FA) are quantitatively the most important substrates for the heart under normal conditions ¹.

Before FA can be used to sustain the cardiac energy need, FA must be transported across several barriers. In blood plasma FA is bound to albumin or incorporated in triacylglycerols (TG) (figure 1.1). FA can be taken up directly from albumin or via release of FA from circulating TG by lipoprotein lipase (LPL). FA have to cross the luminal membrane, the cytoplasm, and the abluminal membrane of the endothelial cells.

After transport over the endothelial cell layer, it is most likely bound to albumin in the interstitial compartment. The next barrier is the sarcolemma of the cardiomyocytes. There are two possible pathways by which FA can cross the membrane of the cardiomyocytes.

It is a matter of debate whether FA is transported across the sarcolemma via passive diffusion ^{2,3} or via protein mediated transport. At present, three proteins are associated which may facilitate FA membrane transport namely, fatty acid translocase (FAT/CD36) ⁴, plasmalemmal fatty acid binding protein (FABPpm) ⁵ and fatty acid transport protein (FATP) ⁶. For the latter protein it is also documented that it possesses fatty acid acyl-CoA synthetase activity ⁷. After FA have crossed the sarcolemma, it is bound to cytoplasmic fatty acid-binding protein (FABPc) ⁸, acting as a sink, and most likely involved in the transport through the cytoplasm to the mitochondria. On the outer mitochondrial membrane FA are converted to FA-CoA by the enzyme acyl-CoA synthetase (ACS). FA-CoA can enter multiple pathways. It is documented that FA-CoA is bound to acyl-CoA binding protein (ACBP), taking care of the further distribution of FA-CoA to the several sites of interaction ⁹.

In the heart, muscle-type carnitine acyl transferase (mCPT1) is involved in the first step of transport of the fatty acyl chains of FA-CoA across the mitochondrial inner membrane, by catalyzing the conversion of FA-CoA in FA-carnitine. Inside the mitochondrial matrix FA-CoA is reconstituted and stepwise degraded during β -oxidation to form acetyl-CoA. Subsequently, acetyl-CoA enters the citric acid cycle where it becomes further metabolised. Furthermore, FA-CoA can be used for synthesis of diacylglycerols (DG) and from thereon to phospholipids (PL) and TG ¹⁰.

In addition to serving as energy carrying, oxidizable substrates, FA are involved in signal transduction pathways. Evidence is accumulating that FA

can interact via the peroxisome proliferator activated receptor (PPAR) with nuclear gene expression resulting in, among others, enhanced production of FA handling proteins^{11, 12}.

Figure 1.1 Schematic representation of fatty acid uptake, transport, and handling in the cardiomyocyte.

FA, long chain fatty acids; LPL, lipoprotein lipase; TG, triacylglycerols; FATP, fatty acid transport protein; FABPpm, plasmalemmal fatty acid binding protein; FAT/CD36, fatty acid translocase; FA-CoA, fatty acyl coenzyme A; FABPc, cytoplasmic fatty acid binding protein; DG, diacylglycerols; PL, phospholipids; ACBP, acyl-CoA binding protein; ACS, acyl-CoA synthetase; mCPT1/2, muscle-type carnitine palmitoyl transferase 1 or 2; CAT, carnitine acyltransferase.

Moreover, FA-CoA can be used for covalent attachment of FA to protein either via N-myristoyl transferase (NMT) leading to myristoylation¹³ or via unknown mechanisms to S-acylation (palmitoylation)¹⁴. The latter processes lead to alterations in cellular localization and/or biological activity of the acylated proteins.

Diabetes Mellitus

Diabetes Mellitus is a clinical syndrome characterized by hyperglycemia. It is caused by a relative or absolute deficiency of insulin or resistance to the action of insulin. These two distinct forms of diabetes are named respectively, diabetes type 1 and type 2. During diabetes the lack (of action) of insulin causes a wide variety of metabolic derangements. This includes abnormal elevation of blood sugar, increases in plasma levels of FA, amino acids, TG, and alterations in hormone levels and plasma ketone bodies¹⁵⁻²⁰. Elevations of tissue FA and TG are associated with the development of diabetic cardiomyopathies, although the exact mechanism is not yet known²¹. Experiments on isolated hearts strongly suggest that cardiac FA oxidation is substantially increased in diabetic heart²²⁻²⁴. These findings are, however, at variance with observations in patients and intact animals, indicating no change or even a decline in cardiac FA utilization²⁵. Besides the discrepancy, in general, novel therapies are aiming to lower plasma FA and TG levels since they are considered the prime targets in preventing diabetic cardiomyopathies.

Aims

In the present thesis, the overall aim was to enlarge our knowledge about abnormalities in cardiac FA handling under diabetic conditions. Experiments were performed on a variety of experimental models, including streptozotocin-treated rats to evoke type 1 diabetes, cardiomyocytes isolated from diabetic rat hearts, and H9c2 cell line, derived from embryonic rat hearts.

The sub-aims of the thesis were:

- Detailed analysis of plasma and cardiac tissue levels of fatty acids (FA), triacylglycerols (TG) and phospholipids (PL) and their relative fatty acyl composition of diabetic rats.
- Assessment of the presence of acylated proteins in cardiac tissue and the effect of diabetes thereon.
- Exploration of the uptake and utilization rate in diabetic cardiomyocytes and the interference of elevated concentrations of ketone bodies.
- Assessment of mechanisms underlying palmitoylation of proteins making use of the embryonic cardiac derived H9c2 cell line

- Exploration of cardiac adaptation to diabetic conditions on the level of cofactors, enzymes and mRNA. Assessment of the effect of ketone bodies and FA on cardiac gene expression making use of the neonatal cardiomyocyte model.

Scope of the thesis

In *chapter 1* a short introduction concerning cardiac FA handling and diabetes is given and the aims of the study are indicated.

In *chapter 2* mechanisms underlying the uptake and oxidation of FA are reviewed. Attention is paid to critical steps in the transport route of FA with special reference to skeletal and heart muscle. Processes underlying protein acylation are discussed in detail.

A sensitive and reliable analytic technique to measure fatty acyl moieties is described in *chapter 3*. This technique is used to determine the total amount and relative composition of the long-chain FA, triacylglycerols, and phospholipids in cardiac tissue and blood plasma of normal and diabetic rats. Capillary gas chromatography was used to determine the content and relative composition of the acyl chains covalently bound to proteins in cardiac tissue, and to assess the effect of diabetes on protein acylation. The findings are presented in *chapter 4*. Furthermore, the influence of ketone bodies on the degree of protein acylation in adult cardiomyocytes derived from control and diabetic rat hearts was investigated.

The H9c2 cell line was used as a model system to explore several aspects of protein acylation in the cardiac muscle. *Chapter 5* describes protein acylation as function of time, relative to incorporation of fatty acyl moieties into various lipid classes. Furthermore, the influence of exogenous palmitate on the extent of S-acylation was investigated. In addition, the effect of two inhibitors of protein acylation, tunicamycin and cerulenin, was studied in H9c2 cells.

In *chapter 6* the effect of ketone bodies, which are substantially elevated in the diabetic state, on cardiac uptake and fatty acid handling was studied. To this end cardiomyocytes were isolated from control and diabetic rat hearts. The cardiomyocytes were exposed to ketone bodies in the presence of FA levels corresponding with those found in plasma of control and diabetic rats.

Chapter 7 explores several facets of experimentally induced diabetes on cardiac metabolism, including tissue content of substrates, cofactors, enzyme activities and mRNA levels. Moreover, in rat neonatal cardiomyocytes the effect of FA and ketone bodies on the expression of metabolic genes was investigated. Special attention is paid to carnitine

palmitoyltransferase (CPT1), an enzyme involved in the first step of transporting fatty acyl chains across the mitochondrial inner membrane.

Finally, a general discussion of the results obtained and the implications of diabetes in cardiac FA uptake and handling as well as protein acylation are given in *chapter 8*.

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Chapter 2

Fatty acid handling in myocardial tissue

Part of this chapter is published in Ger J. van der Vusse, Marc van Bilsen, Jan F. Glatz, Danny. M. Hasselbaink and Joost.J. Luiken. Critical steps in cellular fatty acid uptake and utilization. Mol. Cell. Biochem 239 (2002), 9-15

Abstract

Despite decades of extensive research, the transport routes, mechanisms of uptake, metabolic fate, and points of flux control of long-chain fatty acids (FA) in mammalian organs are still incompletely understood. In non-fenestrated organs such as heart and skeletal muscle, membrane barriers for blood-borne FA are the luminal and abluminal membranes of endothelial cells, the sarcolemma and the mitochondrial membranes. Transport of FA through the phospholipid bilayer of the cellular membrane is most likely accomplished by diffusion of protonated FA. Evidence is accumulating that membrane-associated proteins, such as plasmalemmal Fatty Acid-Binding Protein (FABPpm) and Fatty Acid Translocase (FAT/CD36), either alone or in conjunction with Albumin Binding Protein (ABP), are instrumental in enhancing the delivery of FA to the cellular membrane.

Inside the cell, cytoplasmic Fatty Acid-Binding Proteins (FABPc) are involved in diffusion of FA from the plasmalemma to the intracellular sites of conversion, such as the mitochondrial outer membrane. After conversion of FA to FAcOA, the fatty acyl chain is transported across the mitochondrial inner membrane in a carnitine-mediated fashion.

Uptake and utilization of FA by muscle cells are finely tuned, most likely to avoid the intracellular accumulation of FA, as these are cytotoxic at high concentrations. On a short-term basis, net uptake is, among others, regulated by intracellular translocation of FAT from intracellular stores to the sarcolemma and by the concentration gradient of FA across the sarcolemma. The latter implies that, among others, the rate of FA utilization determines the rate of uptake. The rate of FA oxidation is governed by a variety of factors, including malonyl-CoA, the ratio acetylCoA/CoA and the availability of competing substrates such as glucose, lactate, and ketone bodies. Besides, FA are also involved in co-translational and post-translational modification of proteins, *i.e.*, protein acylation. Long-term regulation of uptake and utilization is accomplished by alterations in the expression of genes, encoding for FA-handling proteins. Recent findings indicates that FA themselves are able to modulate the expression of FA-handling genes via nuclear transcription factors such as peroxisome proliferator-activated receptors (PPARs).

Introduction

Long-chain fatty acids (FA) are serving a variety of roles in cell homeostasis. In addition to constituents of membrane PL they are substrates for mitochondrial energy conversion and act as energy store after esterification into the TG pool. Moreover, FA are involved in co-translational and post-translational modification of proteins, *i.e.*, protein acylation, and modulate gene expression via interaction with members of the PPAR family ¹⁻⁴. Since most organs, including cardiac and skeletal muscle, are not capable of *de novo* synthesizing FA and possess limited amounts of FA stored in the cytoplasmic compartment, they heavily rely on exogenous FA. The mechanisms underlying the uptake and transport of FA are incompletely understood. In this brief review the potentially critical steps in the transport route of blood-borne FA will be discussed with special reference to heart and skeletal muscle. Moreover, attention is paid to processes underlying acylation of intracellular proteins. These subject matters have been reviewed elsewhere recently ^{1, 5-7} and will not be addressed in detail here.

Fatty acid handling in myocardial tissue

Supply of FA to organs

Circulating FA are supplied to the organ either complexed to plasma albumin or esterified into glycerol, forming the TG core of circulating chylomicrons and very low-density lipoproteins ⁵. Prior to uptake by the parenchymal cells, FA are liberated from plasma TG by the catalytic action of lipoprotein lipase, attached to the luminal side of the endothelium ².

Both cellular membranes composed of a phospholipid bilayer, and aqueous spaces such as the interstitial and intracellular compartment represent constraints for FA on their route from blood to the mitochondrial outer membrane due to the amphiphilic nature of the FA molecule. The mitochondrial outer membrane is the site where the first committed step in overall FA metabolism takes place, *i.e.*, the activation of FA to fatty-acylCoA catalyzed by acylCoA synthetase (ACS, figure 2.1).

The endothelium and interstitial compartment

In liver, the FA-albumin complex diffuses through the fenestrae of the endothelium into the spaces of Disse and the interstitial compartment ⁸. However, in organs with non-fenestrated endothelium such as cardiac and

skeletal muscle the endothelial cells lining the microvascular compartment form a serious transport barrier are blood-borne FA. Accordingly, FA needs to be dissociated from their plasma albumin carrier prior to transport through the endothelium⁹.

Figure 2.1 Schematic representation of long-chain fatty acid uptake and transport in organs with non-fenestrated endothelium.

FA, long-chain fatty acid; FABP_c, cytoplasmic Fatty Acid-Binding Protein; ACS, acyl CoA synthetase; ACBP, Acyl CoA-Binding Protein; VLDL, very low density lipoprotein; chylomicrons; LPL, lipoprotein lipase.

Circumstantial evidence indicates that in muscle tissue diffusion of FA through the endothelial clefts is inconsequential⁹. The bulk of FA most likely diffuses through the luminal membrane of the endothelial cell, the endothelial cytoplasm and subsequently through the abluminal membrane to be transmitted to albumin present in the interstitial compartment. Detailed information on the mechanisms underlying transendothelial transport of FA in heart and skeletal muscle is, however, lacking.

Earlier studies suggest that specific interaction of the albumin-FA complex with the outer leaflet of the luminal membrane or with proteins associated with the membrane facilitates the dissociation of the FA-albumin complex and, hence, the transfers of FA from the vascular to the interstitial compartment¹⁰. Endothelial cytoplasmic Fatty Acid-Binding Protein, FABP_c, may play a role in the transport of FA from the luminal to the abluminal endothelial membrane. The diffusion of FA from the abluminal membrane to

the plasmalemma of the parenchymal cell is facilitated by interstitial albumin (figure 2.1).

The sarcolemma

Recent findings indicate that the plasmalemma (sarcolemma) of cardiac and skeletal muscle cells represent a major constraint for FA, bound to albumin in the interstitial compartment ¹¹. The traditional view of transport of FA across biological membranes, including the sarcolemma, composed of a phospholipid bilayer is by passive diffusion of protonated FA driven by the concentration difference of FA in the two compartments separated by the membrane ¹²⁻¹⁴. Studies on experimental membranes revealed that diffusion of FA through the phospholipid bilayer is fast, leading the investigators to conclude that auxiliary proteins are not required for transmembrane movement of FA ¹⁴. Evidence, however, is accumulating that proteins are involved in the overall transport of FA from the interstitial compartment to the cytoplasm of the parenchymal cell.

At present, at least three different membrane-associated proteins have been identified putatively involved in FA trafficking across the membrane. Stremmel and co-workers ¹⁵ identified a 40-43 kDa protein in the plasmalemma of hepatocytes, displaying high affinity for FA. This protein, designated plasmalemmal Fatty Acid-Binding Protein, FABPpm, is a peripherally bound protein and expressed in a variety of tissues, including heart and skeletal muscle. Antibodies against FABPpm significantly inhibit the cellular uptake of FA ¹⁶. Later studies by Abumrad and colleagues ¹⁷ established the existence of an 88kDa integral membrane protein, called Fatty Acid Translocase, FAT, which turned out to be homologous to the human leukocyte differentiation antigen CD36. This protein is expressed in a host of tissues but not in liver ^{17, 18}. Transfection of this membrane protein into fibroblast induced a saturable, high-affinity component of oleate uptake ¹⁹. Thirdly, Schaffer and Lodish ²⁰ discovered a 63kDa protein via expression cloning of an adipocyte cDNA library in COS7 cells. More recent findings ²¹ indicate that this integral membrane protein, designated Fatty Acid Transport Protein, FATP, possesses catalytic properties, promoting the conversion of FA into fatty-acylCoA, and may therefore modulate the uptake of FA by creating an intracellular sink for FA. Finally, a series of membrane-associated proteins has been discovered expressing a relatively high affinity for albumin ²²⁻²⁴.

In addition to the earlier mentioned inhibitory effect of antibodies of FABPpm on cellular FA uptake, the outcome of recently performed experiments points towards a quantitatively important role of membrane-

associated proteins in transmembrane FA transport. Mild treatment of isolated cardiomyocytes with trypsin significantly reduced the uptake of FA²⁵. Phloretin, a universal inhibitor of membrane-associated transport proteins, severely hampered cardiomyocyte FA uptake. Of interest is the fact that also sulfo-N-succinimidyl oleate (SSO), a ligand of FAT/CD36, inhibited FA uptake by isolated cardiomyocytes. More recently it was shown that FA uptake by sarcolemmal giant vesicles prepared from rat heart is also blocked by trypsin, phloretin, SSO, and antibodies against FABPpm²⁶. Since these vesicles lack FA converting enzymes an indirect effect of these inhibitory agents on membrane FA transport is rather unlikely. Interestingly, the inhibitory actions of anti-FABPpm and SSO were found to be non-additive, which indicates that FABPpm and FAT/CD36 cooperate in facilitating the translocation of FA across the plasmalemma.

In order to unify the notion that the transport of FA across the phospholipid bilayer of biological membranes is accomplished by passive diffusion with the recent findings that membrane-associated proteins are involved in cellular FA uptake, we propose the following paradigm. Proteins such as albumin-binding proteins (ABP) are trapping the albumin-FA complex at close vicinity of the outer leaflet of the phospholipid bilayer (figure 2.2).

The interaction of the albumin-FA complex with ABPs facilitates the dissociation of the ligand from the complex. FA accumulating in the stagnant water layer of the extracellular compartment bind to the peripheral membrane protein FABPpm and are subsequently transmitted to the integral membrane protein, FAT/CD36. Alternatively, FA is directly transferred from albumin to FABPpm. This process substantially increases the amount of FA available for transport through the membrane.

The latter is accomplished by passive diffusion of protonated FA, most likely by a flip-flop mechanism of the fatty acyl moieties through the outer and inner leaflet of the phospholipid bilayer. It can, however, not be excluded that FAT/CD36 is physically involved in translocating non-protonated FA through the phospholipid bilayer by facilitated diffusion. Moreover, membrane-associated proteins may also play a crucial role in the release of FA from the inner leaflet of the plasmalemma into the aqueous cytoplasm of the parenchymal cell.

Figure 2.2 Hypothetical transport of long-chain fatty acids across the plasmalemma of a muscle cell.

FA, long-chain fatty acid; ABP, albumin-binding protein; FABP_{pm}, plasmalemmal Fatty Acid-Binding Protein; FAT, Fatty Acid Translocase; FATP, Fatty Acid Transport Protein; ACBP, Acyl CoA-Binding Protein; ACS, acyl CoA synthetase.

The cytoplasm

Pioneering studies of Ockner and co-workers²⁷ have disclosed the presence of cytoplasmic proteins with a high affinity for FA. Mathematical models and *in vitro* studies have later shown that these proteins, designated Fatty Acid-Binding Proteins, FABPc, are indispensable for bulk transport of FA from the plasmalemma to the main intracellular site of conversion, *i.e.*, the mitochondrial outer membrane, home of fatty acylCoA-synthetase^{28,29}. FABPc, a 14-15 kDa protein, consists of a family of at least 13 members, slightly differing in amino acid sequence depending on the cell type of expression. The physiological role of FABPc in cardiac myocytes has recently been firmly established by Binas and co-workers³⁰ and Schaap and colleagues³¹. They investigated the FA uptake in the heart of intact mice carrying a targeted disruption of the gene coding for heart-type FABPc, and FA utilization by cardiomyocytes isolated from H-FABPc deficient mouse

heart, respectively. Other studies on immortalised cell lines and other types of parenchymal cells and experiments performed on immortalized cell lines underlines the notion that FABPc is indeed involved in cytoplasmic FA trafficking³².

It is still a matter of debate whether FA are transmitted from the inner leaflet of the phospholipid bilayer to FABPc, one, by collisional interaction of FABPc with the membrane, or two, after release of FA into the water layer adjacent to the membrane prior to binding to FABPc. Evidence provided by Storch and colleagues³³ indicates that the former mechanism is true for muscle FABPc and the latter for liver FABPc. It is of interest to note that Spitsberg and co-workers observed in the bovine mammary gland physical interaction between soluble FABP and membrane-associated FAT/CD36³⁴.

It remains to be established whether FABPc physically interacts with fatty acylCoA-synthetase or with PL domains in close vicinity of the enzyme in the mitochondrial outer membrane. This kind of interaction may facilitate and regulate the transmission of cytoplasmic FA to the enzyme, which catalyses the first committed step in intracellular FA metabolism. The product, fatty acylCoA, serves as substrate for reacylation of PL, formation of the intracellular lipid store, TG, and/or intramitochondrial oxidation to CO₂ and H₂O after transport through the mitochondrial inner membrane in a carnitine-mediated process (figure 2.1). Moreover, acylCoA is also a substrate for the acylation of proteins, *i.e.* S-acylation and myristoylation. With respect to acylCoA it is important to note that another protein involved in cellular FA handling, namely the cytoplasmic acylCoA-binding protein (ACBP), is thought to be involved in the vectorial delivery of the fatty acyl moieties to the distinct metabolic pathways^{35, 36}.

Acylation of cellular proteins

Besides modifications of proteins via *e.g.* phosphorylation and glycosylation, proteins can also become modified via interactions with hydrophobic moieties. These moieties include isoprenoids (farnesyl and geranylgeranyl), glycosyl-phosphatidyl inositol anchors and, discovered more recently, fatty acids³⁷⁻⁴⁰. Although the initial observation that proteins were modified with lipids was done in 1956⁴¹, it took until 1969 before a detailed study was performed by Braun and Radin⁴². They showed that FA chains were found neither free nor as part of complex lipids, but instead covalently associated to the myelin proteolipid protein. Several experiments showed that modifications with palmitate and those with myristate were of a different

nature. Proteins modified by either fatty acid could be distinguished from one another^{43, 44}, and thus represented two distinct classes of protein modification (table 2.1). The myelin proteolipid protein, earlier mentioned, appeared to be modified by palmitate. Protein myristoylation was first described by Carr and colleagues, who identified the N-terminal blocking group on the catalytic subunit of A kinase and calcineurin B as tetradecanoate (myristate; C14:0)^{45, 46}.

There are a number of differences between modifications with myristate and palmitate. Myristoylation occurs through amide linkages on the N-terminal glycine residue after removal of the initiator methionine, and this attachment takes place during protein synthesis, *e.g.* co-translational modification. Furthermore, myristoylation is highly specific due to the strict substrate selectivity of the enzyme responsible for myristoylation, *e.g.* N-myristoyltransferase^{38, 47}. Generally, the attached myristic acid is stable and has a half-life similar to that of the protein to which it is bound. There are, however, two reports indicating that demyristoylation of particular proteins can occur^{48, 49}. Palmitoylation, or S-acylation, occurs on cysteine residues through a thioester linkage in a wide variety of sequence contexts, and is a post-translational modification. While palmitate is the most common fatty acid involved in this type of modification, other FA can also become incorporated into cellular proteins via a thioester linkage, including myristate, stearate, and arachidonate^{50, 51}. In general, S-acylation is a dynamic modification with a half-life of the fatty acyl moieties shorter than that of the polypeptide backbone, indicating the existence of palmitoylation / depalmitoylation cycles⁵²⁻⁵⁴. Caveolin-1, a protein which is S-acylated, is the only known protein not to undergo depalmitoylation⁵⁵. In contrast to myristoylation, the enzymology underlying S-acylation and deacylation is unknown.

There is still controversy whether S-acylation takes place in a non-enzymatic or in an enzymatic fashion. Although several *in vitro* studies showed that S-acylation can take place in the absence of enzymes^{56, 90-92}, evidence is accumulating that the same does not hold true *in vivo*. Leventis and colleagues⁹³ have shown in an *in vitro* study that non-enzymatic S-acylation was inhibited at physiological ratios of ACBP-acylCoA, leading to a substantial increase in the half time of the turnover rate of the acyl moiety.

Table 2.1 Acylated proteins in the heart

<i>Palmitoylated proteins</i>	
Gα0, Gαs, Gαi1, Gαz ⁵⁶⁻⁵⁸	GLUT1 ⁵⁹
H-ras, N-ras, Ki-ras ⁶⁰	PSD-95 ⁶¹
Src family (Yes, Fgr, Fyn, Lck) ⁶²⁻⁶⁵	CD36 ⁶⁶
α2-adrenergic receptor ⁶⁷	Caveolin-1 ⁶⁸
β2-adrenergic receptor ⁶⁷	Phospholipase-D1 ⁶⁹
Insulin receptor (α and β subunits) ^{70,71}	Phospholipid scramblase ⁷²
Mannose-6-phosphate receptor ⁷³	PKC ⁷⁴
L-type calcium channel (β2α-subunit) ⁷⁵	eNOS ⁷⁶
Sodium channels (α-subunit) ⁷⁷	IGF-1 ⁷¹
GRK6 ⁵⁴	RGS3, RGS4, RGS10, RGS16 ⁷⁸⁻⁸⁰
<i>Myristoylated proteins</i>	
Gα0, Gαi1, Gαt, Gαx ^{58, 81, 82}	Arf family ^{83, 84}
NADH cytochrome b5 reductase ⁸⁵	Calcineurin B ⁸⁶
Nitric oxide synthase ⁸⁷	AKAP 18 ⁸⁸
Src family (Src, Yes, Fyn, Lck, Hck, Fgr, Yrk, Blk) ⁸⁹	

Although no palmitoyl acyl transferase (PPT) protein has been purified, which can account for S-acylation, two groups have reported partial purification of enzymatic activity responsible for palmitoylation of a subset of proteins^{94, 95}. Furthermore, three groups purified PATs responsible for S-acylation of specific proteins, while it is not known whether other proteins can serve as substrates⁹⁶⁻⁹⁸. When considering the enzymology of deacylation, two thioesterases have been identified which are capable to remove palmitate from palmitoylated proteins, namely PPT1 and APT1. PPT1 is localised in the lysosome and thus an unlikely candidate for deacylation of plasmamembrane bound proteins. PPT1 is associated with a lysosomal storage disease, infantile neuronal ceroid lipofuscinosis. During this disease small lipid thioesters, which are presumably substrates for PPT1, accumulate leading eventually to death. APT1, an enzyme which originally had been identified as a lysophospholipase, depalmitoylates Gα subunits and Ras *in vitro* and *in vivo*. Additional information about the substrate specificity of PPT1 or APT1 is lacking. Likely, other palmitoyl protein thioesterases will exist with different substrate specificities of these enzymes and for different classes of proteins.

Although numeral proteins are identified undergoing acylation (table 2.1), the precise role of protein acylation is still not clear. While protein acylation has been associated with a number of functional consequences

such as membrane targeting/binding (ras p21)⁹⁹, conformational changes (rhodopsin)¹⁰⁰, regulation of protein function (RGS, GRK6)^{78, 101}, protection against proteolysis (A1 adenosine receptor)¹⁰², protein-protein interactions (tetraspanin)¹⁰³, for the majority of acylated proteins the role of acylation is not clearly established and remains to be elucidated.

Regulation of cellular FA utilization

In tissues, such as the heart and skeletal muscle, FA are important oxidizable substrates. Over 70% of energy consumed for electro-mechanical activity is covered by mitochondrial oxidation of FA². The accompanying FA flux from the vascular compartment to the mitochondrial matrix is on the order of 100-150 nmol fatty acyl moieties·gram⁻¹ per minute. In resting skeletal muscle blood-borne FA utilization is minor, but during prolonged muscular contractile activity FA uptake readily increases to 200 nmol·g⁻¹ per minute¹⁰⁴.

Under physiological conditions, uptake is in equilibrium to mitochondrial utilization, to prevent intracellular accumulation of FA, being highly toxic at elevated intracellular concentrations². The endogenous TG pool acts most likely as an intracellular capacitor to absorb temporary imbalances in uptake and utilization of exogenous FA. The intracellular FA concentration is also kept as low as possible to create a concentration difference between interstitial and cytoplasmic FA, which is required as driving force for the transition of FA from the extracellular to the intracellular compartment. Noteworthy is the observation of Kiens and co-workers¹⁰⁵ that during skeletal muscle activity at moderate intensity the cellular FA concentration decreases compared to the resting state, promoting the diffusion of blood-borne FA to the muscle cell interior.

Recent findings of Bonen and colleagues¹⁰⁶ indicate that the capacity to extract FA from extracellular sources is also regulated during acute changes in muscle energy requirements. They observed that sarcolemmal giant vesicles prepared from muscles that were electrically stimulated for 25 min display a substantially higher rate of FA uptake than vesicles from non-stimulated muscles. The increase was paralleled by a higher sarcolemmal content of FAT/CD36, most likely due to translocation of the FA transporting protein from an intracellular pool to the cell membrane. Of interest is that insulin is also able to promote the translocation of FAT/CD36 from the intracellular storage site to the sarcolemma¹⁰⁷. In addition to translocation, the biological activity of FAT/CD36 may be acutely modulated by phosphorylation of the membrane-associated protein¹⁰⁸.

Mitochondrial acyl-CoA formation and/or subsequent oxidation of fatty acyl chains via β -oxidation and citric acid cycle activity offers (an) additional site(s) of control of the rate of muscular FA utilization. The actual regulation of mitochondrial FA oxidation is a multifactorial process in which malonyl CoA, the ratio acetyl CoA/CoA and NADH/NAD⁺ play prominent roles ². Moreover, the availability of competing substrates such as lactate has profound effects on the rate of muscular FA oxidation. The collected findings indicate that both the rate of mitochondrial FA utilization and the rate of transport of FA from the interstitial compartment into the myocyte interior contribute to the overall rate of muscle cell FA utilization.

Long-term adaptation of cellular FA uptake and transport

Various lines of evidence suggest that chronic alterations in the cellular utilization of FA can be attributed to adjustment of the expression of genes encoding for proteins involved in FA uptake, transport and metabolism. Experiments performed on adipocytes and hepatocytes indicated that substrates for energy conversion themselves are capable of modulating the transcriptional activity of metabolic genes ¹⁰⁹⁻¹¹². We observed recently in cultured neonatal cardiac muscle cells ^{4, 113} that exogenous FA are also capable to control cardiac transcriptional activity. Chronic exposure of the neonatal cardiomyocytes to physiological concentrations of oleate and palmitate in the incubation medium for 48 h resulted in a 3-fold increase in the mRNA levels of FAT/CD36 and FABP ¹¹³. In addition, the transcription of two mitochondrial FA-handling enzymes, acyl CoA synthetase and long-chain acyl-CoA dehydrogenase increased substantially. In this respect it is of interest to note that in the healthy rat, deprivation of food for 48 h increased the plasma FA concentration almost 2-fold, associated with an increase in the cardiac tissue levels of FAT/CD36 and FABPc mRNA ¹¹⁴. Moreover, the maturational increase in rat cardiac FA oxidation, starting a couple of days after birth ^{115, 116}, was found to be accompanied by an increased content of FAT/CD36 and of cytoplasmic FABP in the myocardium ^{18, 117}.

The finding that chronic exposure of cardiomyocytes to exogenous FA results in the transcriptional upregulation of a variety of FA-handling genes indicates that the cells are capable of sensing changes in the supply of FA and/or FA derivatives. Recent studies ^{3, 118} revealed that the promoter region of FAT/CD36 contains cis-regulatory elements with binding sites for transcription factors such as COUP/TF, PPAR, RXR, and HFN-4 ^{118, 119}. PPAR is a likely candidate for cellular FA sensing, since FA may act as natural ligand of the PPARs ¹²⁰⁻¹²². In particular, the PPAR α and PPAR β isoforms may be involved

in modulating transcriptional activity in cardiomyocytes by FA, as cardiac tissue lacks the expression of PPAR .

Future directions

Despite many attempts to elucidate in full detail the molecular mechanism of cellular fatty acid uptake, transport and utilization, the complete picture of transport routes and regulatory processes is still missing. It is for instance unknown whether FA released from the triacylglycerol core of circulating lipoproteins mix with FA supplied by albumin in the microvascular compartment prior to transport through the endothelium and utilization in the parenchymal cells. The route of FA transport across the endothelium and the mechanisms involved are still enigmatic. The precise mechanism of transport of FA across a biological membrane, consisting of a phospholipid bilayer, is incompletely understood. The question as to whether membrane-associated proteins promote the FA membrane trafficking per se or enhance the availability of FA at close vicinity of the membrane remains to be answered. Moreover, it is unclear whether the putative membrane-associated FA binding proteins are acting in concert. The role of albumin-binding proteins attached the cellular membranes in cellular FA uptake and transport remains to be established.

Since evidence is accumulating that proteins involved in cellular FA uptake, such as FAT/CD36, can be mobilized from intracellular sites, future studies should be performed to elucidate the nature of the signalling pathways resulting in translocation of membrane FA-binding proteins.

The fine-tuning between mitochondrial FA oxidation and cellular FA uptake and transport is only partly understood, but it is becoming evident that factors increasing mitochondrial activity also act on signalling pathways involved in FAT/CD36 translocation. Finally, the investigation on the transcriptional regulation of genes encoding for proteins involved in tissue FA handling state is in its infancy. Detailed information is required to elucidate both the long-term regulation of FA-handling under physiological conditions and the impact of alterations in FA-handling in the diseased state. The latter is of utmost importance to fully appreciate the alterations in FA handling by the diabetic heart and to elucidate the potential role of FA in processes leading to diabetic cardiomyopathy.

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Chapter 3

Determination of long-chain fatty acids, triacylglycerols and phospholipids by capillary gas chromatography in cardiac tissue and blood plasma of normal and diabetic rats

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Abstract

A reliable and sensitive technique is presented to monitor the total content of non-esterified fatty acids (FA), triacylglycerols (TG), phospholipids (PL) and their relative fatty acyl composition in biological material, with special reference to rat cardiac tissue and blood plasma. The various lipid classes were separated with thin-layer chromatography. Quantification of the fatty acyl moieties was accomplished by capillary gas chromatography. Since in muscle tissue the amount of FA represents approximately 0.2% of the total esterified and non-esterified fatty acyl pool, precautions should be taken to avoid hydrolysis and/or transmethylation of esterified fatty acyl moieties during the assay procedure. In particular, attention should be paid to storage of tissue samples, disruption of tissue prior to lipid extraction and contaminants increasing the blank of the assay.

The content of FA in the cardiac ventricles of normal rats was 338 ± 83 nmol per gram dry weight. Streptozotocin-induced diabetes caused a significant 4.8-fold increase in the FA content of rat cardiac ventricular tissue. The content in normal and diabetic cardiac ventricles of TG amounted to 19.6 ± 5.3 and 36.4 ± 4.8 μ mol FA per gram dry weight, respectively. The PL content of normal and diabetic ventricles was comparable (152 ± 14 versus 153 ± 9 μ mol FA per gram dry weight). In blood plasma, the content of non-esterified FA increased from 134 ± 59 to 265 ± 88 μ mol FA per litre, following 4 weeks of diabetes. The TG and PL concentrations increased from 1.67 ± 0.39 and 2.62 ± 0.75 mmol FA per litre plasma to 9.11 ± 1.90 and 5.01 ± 1.11 mmol FA per litre plasma, respectively. Both in cardiac tissue and in blood plasma, streptozotocin-induced diabetes resulted in changes in the relative fatty acyl composition of the three lipid classes studied. The changes in blood and cardiac lipid content as well as fatty acyl profiles indicate profound alterations in lipid homeostasis in the diabetic state. These alterations may contribute to the development of diabetic cardiomyopathies.

Introduction

Non-esterified long-chain fatty acids (FA) are important substrates for energy conversion in cardiac muscle. Moreover, esterified in phospholipids (PL), they serve as building blocks of cellular membranes. Part of FA are stored inside the muscle cells as triacylglycerols (TG), while recent studies have shown that FA are also involved in intracellular signal transduction either via modulation of nuclear gene expression or through covalent binding to proteins¹⁻⁴.

Since muscle tissue lacks the capability of *de novo* synthesis of FA, cardiac myocytes rely on the supply of FA from extracellular sources to cover their need for these substrates. FA are supplied to cardiac muscle either bound to plasma albumin (non-esterified fatty acids) or in the form of TG in the core of circulating lipoproteins³.

To investigate cardiac FA homeostasis in full detail, a sensitive and reliable analytical technique is required providing information on the total amount of FA and the relative fatty acyl composition (variation in chain length, number of unsaturated bonds). Although a variety of methods, including titrimetric, colorimetric, enzymatic and radiochemical analytical techniques⁵⁻¹², has been employed to assess the total content of FA in biological material, capillary gas chromatography and high performance liquid chromatography (HPLC) are the first choice to analyze both the total content and the fatty acyl composition of the FA pool¹³⁻¹⁶. Since in cardiac muscle the majority of fatty acyl moieties are incorporated into the esterified lipid pool, *i.e.*, TG and PL, special attention should be paid to tissue collection, storage and lipid extraction prior to chromatographical analysis in order to avoid any artificial elevation of the amount of FA due to hydrolysis and/or transmethylation of esterified fatty acyl moieties.

Artificial elevation of the tissue content of FA will hamper correct interpretation of findings in physiological studies aiming to unravel, for instance, the mechanisms underlying the transport of FA from the extracellular compartment to the cell interior or to disclose the biological effect of FA in the regulation of gene expression. Moreover, changes in the tissue content of FA most likely indicate profound changes in cardiac metabolism in general and in FA handling in particular. Since the metabolic fate of FA in the myocardium may depend on chain length and degree of unsaturation^{3,4}, it is of importance to analyse the relative composition of the FA pool in this tissue.

Here, a sensitive and reliable technique is presented to monitor the content and relative fatty acyl composition of FA and of the esterified lipid pools, TG and PL, in cardiac and blood plasma. Possible pitfalls in tissue collection, storage and lipid extraction will be indicated. The application of gas capillary chromatography for the analysis of the cardiac muscle lipid content will be discussed. Finally, two examples of the analysis of the content and relative composition of FA in biological material are presented in detail: cardiac ventricular muscle and blood plasma of both normal and diabetic rats. Diabetes was studied, since evidence is accumulating that in addition to carbohydrate handling, lipid metabolism is affected in this pathological condition ¹⁷.

Experimental procedures

Induction of diabetes by streptozotocin

Male Lewis rats were fed *ad libitum*, had free access to water, and were kept under a light-dark cycle of 12 hours ¹⁸. The Institutional Animal Care and Use Committee, Maastricht University, approved the experiments.

Animals were anaesthetised with ketamine and xylazine (Eurovet, Bladel, The Netherlands), 37.5 and 7.5 mg·kg⁻¹, respectively, via intraperitoneal injection. Subsequently, the animals (*n*=10) were treated with 70 mg·kg⁻¹ streptozotocin (STZ) in disodium citrate (100 mM, pH 4.5) via intravenous injection in the tail vein to induce type-1 diabetes, and received a subcutaneous injection of 2.5 ml 20% glucose on both lateral sides. Controls (*n*=12) received vehicle only. STZ-treated animals were given daily a 5% glucose (w/v) solution to drink. The body weight of the animals was measured prior to the injection of STZ or vehicle, and 4-5 weeks later when the animals were sacrificed (table 3.1).

Tissue sampling

Rats were anaesthetized with nembutal (sodium pentobarbital, 75 mg·kg⁻¹ body weight), and weighed (table 3.1). After thoracotomy, 1 ml of blood was removed from the left ventricular cavity with a syringe, rinsed with citrate. Thereafter, the heart was rapidly excised and rinsed in ice-cold 0.9% NaCl solution and thereafter weighed. Then ventricles were cut from the heart, weighed (table 3.1), and frozen between the tongues of a liquid nitrogen cooled aluminium clamp ¹⁰ and transferred to aluminium canisters. Blood

was centrifuged to separate plasma from blood cells, after which plasma was transferred to glass tubes. Both plasma and the frozen tissue samples were stored at the temperature of -80°C .

Table 3.1

Characteristics of control and streptozotocin-treated rats

	Normal	Diabetic
Body weight-start (gram)	203 ± 6.9	203 ± 8.0
Body weight-end (gram)	310 ± 33.3	210 ± 23.1
Ventricles (gram wet weight)	0.9 ± 0.1	0.7 ± 0.1

Bodyweight was measured just prior to injection of streptozotocin (diabetic) or vehicle (normal), *i.e.* body weight-start, and 4-5 weeks later, *i.e.* body weight-end. Ventricles were weighed after removing the heart from the thorax and separation from atrial, lung and fat tissue.

Handling of tissue samples

Aliquots of muscle tissue ranging from 100 to 300 mg wet weight were pulverized in an aluminium mortar with a stainless steel pestle, both cooled in liquid nitrogen. The tissue powder was transferred to glass test tubes cooled in liquid nitrogen.

Extraction of lipids

The test tubes containing pulverized tissue were placed at -21°C in a mixture of melting ice and NaCl. The tissue powder was wetted with 2 ml ice-cold methanol. The content of the test tubes was allowed to warm up to room temperature and weighed. According to the extraction procedure of Folch and co-workers¹⁹ 4 ml chloroform was subsequently added. Butylated hydroxytoluene (0.01%) was present in both methanol and chloroform to prevent auto-oxidation of lipids. Thereafter, heptadecanoic acid (8 nmol) was added to correct for losses of FA during the extraction and assay procedure (recovery standard). Two hundred microlitre of blood plasma was extracted with a similar protocol.

Separation of lipids and gas chromatographical analysis

The above mentioned lipid extracts were evaporated and subjected to one-dimensional thin-layer chromatography (TLC), as described previously ^{3,20}. The spots corresponding with fatty acids (FA), triacylglycerols (TG) and phospholipids (PL) were scraped from the plate and transferred into test tubes containing 0.5 ml boron trifluoride (BF₃)-methanol solution (7% BF₃) according to Morrison and Smith ²¹. The fatty acyl moieties of the lipid classes were methylated at 20°C for 15 min (FA), at 100 °C for 30 min (TG) and at 100°C for 20 min (PL). Identification and quantification of the fatty acyl moieties in the various lipid classes were achieved with the use of a standard solution of methyl esters of fatty acids, consisting of (by their chemical notation) 14:0, 15:0, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 18:3n6, 20:0, 20:4, 22:0, 22:4, 22:6 and 24:0. The methyl ester mixtures were analyzed by gas chromatography using a WCOT fused-silica capillary column coated with 0.2 µm SP-2330 (30 m x 0.25 mm ID; Supelco, Bellefonte, PA) as described earlier ²². The overall recovery of FA ranged from 90% to 105% after correction for losses during the assay procedure with the use of the recovery standard.

Statistical analysis

Data obtained from control and diabetic hearts were statistically analysed with student's *t* test. *P* values <0.02 were considered to be statistically significant.

Results and Discussion

Non-esterified long-chain fatty acids (FA) are important substrates for mammalian organs, including cardiac muscle ^{3,4}. FA not only substantially contribute to muscle oxidative energy conversion, but also serve as substrates for biologically active compounds, such as eicosanoids, and for membrane phospholipid synthesis, and act as ligands for protein factors involved in signal transduction and gene expression. To explore the precise role of FA in these physiologically important processes a sensitive and reliable analytical technique is required to analyse and quantify the content of total and individual FA in biological structures, such as cardiac muscle. The present paper focuses on a capillary gas chromatographical technique to quantify FA in muscle samples obtained from rat cardiac ventricles and blood plasma, both from control and diabetic animals. Special attention was paid

to potential pitfalls in tissue handling and sample preparation prior to gas chromatography. The diabetic heart was included in the study since this pathological condition is associated with profound changes in FA metabolism.

Sampling, storage, and handling of cardiac tissue specimens

A time interval of warm ischemia inevitably occurs between taking a tissue specimen from cardiac muscle and freezing of the sample in liquid nitrogen. For some biochemical components, such as phosphocreatine, the time interval should be kept as short as possible, *i.e.*, less than 2 seconds, to avoid ischemia-induced degradation of the high-energy phosphate. In contrast, we observed that no measurable change occurred in the FA content of cardiac muscle tissue with a time interval up to 15 seconds²³. The deeply frozen tissue specimen can be stored up to 6 months at -80°C without any change in the content of FA. In contrast, storage at -20°C for 4 weeks resulted in a 30-fold increase in tissue FA content due to autolysis or (phospho)lipase-induced hydrolysis of the endogenous esterified lipid pool²³.

Thawing of the frozen tissue sample should be avoided at any rate. We observed a 2.5-fold increase in the FA content of cardiac muscle when the temperature of the frozen tissue specimen is raised from -80°C to room temperature. The increase was most likely caused by activation of endogenous (phospho)lipases in muscle cells due to the freeze-thawing procedure. Lipolysis can be prevented when the frozen tissue powder, prepared at the temperature of liquid nitrogen, is wetted with ice-cold methanol at -21°C prior to lipid extraction (see Experimental procedures). Homogenization of aliquots of cardiac muscle in homogenization buffers with the use of tissue blenders should be discouraged²⁴. In our hands, the homogenization of freshly obtained cardiac tissue in a sucrose-EDTA-TRIS buffer (pH 7.4), even when performed at 0-4°C, resulted in a significant 2.8-fold increase in the tissue FA content, most likely due to activation of (phospho)lipases during homogenization.

Extraction of lipids

A variety of procedures has been introduced to extract lipids from muscle tissue. The overall requirements of any technique should be that extraction of lipids is as complete as possible and that unwanted hydrolysis and/or transmethylation of esterified fatty acyl moieties are avoided. Satisfying

results were obtained with the extraction method originally described by Folch and co-workers¹⁹. In this procedure, pulverized tissue was treated with methanol-chloroform (1:2, by volume) at -21°C (see for detail Experimental procedures). Alternatively, when the extraction procedure was applied as originally described by Dole and Meinertz²⁵ a 140% increase in tissue FA content was observed²⁶. It was found that the difference in outcome between the Folch and Dole methods was not caused by incomplete extraction of FA by the former method but by autolysis of endogenous lipids in the Dole extraction procedure²⁶. When the Hagenfeldt modification of the Dole extraction method was applied²⁷, extremely high tissue FA levels were monitored mainly due to excessive transmethylation of esterified fatty acyl moieties during the extraction procedure²⁶.

Separation and quantification of muscle tissue FA

Prior to methylation of the fatty acyl moieties, the total lipid extract is routinely separated into the FA, TG and PL fraction using a standard TLC method^{20,22}. The application of capillary gas chromatography allows for assessment of long-chain FA with up to 6 unsaturated carbon bonds commonly present in cardiac muscle. Figure 3.1A shows an example of the separation of the methyl esters of the standards used. In figure 3.1B a typical example of a FA chromatogram of a normal rat heart is shown.

Blank values

The sensitivity of the overall technique to assess FA in muscle tissue is, among others, determined by the blank value of the assay system. Despite the fact that all glassware was carefully cleaned, all solvents of high purity analytical grade and precautions were taken to minimize contamination by finger prints, laboratory greases, etc., the blanks of the overall FA assay were on the order of 3 nmol per sample²³. To obtain a reliable estimation of the FA content in cardiac muscle tissue, the amount of FA in the extract of the biological material should be at least 2.5-fold higher than the blank. To fulfil this requirement, at least 100 mg of cardiac muscle or 200 µl blood plasma was used for lipid extraction.

Figure 3.1 A Chromatogram of a standard mixture of methyl esters of long-chain FA.

(1) 14:0; (2) 15:0; (3) 16:0; (4) 16:1; (5) 17:0; (6) 18:0; (7) 18:1; (8) 18:2; (9) 18:3; (10) 20:0; (11) 22:0; (12) 20:4; (13) 22:4; (14) 24:0 and (15) 22:6 (fatty acyl moieties according to their chemical notation). BHT refers to butylated hydroxytoluene; a.u. refers to arbitrary units.

Figure 3.1 B Typical examples of a chromatogram of fatty acyl methylesters derived from the cardiac non-esterified FA pool in normal rat cardiac ventricles.

The weight of the tissue specimen used for the measurement was 115 mg wet weight. See for details citations to **figure 3.1 A**.

The content of FA and relative fatty acid composition in rat cardiac ventricles and blood plasma

The content of FA in normal and diabetic rat ventricular tissue is shown in table 2A. The content of total FA was 338 ± 83 nmol per gram dry weight of tissue in normal rat ventricles. Since in rat cardiac tissue the dry/wet weight ratio is 4:1, the FA content expressed per gram wet weight is approximately $65 \text{ nmol} \cdot \text{g}^{-1}$ wet weight. The main constituents of the FA pool in normal rat heart were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and docosahexaenoic (22:6) acids. The total amount of fatty acyl moieties in PL and TG in normal rat cardiac ventricles was 152 ± 14 and 19.6 ± 5.3 μmol per gram dry weight, respectively, indicating that the percentage fatty acyl moieties in the non-esterified form in control rat cardiac ventricles is in the order of 0.2%.

In the diabetic rat, the cardiac content of FA increased significantly 4.8-fold (table 3.2A). This finding corroborates earlier observations indicating that FA accumulate in the diabetic heart ^{28,29}, but are in contrast with previously published data by Han and colleagues. ³⁰, who reported a statistically insignificant 20-30% increase in cardiac FA content, 6 weeks after streptozotocin treatment. In our study, all individually measured FA contributed to the increase in cardiac FA content under diabetic conditions. Of interest is the observation that the relative contribution of stearic (18:0) and oleic (18:1) acid to total FA increased, but that the relative contribution of the polyunsaturated fatty acids arachidonic (20:4) and docosahexaenoic (22:6) acid decreased (table 3.2A).

The TG pool significantly expanded from 19.6 ± 5.3 to 36.4 ± 5.3 μmol per gram dry weight in the diabetic rat heart. Almost all fatty acyl moieties contribute to the increase in the tissue pool. Only minor changes in the relative fatty acyl composition of this lipid pool in the diabetic heart were found (table 3.2B).

The total amount of fatty acyl moieties in the PL fraction of diabetic cardiac tissue did not change as compared to non-diabetic controls (table 3.2C). With respect to the individual fatty acyl moieties in the cardiac PL pool, significant changes were observed in the content of 14:0, 16:1, 18:1 and 22:4, all showing a decline. In contrast, the contribution of 22:6 to the cardiac PL pool significantly increased. A number of studies have shown alterations in the relative fatty acyl profiles between control and diabetic rats in cardiac myocytes ³¹⁻³⁵ as well as in liver ^{36,37}.

Table 3.2 A. The content of non-esterified fatty acids (FA) in cardiac ventricular tissue of normal and diabetic rats

Fatty acid	Control		Diabetic	
	nmol/g dw ^a	% of total	nmol/g dw ^a	% of total
total	338 ± 83	100	1,631 ± 315 *	100
14:0	7.6 ± 1.6	2.3 ± 0.5	36 ± 17 *	2.2 ± 0.7
16:0	77 ± 22	22.7 ± 2.0	396 ± 89 *	24.2 ± 1.0
16:1	5.6 ± 1.3	1.7 ± 0.6	23 ± 9 *	1.4 ± 0.3
18:0	47 ± 12	13.9 ± 0.8	264 ± 38 *	16.4 ± 1.5 *
18:1	45 ± 16	13.1 ± 2.0	271 ± 60 *	16.6 ± 1.3 *
18:2	86 ± 27	25.2 ± 3.1	443 ± 93 *	27.1 ± 2.0
18:3	n.d.	n.d.	6.3 ± 2.1 *	0.4 ± 0.1 *
20:0	1.0 ± 0.5	0.3 ± 0.1	8.2 ± 1.0 *	0.5 ± 0.1 *
20:4	15 ± 2	4.8 ± 0.8	31 ± 5 *	1.9 ± 0.3 *
22:0	n.d.	n.d.	6.3 ± 0.8 *	0.4 ± 0.1 *
22:4	0.6 ± 1.6	0.2 ± 0.5	3.8 ± 2.1 *	0.2 ± 0.2
22:5	12 ± 3	3.9 ± 1.6	19 ± 5 *	1.2 ± 0.3 *
22:6	36 ± 6	11.3 ± 2.8	113 ± 24 *	7.0 ± 1.1 *
24:0	2.6 ± 1.3	0.7 ± 0.4	7.1 ± 1.3 *	0.4 ± 0.1

Mean ± SD is presented; n.d., not detectable. The FA content is normalized to the dry weight mass of the cardiac muscle. Rats were made diabetic by intravenous injection of streptozotocin (70 mg/kg body weight) and sacrificed 4-5 weeks later. Total amount of FA was obtained by adding up the amounts of individual FA.

Control (n=6) and diabetic (n=5). * significant and different from control.

Table 3.2 B. The content of triacylglycerols (TG) in cardiac ventricular tissue of normal and diabetic rats

Fatty acid	Control		Diabetic	
	nmol/g dw ^a	% of total	nmol/g dw ^a	% of total
total	19.8 ± 5	100	36.4 ± 5.3 *	100
14:0	0.2 ± 0.1	1.1 ± 0.3	0.3 ± 0.1	0.7 ± 0.1 *
16:0	5.3 ± 1.4	27.2 ± 0.5	5.3 ± 1.4 *	28.6 ± 0.6 *
16:1	0.2 ± 0.1	1.1 ± 0.4	0.2 ± 0.1	0.6 ± 0.1 *
18:0	1.6 ± 0.3	8.2 ± 0.8	3.6 ± 0.4 *	9.8 ± 0.3 *
18:1	4.8 ± 1.5	24.5 ± 2.3	8.1 ± 1.1 *	22.3 ± 0.7
18:2	5.0 ± 1.6	25.3 ± 1.8	8.6 ± 1.1 *	23.6 ± 0.9
18:3	n.d.	n.d.	0.02 ± 0.02	0.06 ± 0.05
20:0	0.06 ± 0.02	0.4 ± 0.1	0.3 ± 0.0 *	0.7 ± 0.1 *
20:4	0.5 ± 0.2	2.4 ± 0.8	0.6 ± 0.1	1.7 ± 0.2
22:0	0.03 ± 0.02	0.2 ± 0.1	0.12 ± 0.0 *	0.3 ± 0.1 *
22:4	0.08 ± 0.03	0.4 ± 0.1	0.1 ± 0.02	0.3 ± 0.0
22:5	0.5 ± 0.2	2.5 ± 0.9	1.0 ± 0.2 *	2.7 ± 0.2
22:6	1.3 ± 0.4	6.7 ± 2.1	3.0 ± 0.6 *	8.3 ± 1.1
24:0	0.01 ± 0.02	0.03 ± 0.05	0.05 ± 0.02 *	0.1 ± 0.1 *

Mean ± SD is presented; n.d., not detectable. The TG content is normalized to the dry weight mass of the cardiac muscle. Rats were made diabetic by intravenous injection of streptozotocin (70 mg/kg body weight) and sacrificed 4-5 weeks later. Total amount of TG was obtained by adding up the amounts of individual TG.

Control (n=6) and diabetic (n=5). * significant and different from control.

Table 3.2 C The content of phospholipids (P_i) in cardiac ventricular tissue of normal and diabetic rats

Fatty acid	Control		Diabetic	
	mmol g dw ⁻¹	% of total	mmol g dw ⁻¹	% of total
total	152 ± 14	100	152.9 ± 9	100
14:0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.02 *	0.07 ± 0.01 *
16:0	234 ± 2.2	15.4 ± 0.3	23.4 ± 1.8	15.3 ± 0.5
16:1	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0 *	0.2 ± 0.0 *
18:0	29.7 ± 3.0	19.2 ± 0.2	30.3 ± 1.9	19.8 ± 0.2 *
18:1	16.1 ± 1.4	10.6 ± 0.5	13.8 ± 0.7 *	9.0 ± 0.4 *
18:2	31.2 ± 3.9	20.5 ± 0.9	30.3 ± 2.5	19.8 ± 0.7
18:3	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01
20:0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:4	23.6 ± 2.6	15.5 ± 0.3	23.8 ± 1.7	15.6 ± 0.7
22:0	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
22:4	0.8 ± 0.1	0.5 ± 0.1	0.5 ± 0.0 *	0.3 ± 0.0 *
22:5	4.4 ± 0.2	2.9 ± 0.2	4.8 ± 0.4	3.1 ± 0.3
22:6	21.4 ± 1.8	14.1 ± 0.7	24.6 ± 1.3 *	16.1 ± 0.5 *
24:0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.01

See for details legend table 3.2 A.

Table 3.3 A The concentration of non-esterified fatty acids (FA) in blood plasma of normal and diabetic rats

Fatty acid	Control		Diabetic	
	mmol l ⁻¹	% of total	mmol l ⁻¹	% of total
total	134 ± 59	100	265 ± 88 *	100
14:0	2.1 ± 1.0	1.8 ± 0.7	3.0 ± 1.0	1.1 ± 0.1
16:0	37.3 ± 16.3	27.9 ± 0.3	73.1 ± 26.0	27.4 ± 0.9
16:1	4.4 ± 2.0	3.2 ± 0.3	4.4 ± 1.0	1.7 ± 0.2 *
18:0	13.2 ± 4.6	10.4 ± 1.6	37.8 ± 10.5 *	14.8 ± 1.1 *
18:1	27.6 ± 13.9	19.9 ± 2.7	55.8 ± 18.0	21.0 ± 0.3
18:2	32.5 ± 17.2	23.2 ± 3.1	70.2 ± 21.3 *	26.2 ± 1.1
18:3	4.0 ± 1.2	3.9 ± 3.0	1.6 ± 1.5 *	0.6 ± 0.7
20:0	0.3 ± 0.5	0.3 ± 0.4	0.7 ± 0.2	0.3 ± 0.05
20:4	5.4 ± 2.1	4.2 ± 0.5	6.0 ± 3.0	2.2 ± 0.6
22:0	n.d.	n.d.	n.d.	n.d.
22:4	n.d.	n.d.	n.d.	n.d.
22:5	1.3 ± 1.2	0.7 ± 0.7	1.1 ± 1.6	0.3 ± 0.4
22:6	6.0 ± 2.5	4.6 ± 0.4	11.3 ± 5.0	4.2 ± 0.6
24:0	n.d.	n.d.	0.4 ± 0.9	0.1 ± 0.2

Mean ± SD is presented; n.d., not detectable. The FA content is normalized to the dry weight mass of the cardiac muscle. Rats were made diabetic by intravenous injection of streptozotocin (70 mg kg⁻¹ body weight) and sacrificed 4-5 weeks later. Total amount of FA was obtained by adding up the amounts of individual FA. Control (n=5) and diabetic (n=5). * Significantly different from control.

Table 3.3 B The concentration of triacylglycerols (TG) in blood plasma of normal and diabetic rats

Fatty acid	Control		Diabetic	
	mmol l ⁻¹	% of total	mmol l ⁻¹	% of total
total	1,678 ± 391	100	9,113 ± 1903 *	100
14:0	20 ± 7	1.2 ± 0.3	161 ± 53 *	1.7 ± 0.3 *
16:0	438 ± 101	25.8 ± 0.8	2,436 ± 530 *	26.7 ± 0.6 *
16:1	30 ± 11	1.7 ± 0.3	167 ± 56 *	1.8 ± 0.3
18:0	62 ± 24	3.6 ± 0.9	689 ± 178 *	7.5 ± 0.7 *
18:1	374 ± 100	22.2 ± 1.2	2,048 ± 341 *	22.6 ± 1.1
18:2	526 ± 105	31.6 ± 1.3	2,478 ± 399 *	27.4 ± 1.2 *
18:3	5 ± 1	0.3 ± 0.1	53 ± 31 *	0.6 ± 0.2
20:0	2 ± 2	0.1 ± 0.1	21 ± 11 *	0.2 ± 0.1
20:4	32 ± 5	1.9 ± 0.3	198 ± 70 *	2.1 ± 0.3
22:0	8 ± 3	0.5 ± 0.3	15 ± 5	0.2 ± 0.0
22:4	n.d	n.d	13 ± 8 *	0.1 ± 0.0 *
22:5	50 ± 11	3.1 ± 0.6	160 ± 52 *	1.8 ± 0.3 *
22:6	131 ± 35	7.8 ± 0.8	623 ± 222 *	6.8 ± 1.3
24:0	n.d	n.d	17 ± 2 *	0.2 ± 0.1 *

See for details legend **table 3.3 A**.**Table 3.3 C** The content of phospholipids (PL) in blood plasma of normal and diabetic rats

Fatty acid	Control		Diabetic	
	mmol l ⁻¹	% of total	mmol l ⁻¹	% of total
total	2,625 ± 754	100	5,015 ± 1,108 *	100
14:0	12 ± 4	0.5 ± 0.05	12 ± 2	0.3 ± 0.04 *
16:0	642 ± 186	24.4 ± 0.2	1,253 ± 322 *	24.8 ± 1.2
16:1	11 ± 5	0.4 ± 0.05	17 ± 3	0.3 ± 0.05 *
18:0	462 ± 119	17.7 ± 0.7	1,062 ± 212 *	21.6 ± 0.9 *
18:1	133 ± 43	5.01 ± 0.2	245 ± 56 *	4.9 ± 0.3
18:2	701 ± 223	26.6 ± 1.6	1,468 ± 291 *	29.4 ± 0.7
18:3	1 ± 2	0.02 ± 0.04	7 ± 2 *	0.1 ± 0.05 *
20:0	4 ± 3	0.2 ± 0.2	12 ± 2 *	0.3 ± 0.09
20:4	508 ± 116	19.4 ± 1.3	684 ± 143	13.7 ± 0.5 *
22:0	4 ± 1	0.2 ± 0.04	7 ± 1	0.2 ± 0.04
22:4	4 ± 6	0.2 ± 0.3	3 ± 1	0.08 ± 0.04
22:5	24 ± 9	0.9 ± 0.1	27 ± 5	0.5 ± 0.05 *
22:6	115 ± 46	4.3 ± 0.4	194 ± 71	3.8 ± 0.5
24:0	4 ± 5	0.2 ± 0.25	16 ± 3	0.3 ± 0.04

When considering the studies performed in cardiac myocytes, four studies showed a decrease in the relative amount of 20:4^{31,33-35}. This is in contrast with our observations concerning arachidonic acid showing no differences either in the absolute amount of 20:4 or in the relative amount (see table 3.2C). Other alterations were also reported, namely an increase in the relative amount of 18:2^{33,34} and 22:6^{32,35} of which the latter was in accordance with our results. Similar results are also reported for the liver phospholipid fatty acid composition^{36,37} reporting a decline in 18:0 and 20:4 and an increase in 16:0, 18:2 and 22:6. The apparent discrepancy between previous findings and the present results is incompletely understood and requires further experimentation.

Table 3.3 A, B and C provides information on the content and relative fatty acyl composition of FA, TG and PL in blood plasma of normal and diabetic rats. Table 3.3A indicates that the concentration of FA almost doubled in the diabetic animals. All major fatty acyl moieties contributed to this diabetes-induced increase in circulating FA. The relative composition of 18:0 increased, while the percentage of 16:1 and 20:4 significantly decreased in the FA fraction of diabetic blood plasma. The fact that the increase in cardiac FA content was about 4.8 and in blood plasma only 2-fold suggests that FA specifically accumulates in the myocardium of the diabetic animals (figure 3.2). The precise reason is unknown, but one may speculate that in the diabetic heart uptake of FA surpasses the rate of consumption of this important substrate.

Circulating TG significantly increased from 1.68 ± 0.39 to 9.11 ± 1.90 mmol FA per litre in diabetic animals reflecting a 5.4-fold increase (table 3.3 B and figure 3.2). All major fatty acyl moieties contributed to this substantial increase (table 3.3B). The relative fatty acyl composition showed some minor changes, e.g. a relative decline in 18:2 and a relative increase in 16:0.

The PL concentration in blood plasma of diabetic rats increased from 2.62 ± 0.75 to 5.01 ± 1.11 mmol fatty acyl moieties per litre (table 3.3C and figure 3.2). All major fatty acyl moieties contributed to this increase with the exception of arachidonic acid (20:4). The relative contribution of this fatty acyl chain to the circulating PL significantly decreased from 19.4 to 13.7% (table 3.3 C).

Figure 3.2 Cardiac tissue content and blood plasma concentration of FA, triacylglycerols (TG) and phospholipids (PL) in normal (C) and diabetic rats (DM).

Diabetes was induced by streptozotocin treatment 4 weeks prior to sacrifice. * Indicates significantly different from corresponding control values. See tables for number of experiments

The alterations in blood plasma lipid concentration and in the relative fatty acyl composition clearly indicate that in streptozotocin-induced diabetes total body FA metabolism is profoundly changed. This is most likely caused by the almost complete lack of insulin in these animals, hampering proper control of lipid metabolism in adipose and hepatic tissue.

The collected findings in the diabetic animal, *i.e.* increased cardiac FA and TG contents, strongly suggest that FA handling is altered in the heart of diabetic animals in addition to overall changes in lipid homeostasis in the

body. It is of interest to note that earlier studies have shown ³ that elevated concentrations of FA are detrimental in biological structures, suggesting that changes in cardiac FA handling in the diabetic state may contribute to diabetic cardiomyopathy commonly seen in patients suffering from diabetes mellitus. Future studies should be performed to elucidate the cause(s) of FA accumulation in the diabetic heart and to explore the pathophysiological consequences of the increased FA burden on the diseased heart. These studies require reliable and sensitive analytical techniques to monitor the content and alteration therein of FA in both cardiac muscle and blood plasma. The present study offers a technique based on capillary gas chromatography to assess the content of individual and total FA in relatively small amounts of cardiac muscle tissue and in blood plasma.

Conclusions

An analytical method is discussed to determine the (non-esterified) FA content and relative fatty acyl composition in biological material with special reference to cardiac muscle and blood plasma. The fatty acyl moieties were quantified with capillary gas chromatography. Since non-esterified FA represent only a minor fraction of the total amount of fatty acyl moieties (esterified and non-esterified) in muscle tissue, *i.e.*, on the order of 0.2%, precautions should be taken to avoid hydrolysis and/or transmethylation of esterified FA during tissue sampling, storage, and lipid extraction. Comparison of cardiac tissue and blood plasma of streptozotocin-treated rats with age-matched normal rats clearly indicates that FA homeostasis is substantially affected in the diabetic animal.

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Chapter 4

Protein acylation in the cardiac muscle like cell line, H9c2.

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Abstract

Besides serving as oxidizable substrates, fatty acids (FA) are involved in co- and post-translational modification of proteins (protein acylation). Despite the high rate of fatty acid utilisation in the heart, information on protein acylation in cardiac muscle is scarce. To explore this subject in more detail, we used the H9c2 cell line as an experimental model. After incubation with ^3H -palmitate or ^3H -myristate, cells were lysed and proteins precipitated, followed by extensive delipidation. The delipidated proteins were subjected to SDS-PAGE and transferred to nitro-cellulose prior to autoradiography. In addition, TLC was used to separate the various lipid classes. The first aspect we addressed was the extent of protein acylation as a function of time, relative to fatty acid incorporation into various lipid classes. Cells were incubated for 30 min, 1 h and 2 h with 100 μCi palmitate (PA, 2.3 nmol) or 125 μCi myristate (MA, 2.5 nmol). Palmitoylation increased from 0.48 ± 0.25 to $1.25 \pm 0.56 \mu\text{Ci}\cdot\text{mg protein}^{-1}$ between 30 min to 2 h, while myristoylation increased from 0.25 ± 0.12 to $0.77 \pm 0.36 \mu\text{Ci}\cdot\text{mg protein}^{-1}$. Furthermore, delipidated proteins subjected to autoradiography showed that a set of distinct proteins was labelled with ^3H -palmitate. Incorporation into phospholipids (PL) increased from 40% to 60% of the total amount of radio-labelled PA or MA supplied between 30 minutes and 2 hours. Only the FA pool differed between MA and PA, with a higher FA content present after incubations with MA. Second, we investigated palmitoylation and incorporation into cellular lipids as a function of the amount of PA applied. Palmitoylation showed saturation at high PA concentrations. The percentage incorporation of ^3H -PA in the various lipids depended on the amount of PA added: a decline in the PL pool with a concomitant increase in the size of the diacylglycerol pool at high PA concentrations. Third, inhibition of palmitoylation by cerulenin and tunicamycin was investigated. While both were able to inhibit palmitoylation, cerulenin also inhibited the incorporation of PA into various lipid classes, indicating differences in inhibitory action.

Introduction

Under normal physiological circumstances, blood-borne fatty acids are the major energy source for cardiac muscle, their oxidation providing about 70% of the energy demand of the heart¹. In addition, they serve as building blocks of cellular membranes after esterification into phospholipids or they are incorporated in the intracellular triacylglycerol pool. Recently evidence is accumulating that fatty acids also partake in signal transduction pathways²⁻⁵. In this respect, it is noteworthy that fatty acids are involved in co- and post-translational modifications of proteins.

Covalent attachment of fatty acids to proteins is now a widely recognised form of protein modification. This modification is thought to be important for protein-protein interaction, conformation, protein localisation, and activity⁶⁻¹⁰. The two most common forms of protein acylation are modifications with myristate (C14:0) and palmitate (C16:0), both saturated fatty acids. Despite the high rate of fatty acid utilisation in the heart, information on the physiological and pathophysiological significance of protein acylation in myocardial tissue is scarce. In 1999 Van der Vusse and Roemen showed for the first time that acylated proteins are present in normoxic cardiac tissue and were able to quantify the amount of acylated proteins in this tissue¹¹.

Protein acylation most likely depends on a number of factors which could include the availability of exogenous fatty acids, the energy demand of the cell, and incorporation and release of fatty acids into or from the endogenous phospholipid or triacylglycerol pool. This indicates that under circumstances in which fatty acid metabolism is altered, like hypertrophy, diabetes and ischemia/reperfusion, myocardial protein acylation may be changed.

In the present study, we explored various aspects of protein acylation in cardiac muscle cells. To this end, we used the clonal rat muscle cell line H9c2 as a cell model of cardiac myocytes. This cell line is derived from embryonic rat heart tissue and has properties of skeletal muscle¹² as well as cardiac muscle^{13, 14}. As H9c2 cells possess properties of cardiac muscle, they are commonly used as a model system to study metabolic properties of myocardial tissue¹⁵⁻¹⁸.

To further investigate protein acylation in cardiac tissue three separate goals were addressed in the present study. The first aspect of this study was to explore protein palmitoylation and myristoylation in H9c2 cells as function of time in relation to fatty acid incorporation into various lipid classes, such as triacylglycerol, phospholipids, and unesterified fatty acids. Second, the extent of protein palmitoylation and incorporation in cellular

lipids as a function of the amount of exogenous palmitate applied was investigated. The final aspect was to explore whether protein acylation in the experimental cell line could be inhibited by cerulenin or tunicamycin, two known inhibitors of protein acylation^{19, 20}.

Experimental procedures

Materials

[9,10-³H] Palmitic acid (43 Ci/mmol) and [9,10-³H] myristic acid (49 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, USA). Protease inhibitor cocktail for mammalian cell extracts (P8340), palmitate, cerulenin and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hyperfilm™-³H was acquired from Amersham Pharmacia Biotech (Buckinghamshire, UK). The scintillation fluid Opti-fluor® was obtained from Packard Instrument Company (Meriden, CT, USA)

Cell culture

H9c2 cells were obtained from the American Type Culture Collection (ATCC CRL1446; Rockville, MD, USA) and were used from passages 22 to 25. These cells were cultured in DMEM (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% foetal calf serum (FCS, Life Technologies). Cells were plated in a 25 cm² flask (Falcon, Becton Dickinson, Plymouth, UK) under 5% CO₂ in water saturated air. The experiments were performed two days after the cells were confluent, but still in the myoblast form.

Incubations of H9c2 cells

I. ³H-labelled fatty acid incorporation as a function of time

Prior to the start of the experiments, the H9c2 cells were washed three times with PBS. Subsequently, cells were incubated for 0, 0.5, 1 or 2 hours with 100 µCi ³H-labelled palmitate (43 Ci/mmol) or 125 µCi ³H-labelled myristate (49 Ci/mmol), each dissolved in 10 µl ethanol and transferred to 1 ml DMEM per flask. At the end of the incubation, cells were washed three times with ice-cold PBS. Cells were lysed using 1 ml harvesting buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (w/v), 10 µl protease inhibitor cocktail) for 30 minutes on ice. Subsequently, cell remnants were

scraped from the bottom of the flask using a rubber-policeman, after which the lysate was transferred to a glass tube.

II. Addition of an increasing amounts of non-radiolabelled palmitate

H9c2 cells were incubated for 0, 1, or 2 hours with 100 μCi (2.3 nmol) ^3H -labelled palmitate in the presence of 0, 25, 50, 75 or 150 nmol non-radiolabelled palmitate per incubation. The mixture of labelled and non-labelled palmitate was evaporated to complete dryness under nitrogen and subsequently dissolved in 10 μl ethanol and transferred to 1 ml DMEM per flask. The same procedure as described above was performed at the end of the incubation period.

III. Inhibitors (cerulenin and tunicamycin)

The stock solution of cerulenin in ethanol contained 1 mM inhibitor. An appropriate volume of the stock solution was transferred to a glass tube and dried under nitrogen. Subsequently, cerulenin was dissolved in 10 μl ethanol, leading to final concentrations of 0.1 or 0.3 mM cerulenin in 1 ml DMEM per incubation. H9c2 cells were washed three times with PBS, after which the cells were pre-incubated with medium supplemented with cerulenin for 90 minutes. At the end of the pre-incubation period, the medium was removed and cells were washed three times with PBS. Medium supplemented with 100 μCi ^3H -labelled palmitate per ml was added to the cells for up to 30 and 60 minutes. The same procedure as described above was performed at the end of the incubation period.

A stock solution of 1 $\text{mg}\cdot\text{ml}^{-1}$ tunicamycin in 10 mM NaOH was prepared. H9c2 cells were incubated with 100 μCi ^3H -labelled palmitate in combination with 0, 10 or 20 $\mu\text{g}\cdot\text{ml}^{-1}$ tunicamycin per 1 ml DMEM for up to 30 or 60 minutes. Addition of the tunicamycin solution to the medium did not lead to an alteration of the pH (data not shown). The same procedure as described above was performed at the end of the incubation period.

Protein precipitation

Proteins were precipitated according to Wessel and Flügge²¹. In short, cell lysates were subjected to the following protocol. To the lysate, 4 ml methanol was added and the samples were vortexed and centrifuged (1 minute, 4500 g , 20°C). Then 2 ml chloroform was added after which the samples were vortexed and centrifuged again (1 minute, 4500 g). For phase

separation, 3 ml milliQ water was added, and the samples were vortexed vigorously and centrifuged for 5 minutes at 4500 g. The upper phase was carefully removed and transferred to a glass tube. A further 3 ml methanol was added to the lower chloroform phase and interphase. The sample was vortexed and centrifuged once more (15 minutes, 4500 g, 20°C) to precipitate the protein. The supernatant was removed and combined with the earlier obtained upper phase. The precipitated proteins were subsequently subjected to extensive delipidation to remove all non-covalently bound fatty acids.

Removal of non-covalently bound fatty acids

The method for extraction of non-covalently bound fatty acids was adapted from Muszbek and Laposata²². The precipitated proteins were extracted with 3 ml chloroform/methanol (2:1). The sample was vigorously vortexed and incubated for 30 minutes at room temperature. To pellet the protein precipitates, 3 ml methanol was added to the sample and centrifuged (15 minutes, 4500 g, 20°C). The protein precipitate was extracted twice more with chloroform/methanol (2:1) and thereafter twice with chloroform/methanol (1:2). After the last extraction, the precipitated protein fraction contained no radioactivity removable by lipid extracting solvents. The supernatant of each extraction step was collected and combined with the previously obtained supernatants in a glass tube. The protein precipitates as well as the combined lipid extracts were dried completely under nitrogen.

Separation of lipid classes by thin layer chromatography (TLC)

The dried lipid extract was dissolved in 2 ml chloroform/methanol (2:1). On TLC plate coated with a 0.25 mm thick layer of silica gel 60 (Merck), 10 µl of the extract was applied. The lipid spots were pre-developed in chloroform/methanol/milliQ water/acetic acid (10:10:1:1, by volume) until the liquid front reached a level of 1 cm above the site of application of the lipid extract^{23, 24}. After the plate was dried under nitrogen, the lipid spots were developed in petroleum ether/diethylether/acetic acid (120:25:1.5, by volume). After development the plate was dried under nitrogen and measured using Tracemaster 20 with detector LB2821 (Berthold, Benelux Analytical Instruments, Vilvoorde, Belgium). Lipid spot were scraped from the plate and counted with a scintillation counter.

Total lipid content of H9c2 cells

The technique for quantitative analysis of fatty acyl moieties present in the various lipid classes are based on analytical techniques published earlier by Roemen and co-workers²³. In short, H9c2 cells were incubated with 77.3 nmol non-labelled palmitate for 0, 0.5, 1, and 2 hours. Subsequently, cells were scraped from the plate with 2 ml suprasolve methanol using a rubber-policeman and transferred to a glass tube. Thereafter 4 ml chloroform was added. At this point, a mixture of heptadecanoic acid (8nmol) and triheptadecanoyl glycerol (125 nmol) was added to correct for losses during extraction and assay procedures. The chloroform/methanol (2:1, by volume) extraction step was repeated two times. Subsequently, the extracts were combined, dried under a stream of nitrogen and the lipid residue was taken up in 150 μ l chloroform/methanol (2:1, by volume). Thereafter the concentrated lipid extract was subjected to the same TLC procedure as described above. The lipid moieties were made visible with rhodamine B-6G and fluorescein in methanol. The lipid spots corresponding to unesterified FA, triacylglycerols, and phospholipids were scraped from the plate and transmethylated according to Morrison and Smith²⁵. Subsequently, the methylesters were subtracted with pentane and after evaporation of pentane, dissolved in 2,2,4-trimethylpentane containing appropriate amounts of the methyl ester of pentadecanoic acid (recovery standard). Quantification of the fatty acyl moieties in the various lipid classes were achieved with the use of a standard solution of methyl esters of fatty acids, composed of (by their chemical notation) 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3n-3, 18:3n-6, 20:0, 20:4, 22:0, 22:4, 22:6, 24:0 and 24:1. The methyl esters were analysed by gas chromatography using a fused-silica capillary column coated with 0.2 μ m SP-2330 (30 x 0.25 mm ID; Supelco, Bellefonte, PA, USA). The starting temperature of the column was 150°C for 2 minutes and the column temperature was increased to 190°C at 4°C·minute⁻¹. Thereafter, temperature was kept at 190°C for 10 minutes and subsequently increased to 210°C at a rate of 10°C·minute⁻¹. The helium flow rate was 20 cm·s⁻¹. Detection was accomplished by flame ionisation.

Protein separation and autoradiography

Delipidated protein was solubilized in 90 μ l lysis buffer (40mM Tris, pH7.4, 2%SDS, 1mM EDTA). A single aliquot of 10 μ l was used to determine the protein content and the incorporated ³H-labelled fatty acids in the sample. To the remaining fraction, 20 μ l sample buffer was added (0.5mM Tris-HCl, 20% glycerol (v/v), 4% SDS (w/v), 0.02% bromophenol blue, milliQ,

2.5% (β -mercapto-ethanol(v/v)). Proteins were separated by SDS-PAGE in 4-15% gradient gels according to Laemmli ²⁶. Subsequently, proteins were blotted onto nitro-cellulose (pore size 0.45 μ m) using the Electrophoresis System from Bio-Rad Laboratories (Hercules, CA, USA). The blots were dried and exposed to Hyperfilm™-³H for 2 to 3 weeks.

Quantification

For quantification analysis, autoradiography films were scanned with the Fluor-S imaging system (Bio-Rad Laboratories) and images were analysed with the program Quantity One (Bio-Rad Laboratories) to obtain densitometric values. For each band detected with the Quantity One software, the optical density x mm was measured and from this the relative quantity of each detected band was assessed.

Miscellaneous techniques

Total protein content of H9c2 cells was quantified using the Micro BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. Radioactivity was counted in a liquid scintillation counter.

Statistical analysis

Results are expressed as the mean $\pm$ SD for at least four data points, unless otherwise indicated. Analysis of experiments with two groups was performed with Two-sample Student's *t* tests. Differences between various groups at one time point were assessed using a one-way ANOVA. For all analyses, the level of significance was set at 0.05.

Results

Protein acylation and ^3H -fatty acid incorporation in cellular lipids as a function of time

The amount of non-extractable radioactivity in the protein fraction after incubation with either ^3H -palmitate or ^3H -myristate, was determined for several time points over a period of up to 2 hours. Figure 4.1 shows that the amount of activity bound to the protein fraction after incubation with ^3H -palmitate increased in a non-linear fashion to $1.25 \pm 0.56 \mu\text{Ci}$ per mg protein. The time-related pattern of the covalent attachment of ^3H -myristate to proteins showed a more linear relationship. The amount of covalently bound ^3H -myristate increased from 0.36 to $0.77 \mu\text{Ci}\cdot\text{mg protein}^{-1}$ after 1 and 2 hours, respectively. Over 99% of the labelled fatty acids present in the lysate were found in the combined lipid pools, while less than 0.3 percent became covalently attached to proteins.

Figure 4.1 Protein acylation as function of incubation time

H9c2 cells were incubated for up to 2 hours with $100 \mu\text{Ci}\cdot\text{ml}^{-1}$ ^3H -palmitate or $125 \mu\text{Ci}\cdot\text{ml}^{-1}$ ^3H -myristate. Data are mean $\pm$ SD ($n=6$); * $p < 0.05$, 0.5 hour versus 2 hours

Figures 4.2 and 4.3 show that both palmitate and myristate were preferentially incorporated in the phospholipid (PL) pool, increasing from 40% of the total activity found in this lipid fraction after 30 minutes to 60% after 2 hours. Furthermore, there was a slight difference between the distribution over diacylglycerol (DG) and fatty acid (FA) pools when PA and MA are compared. Although not significant, incubations with ^3H -myristate

led to a higher level of this fatty acid in the cellular non-esterified fatty acid pool when compared with ^3H -palmitate, while after 0.5h significantly less ^3H -myristate was incorporated in the DG pool.

Figure 4.2 ^3H -palmitate incorporation in various lipid classes in H9c2 cells

H9c2 cells were incubated with $100 \mu\text{Ci}\cdot\text{ml}^{-1}$ ^3H -palmitate for up to 2 hours. PL-phospholipids, DG-diacylglycerols, TG-triacylglycerols, FA-unesterified fatty acids. Data are mean $\pm$ SD ($n=4$); * $p<0.05$, 0.5 hour versus 2 hours.

Figure. 4.3 ^3H -myristate incorporation in various lipid classes in H9c2 cells

H9c2 cells were incubated with $125 \mu\text{Ci}\cdot\text{ml}^{-1}$ ^3H -myristate for up to 2 hours. PL-phospholipids, DG-diacylglycerols, TG-triacylglycerols, FA-unesterified fatty acids. Data are mean $\pm$ SD ($n=4$); * $p<0.05$, 0.5 hour versus 2 hours.

Autoradiography of ^3H -palmitate labelled proteins

Figure 4.4 illustrates that routine incubation of H9c2 cells with ^3H -labelled palmitate resulted in a pattern in which distinct proteins could be identified. Comparison of a coomassie blue stained gel (panel-A) with the autoradiogram (panel-B) shows that only a selected number of proteins present in the H9c2 cell became modified by covalent attachment of ^3H -palmitate.

Figure 4.4 A representative Coomassie Blue staining of H9c2 proteins and a autoradiogram of H9c2 cells incubated with ^3H -palmitate for 0.5, 1 and 2 hours.

H9c2 cells were labelled with $100 \mu\text{Ci}\cdot\text{ml}^{-1}$ ^3H -palmitate for up to 2 hours. **Panel-A** shows a Coomassie Blue staining of a 12% gel and **Panel-B** shows an autoradiogram obtained after separation of H9c2 proteins using a 12% gel, blotted onto nitro-cellulose, and subjected to 3 weeks exposure to a film. Arrows indicate bands quantified as summarised in **table 4.1**.

Furthermore, it can be seen from figure 4.4, panel B that the density of several distinct proteins varied in time. The variation in relative quantity of the most prominent bands is shown in table 4.1. It can be seen that, for instance, the relative quantity of the 78 kDa band gradually increased in time, while the quantity of the 198 and 123 kDa bands remained stable.

Table 4.1 Quantification of covalently bound ^3H -palmitate to a distinct set of proteins in H9c2 cells

distinct proteins Mw (kDa)	incubation time (h)		
	0.5	1	2
	relative quantity (%)		
194	7.1	4.0	4.5
168	3.4	3.3	3.4
123	2.6	2.1	2.9
93	6.9	4.3	4.4
78	5.6	6.4	7.2
69	4.9	4.0	4.2
42	5.0	7.9	3.6
36	4.8	6.5	5.3

The autoradiogram was scanned with the Fluor-S imaging system (Biorad) and analysed with the software program Quantity-One (Biorad). The values shown represent the relative quantity (%) of the OD x mm of each selected band, after background subtraction. The sum of OD x mm of all detectable bands is set at 100%. Only the most prominent bands with a relative quantity > 2% are shown.

The effect of the concentration of exogenous palmitate on palmitoylation

To study whether protein acylation could be saturated, we incubated H9c2 cells with 100 μCi ^3H -palmitate (2.3 nmol) in the presence of increasing amounts of non-labelled palmitate, varying from 0 nmol palmitate to 150 nmol palmitate. Table 4.2 shows the metabolic fate of the ^3H -fatty acids supplied with increasing concentrations of non-radiolabelled palmitate. The amount of radioactive label present in the lysate diminished with increasing concentrations of non-radiolabelled palmitate. At the same time, the amount of radioactive label present in the medium at the end of the incubation period was enlarged.

Taking into account the differences in specific activity, the amount of nmol palmitate per incubation can be calculated. Figure 4.5 shows that palmitoylation of cellular proteins was not only time dependent (as already shown in figure 4.1) but more importantly, that the amount of fatty acids that became covalently attached to proteins increased with the supplied

amount of palmitate. At increasing concentrations of exogenously supplied palmitate, the amount of palmitate incorporated in the protein fraction levelled off at approximately $0.35 \text{ nmol} \cdot \text{mg protein}^{-1}$.

Table 4.2 Quantitative distribution of ^3H -palmitate with increasing concentrations of non-labelled palmitate

<i>amount of palmitate (nmol)</i>	2.3	27.3	52.3	77.3	152.3
fraction	<i>amount of recovered ^3H-palmitate (μCi)</i>				
medium	11.5 ± 0.9	16.2 ± 2.1	41.5 ± 9.3	43.5 ± 4.6	58.9 ± 6.8
lysate	91.1 ± 11.6	74.6 ± 9.6	72.6 ± 12.8	67.5 ± 8.3	54.4 ± 9.9
lipid	87.3 ± 2.3	77.5 ± 3.5	66.9 ± 1.9	60.7 ± 1.1	52.8 ± 2.4
protein	0.23 ± 0.04	0.11 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.05 ± 0.01

H9c2 cells were incubated with $100 \mu\text{Ci}$ ^3H -palmitate in the presence of increasing amounts of non-labelled palmitate. Aliquots were taken from the medium at the end of the incubation period. Furthermore, aliquots were taken from the lysate, lipid fraction, and protein fraction. The lipid fraction includes the phospholipids, diacylglycerols, triacylglycerols, and non-esterified fatty acids. The values shown represent the mean $\pm$ SD of ^3H -label recovered in each fraction in μCi . ($n=4$)

Figure 4.6 shows that the amount of palmitate present in the incubation medium also influenced the amount of palmitate incorporated into the several lipid fractions. Panel-A shows that increasing amounts of palmitate led to an increased incorporation of palmitate into the PL, DG and TG pool. Panel-B shows that the amount of fatty acid, which remained unesterified in the intracellular lipid pool, increased linearly with the amount of exogenously supplied palmitate.

In parallel experiments, H9c2 cells were incubated with 77.3 nmol non-labelled palmitate after which the cellular content of PL, TG, and FA was measured by capillary gas chromatography (figure 4.7, panels A, C and E). It is shown that the amount of fatty acyl moieties present in the PL pool remained in the order of 220 nmol per incubation. The amount of unlabelled palmitate present in the PL pool remained relatively stable, varying from 47.5 to 53.9 nmol between 0 and 2 hours, respectively (panel-A).

Figure 4.5 Protein palmitoylation as function of the amount of palmitate applied

H9c2 cells were incubated with 100 μCi ^3H -palmitate in the presence of increasing amounts of non-labelled palmitate, varying from 0 to 150 nmol. The amount ($\text{nmol-incubation}^{-1}$) of covalently attached palmitate per mg protein was calculated by taking into account the specific activity of radiolabelled palmitate for each condition. Data are mean $\pm$ SD ($n=4$); * $p<0.05$, 2.3 nmol and 27.3 nmol versus 152.3 nmol; # $p<0.05$, 1 hour versus 2 hours.

Furthermore, the total fatty acid content present in the TG pool was raised from 7.4 to 14.0 nmol per incubation after 0 and 2 hours, respectively. The amount of unlabelled palmitate increased from 1.9 to 6.3 nmol between 0 and 2 hours (Panel-C). The fatty acyl moieties in the FA pool increased from 2.7 to 12.8 nmol per incubation after 0 and 2 hours, respectively. The amount of palmitate in the FA pool first increased from 1.1 to 8.9 nmol palmitate after 0 and 1 hour, respectively, and stabilised thereafter (Panel-E). These results were compared with the amounts of palmitate incorporated per incubation of H9c2 cells in the presence of 77.3 nmol (75 nmol non-labelled + 2.3 nmol ^3H -labelled) palmitate after taking into account specific activity (figure 4.7, panels B, D and F). The amount of palmitate incorporated into the PL pool increased from 7 to 12.5 nmol after 1 and 2 hours, respectively (panel-B). Panel-D shows that the amount of palmitate incorporated into the TG pool increased from 4.1 to 6.1 nmol after 1 and 2 hours, respectively. The amount of palmitate remaining in the lipid pool as unesterified palmitate remained stable, varying from 19.0 to 21.1 nmol after 1 and 2 hours, respectively (panel-F).

Fig. 4.6 Effect of the amount of non-labelled palmitate on the rate of palmitate incorporation in lipid classes

H9c2 cells were incubated with 100 μCi ^3H -palmitate in the presence of increasing amounts of non-labelled palmitate, varying from 0 nmol to 150 nmol non-radiolabelled palmitate for 2 hours. **Panel-A** depicts the amount of esterified palmitate in the phospholipid (PL), diacylglycerol (DG) and triacylglycerol (TG) pools; **Panel-B** shows the amount of palmitate in the unesterified fatty acid (FA) pool. Data are mean $\pm$ SD ($n=4$)

Figure 4.7 Total fatty acid and palmitate content versus ^3H -fatty acid incorporation in distinct lipid classes

Panels-A, C and E show the total amount of fatty acyl moieties (striped bars) and that of palmitate (black bars) present in a particular lipid class per incubation. H9c2 cells were incubated in the presence of 77.3 nmol non-labelled palmitate for 0, 0.5, 1 and 2 hours (no radioactivity added). For the phospholipids (PL), triacylglycerols (TG) and non-esterified fatty acids (FA) the fatty acyl composition was determined by capillary gas chromatography. **Panel-B, D and F** represent the amount of palmitate (grey bars) that became incorporated in a particular lipid class per incubation during 1 and 2 hours. The amount of palmitate incorporated was determined after incubation of H9c2 cells with 100 μCi ^3H -palmitate (2.3 nmol) in the presence of 75 nmol non-labelled palmitate, taking into account the final specific activity of the palmitate present in the incubation mixture. Data represent the mean of two independent experiments.

Autoradiography of ^3H -palmitate labelled proteins after incubation with increasing amounts of exogenous palmitate

Figure 4.8 illustrates that the amount of radioactivity covalently attached to proteins decreased with increasing amounts of non-labelled palmitate. Although this is most prominently seen between 2.3 and 27.3 nmol palmitate, higher amounts of non-labelled palmitate further diminished the number of bands that remained visible.

Table 4.3 shows the change in density of the most prominent bands. The intensity of most protein bands became substantially weaker with increasing palmitate concentrations. The 22 kDa protein band was the most striking exception. The band of this protein showed only a relatively minor decline from 0.60 to 0.45 arbitrary units (a.u.) when the exogenous palmitate concentration was increased from 2.3 to 152.3 nmol.

Figure 4.8 Autoradiogram of H9c2 cells incubated with ^3H -palmitate in the presence of increasing amounts of non-labelled palmitate, varying from 0 nmol to 150 nmol

Shown is an autoradiogram obtained after separation of H9c2 proteins using a 4-15% gradient gel, blotted onto nitro-cellulose, and subjected to 3 weeks exposure to a film. Arrows indicate distinct bands, the quantification of which are summarised in **table 4.2**.

The effect of cerulenin or tunicamycin on inhibition of protein palmitoylation and ^3H -fatty acid incorporation into cellular lipids

Figure 4.9 shows that both cerulenin and tunicamycin were capable of inhibiting protein acylation in the H9c2 cell line. Increasing the concentration of cerulenin from 0.1 to 0.3 mM led to an inhibition of 32% and 54%, respectively, after 1 hour, while increasing the tunicamycin concentration from $10\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ to $20\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ inhibited protein acylation by 16% and 29%, respectively, after 1 hour.

Figure 4.9 Inhibition of protein palmitoylation by cerulenin and tunicamycin

Panel-A H9c2 cells were pre-incubated for 90 minutes with either 0.1 or 0.3 mM cerulenin followed by incubation with $100\text{ }\mu\text{Ci}$ ^3H -palmitate for 30 and 60 minutes. **Panel-B**, H9c2 cells were incubated with $100\text{ }\mu\text{Ci}$ ^3H -palmitate in the presence of either 10 or $20\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ tunicamycin for 30 and 60 minutes. The values shown represent the mean $\pm$ SD and are expressed as percentage of the respective control incubations. ($n=4$); * $p<0.05$ versus control.

The effect of cerulenin and tunicamycin on ^3H -fatty acid incorporation in cellular lipids was determined by measuring the radioactive label present in each lipid class. Incubations with increasing concentrations tunicamycin did not have any effect on the degree of incorporation (figure 4.10).

Figure 4.10 Effect of cerulenin and tunicamycin on incorporation of ^3H -palmitate in endogenous lipid pools

Panel-A, H9c2 cells were pre-incubated for 90 minutes with either 0.1 or 0.3 mM cerulenin followed by incubation with $100\ \mu\text{Ci}\ ^3\text{H}$ -palmitate for 60 minutes. **Panel-B**, H9c2 cells were incubated with $100\ \mu\text{Ci}\ ^3\text{H}$ -palmitate in the presence of either 10 or $20\ \mu\text{g}\cdot\text{ml}^{-1}$ tunicamycin for 60 minutes. PL-phospholipids, DG-diacylglycerols, TG-triacylglycerols, FA-unesterified fatty acids; * $p < 0.05$ versus control; # $p < 0.05$, 0.3mM cerulenin versus $20\ \mu\text{g}\cdot\text{ml}^{-1}$ tunicamycin

This is in contrast with incubations in the presence of cerulenin. With increasing cerulenin concentrations, a decreasing percentage of ^3H -palmitate was incorporated into the PL pool, while the percentage of this fatty acid remaining in the FA pool increased. There was no difference in the percentage of incorporation into the DG and TG pool with increasing cerulenin concentrations.

Discussion

The choice of the H9c2 cell line

The main aim of this study was to investigate some basic properties of protein acylation in cardiac muscle. The H9c2 cell line was chosen as experimental model, since this cell line is derived from embryonic rat heart tissue and thus exhibits several characteristics of cardiomyocytes, such as the expression of the L-type calcium channel^{14, 27}, and the tissue specific splicing protein SmN 13. Besides properties of the cardiac muscle, this cell line also possesses properties that are present in skeletal muscle¹². An additional advantage of this cell line is that the amount of cells that can be obtained is virtually unlimited, in contrast with freshly isolated cardiomyocytes.

Experimental conditions

For this study, we chose to incubate the cells with 2.3 nmol ³H-labelled palmitate, corresponding with 100 μ Ci. This amount was sufficient to enable us to reliably measure incorporation of radioactivity into the protein fraction.

Although myristate is not as abundantly present in cells as is palmitate, we chose to incubate the cells with an amount of myristate comparable with that of palmitate, namely 2.5 nmol myristate, corresponding with 125 μ Ci.

From a pilot study it was known that 2 hours of incubation was an adequate time point to measure protein acylation, while there was still a measurable amount of radioactively labelled unesterified fatty acids left (unpublished result). Therefore, we routinely incubated H9c2 cells for 2 hours or less.

In the experiment with increasing amounts of exogenous palmitate, we chose for additional amounts of non-labelled palmitate ranging from 0 to 150 nmol, to explore whether acylation of proteins shows saturation characteristics.

Protein acylation and ^3H -fatty acid incorporation in cellular lipids as function of time.

Protein acylation

After the tritiated fatty acids entered the cell only a small percentage of the fatty acids, namely 0.3% or less became covalently attached to proteins. The bulk of ^3H -fatty acids was found to be incorporated in the phospholipids (PL), diacylglycerols (DG), and triacylglycerols (TG), or stayed unmetabolised as unesterified fatty acids (FA).

Several lines of evidence proved the covalent binding of ^3H -labelled fatty acids to proteins. First, the tritiated fatty acid was recovered with the protein fraction after extensive delipidation. Furthermore, the tritiated fatty acids could not be separated from the proteins by SDS-PAGE.

Protein synthesis influences the rate of palmitoylation as well as myristoylation, although the impact of protein synthesis of the latter is of more importance due to the co-translation aspects of this process. The finding that comparable amounts of myristate and palmitate became covalently attached to proteins after incubation with these tritiated fatty acids, strongly suggests a relatively high rate of protein synthesis in these cells, even though the cells are two days after reaching confluency.

Autoradiography

The differences observed in the degree of covalent attachment of tritiated fatty acids to distinct proteins bands are remarkable. The relative quantity fluctuates not only in time, but also between the different proteins. When reviewing the development of the relative quantity in time, it is most likely that different proteins have different steady state situations. Besides differences in the turnover rate of the covalently attached fatty acid, preferential incorporation into certain proteins and/or the availability of multiple acylation sites could also explain the observed results.

Incorporation into cellular lipids

After fatty acids are taken up, they are converted to fatty acyl-CoA's. These on their turn can be used for protein acylation or for incorporation of the fatty acyl moiety into diacylglycerols, triacylglycerols or phospholipids via the formation of phosphatidate (diacylglycerol 3-phosphate) ²⁸. After incubation

of H9c2 cells with ^3H -labelled fatty acids radioactive label will therefore be recovered in the PL, DG, or TG pool. When no conversion of the ^3H -fatty acid to fatty acyl-CoA takes place, and thus the fatty acid remains unesterified, it can be recovered in the FA pool.

Incubation of H9c2 cells with ^3H -palmitate as well as with ^3H -myristate led to incorporation into the PL-pool from 40% to 60% between 30 minutes to 2 hours. It is rather surprising that the incorporation of myristate in the PL pool is on the same order of magnitude as palmitate. This is most striking, especially when taking into account the relative fatty acyl composition of the PL pool in the rat heart ¹¹, which shows that myristate is a relatively uncommon fatty acid in the PL pool. The unexpected high percentage of labelled myristate in the PL pool could be due to the surplus of myristate in our experimental set-up as compared to normal *in vivo* circumstances in the rat heart. This would indicate that the enzymes involved in phospholipid synthesis make no distinction between palmitate and myristate, and thus that the relative fatty acid composition of the PL pool strongly depends on the species of fatty acids that are available.

The effect of the concentration of exogenous palmitate

Palmitoylation and autoradiography

Increasing exogenous palmitate concentrations does not only lead to an increased amount of palmitate covalently attached to proteins, but more importantly, it shows that protein palmitoylation is *also a saturatable* process. At 0.35 nmol palmitate per mg protein, the protein palmitoylation seems to be saturated. This could mean that the acylation of the majority of proteins that can be modified by covalent attachment of fatty acids have reached a steady state situation.

From figure 4.8 and table 4.3 it can be concluded that there is a remarkable difference in the effect of increasing the amount of exogenous palmitate on the degree of covalent binding of ^3H -palmitate to proteins. Dilution of labelled palmitate by non-labelled fatty acids resulted, as expected, in an appreciable decline of intensity of the protein bands under investigation. The 22 kDa band, however, showed to be relatively insensitive to the increasing amount of non-labelled palmitate. This indicates that this particular protein has a relatively high capacity for covalent binding of fatty acids such as palmitate.

Incorporation into cellular lipids

Figure-4.6 shows that the incorporation of palmitate into the various lipid classes saturates at higher exogenous palmitate levels. In contrast, the content of unesterified palmitate linearly increased when the concentration of palmitate in the incubation mixture was enhanced. This indicates that the capacity of the H9c2 cells to incorporate exogenous fatty acids into their esterified lipid pools is exceeded when the supply of extracellular fatty acids is increased. The increase in FA and DG content at high extracellular palmitate levels in the H9c2 cells could have a major impact on intracellular signal transduction pathways leading, for example, to apoptosis²⁹⁻³¹, activation of protein kinase C^{32, 33} as well as numerous other signal transduction cascades^{2, 34-38}.

The increase of the amount of palmitate in the TG pool during 2 hours of incubation with a concomitant increase in the total amount of fatty acids in this particular pool (figure 4.7, panel C) can be explained by the increase in incorporation of exogenous palmitate (figure 4.7, panel D). In contrast, the fact that the palmitate content in the phospholipid pool did not measurably change during the two hours of incubation (figure 4.7, panel A) while the amount of exogenous palmitate incorporated increased substantially during the incubation period (figure 4.7 panel B), suggests that in this particular esterified lipid pool the palmitoyl moieties are subjected to a turnover process without a net increase in the amount of cellular phospholipids.

Inhibition of protein acylation by cerulenin or tunicamycin

Palmitoylation

In order to study whether protein palmitoylation could be inhibited in the H9c2 cell line we used cerulenin and tunicamycin, previously described as inhibitors of protein palmitoylation^{19, 20, 39} in, for instance, adipocytes and PC12 cells. The present findings indicate that also in the cardiac-like H9c2 cells these compounds are able to inhibit palmitoylation of proteins.

Incorporation into cellular lipids

The effect on ³H-palmitate incorporation in the PL, DG, and TG pool differed between cerulenin and tunicamycin. There was no difference in pattern and percentage of incorporation between control incubations and incubations

with increasing tunicamycin concentrations. This was in contrast with incubations with increasing concentration of cerulenin which led to a decrease of incorporation into the PL and DG pool, and increase of incorporation into the TG pool, while more fatty acids remained unesterified. This difference indicates that the underlying, inhibitory mechanism differs between cerulenin and tunicamycin. Cerulenin may inhibit the lipid-esterification pathway beyond phosphatidate (diacylglycerol 3-phosphate), a common intermediate in the synthesis of phospholipids and triacylglycerols. This notion is based on the fact that incorporation of label into phospholipids was decreased, but not into triacylglycerols.

In summary, the present study showed that the H9c2 model system is suited for studying basic properties of protein acylation in cardiac-like muscle cells. We showed that the amount of fatty acid covalently attached to proteins increased with time. The majority of the fatty acids applied were incorporated into the various lipid classes with phospholipids as the most prominent pool. Furthermore, protein acylation can be saturated by increasing the amount of fatty acids applied to the cells. This also leads to redistribution of the fatty acid over the various lipid pools. Incubations with two known protein inhibitors, cerulenin and tunicamycin, in the H9c2 cell line also led to a significant decrease in covalent attachment of fatty acids to proteins.

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Chapter 5

**Acylated proteins in control and
streptozotocin-induced diabetic rat myocardium**

SUMMARY

In addition to a prominent role in tissue energy conversion, long-chain fatty acids (FA) are involved in signal transduction and modulation of cellular protein localisation and function. The latter is accomplished by acylation of a variety of cellular proteins. In the present study the total amount of fatty acyl moieties covalently bound to cardiac proteins and the effect of streptozotocin-induced diabetes on the degree and relative fatty acyl composition of cardiac proteins have been investigated in isolated rat hearts. In the control hearts circa 0.43% of the cellular fatty acyl pool is covalently bound to proteins. Approximately 93% of these fatty acyl chains are thio-esterified, whereas a relatively minor part is attached to cardiac proteins through amide linkage. Thio-esterified fatty acyl chains are derived from palmitic, stearic, oleic, linoleic, arachidonic and docosahexaenoic acid. In contrast, amide linked protein acylation shows a strong preference for myristic acyl chains.

Streptozotocin-induced diabetes did significantly enhance the myocardial content of FA, but did not affect the degree of myristoylation of cardiac proteins. In contrast, the total content of thio-esterified fatty acyl chains significantly increased by 22%.

In incubations with isolated myocytes derived from control and diabetic rat hearts, the amount of labelled ^3H -palmitate (2.3 nmol per incubation) incorporated in the cellular protein fraction increased significantly from 0.02 ± 0.01 (control) to 0.05 ± 0.01 nmol palmitoyl moieties per mg protein (diabetic). Ketone bodies did not influence the rate of palmitoylation. However, when the palmitate concentration was raised from 2.3 nmol to 152.3 nmol palmitate per incubation ketone bodies significantly increased the rate of protein palmitoylation in diabetic and not in control cells.

INTRODUCTION

In the myocardium adequate oxidative energy conversion is an absolute requirement for proper mechanical function. Blood-borne long-chain fatty acids (FA) are an important source of oxidisable substrates, since under control conditions approximately 70% of the cardiac energy need is fulfilled by oxidation of lipids¹. Previous studies^{2, 3} have shown that the tissue content of FA in the non-esterified form is very low, whereas the bulk of cellular fatty acyl moieties is present in phospholipids, being crucial building blocks of biological membranes. In addition, appreciable proportions of the cellular fatty acyl chains are incorporated in the neutral lipid pool (triacylglycerols)¹.

During the past decade evidence is accumulating that FA, apart from playing a prominent role in cellular energy conversion and being important constituents of membrane phospholipids, act as intracellular signal transducers ^{4, 5}. Moreover, FA are able, among others, to modulate the biological activity and intracellular localization of a number of proteins ^{6, 7}. The latter was found to be accomplished by co-translational or post-translational modification of these proteins by covalent attachment of fatty acyl chains to specific amino acid residues in the protein molecule. Acylation of proteins either occurs via linkage of the fatty acyl chain to the α -amino group of an N-terminal glycine residue through an amide bond or via a linkage to a cysteine residue through a thio-ester bond ⁸.

Despite the importance of FA for cardiac functioning, there is a paucity of information on the degree of protein acylation in cardiac structures under control ⁹ and diabetic conditions, as the latter is known to have severe implications for cardiac fatty acid handling ¹.

This lack of information prompted us to address the following aims:

- assessment of the content of acylated proteins in control rat cardiac tissue (both thio-esterified and amide linked)
- assessment of the effect of diabetes, streptozotocin-induced, on the rat cardiac content of acylated proteins
- elucidation of the relative fatty acid fatty acyl composition of acyl moieties covalently bound to cardiac proteins, in control and diabetic rat hearts.
- clarification of the relationship between the degree of protein acylation and the cardiac tissue content of non-esterified and glycerol-esterified FA.
- Assessment of the rate of protein palmitoylation and the effect of diabetes and ketone bodies thereon in isolated adult cardiomyocytes.

METHODS

Induction of diabetes by streptozotocin

Male Lewis rats were fed *ad libitum*, had free access to water, and were kept under a light-dark cycle of 12 hours ¹⁰. The Institutional Animal Care and Use Committee, Maastricht University, approved the experiments.

Animals (200-250 g body weight) were anaesthetized with ketamine and xylazine (Eurovet, Bladel, The Netherlands), 37.5 and 7.5 mg·kg⁻¹, respectively, via intraperitoneal injection. Subsequently, the animals (*n*=10) were treated with 70 mg·kg⁻¹ streptozotocin (STZ) in disodium citrate (100 mM, pH 4.5) via intravenous injection in the tail vein to induce type-1 diabetes, and received a subcutaneous injection of 2.5 ml 20% (w/v) glucose on both lateral sides. Controls (*n*=12) received vehicle only. STZ-treated animals were given daily a 5% glucose solution to drink. Four weeks after STZ treatment, rats were anaesthetized with a subcutaneous injection of 48 mg sodium pentobarbital (CEVA Sante Animale B.V., Maassluis, The Netherlands). After thoracotomy, hearts were removed from the thorax, rinsed in ice-cold saline solution and ventricles were freeze-clamped with the used liquid N₂ chilled aluminium tongues.

Analysis of fatty acyl moieties in cardiac tissue

Aliquots of deeply frozen tissue (100-200 mg wet weight) were pulverised in an aluminium mortar with a stainless steel pestle, both previously cooled in liquid nitrogen. The tissue powder was transferred to test tubes cooled with liquid nitrogen. The techniques to extract lipids from powdered cardiac tissue and the quantitative analysis of fatty acyl moieties present in the various lipid fractions and acylated protein pool are based on a combination of modified analytical techniques published earlier by Hasselbaink and colleagues ¹¹ and Muszbek and Laposata ¹². In short, test tubes with pulverised tissue were placed at -21°C and the tissue powder was wetted with 2 ml of ice-cold methanol. The content of the test tubes was allowed to warm up to room temperature and was subsequently weighed. Thereafter, 4 ml of chloroform was added and lipids were extracted. The chloroform/methanol (2:1, by volume) extraction step was repeated 2 times. Lipids were analysed as described in details according to Hasselbaink and co-workers ¹¹ and Van der Vusse and Roemen ¹⁰. The remaining tissue residue was used for the assessment of acylated proteins.

The tissue residue obtained after extraction with chloroform/methanol (2:1, by volume) was further extensively extracted with 6 ml chloroform/methanol (1:2, by volume), 6 ml chloroform/methanol/H₂O (1:1:0.3, by volume) and finally with 6 ml methanol. The latter extracts were discarded, since it was found that they contained only minute amounts of fatty acyl moieties (data not shown).

The remaining tissue residue was subsequently dried at 37° C under a gentle stream of nitrogen. Thereafter, the dried residue was subjected to alkaline hydrolysis to transmethylate fatty acyl moieties attached to tissue proteins via thio-esterification. To this end, 2 ml 0.2 M KOH in methanol was added to the dried material and the mixture was incubated in a tightly capped test tube under N₂ atmosphere at 37°C for 30 minutes. The content of the test tube was subsequently centrifuged at 4500 g, at 20°C for 10 minutes. The pellet was used for acid hydrolysis (see below). The supernatant was mixed with 0.25 ml 2 M HCl in H₂O and subsequently extracted with 1 ml hexane (3 times). The combined hexane extracts were subjected to the above mentioned methylation procedure and the amount of methylesters obtained was quantified by capillary gas chromatography¹¹.

The pellet obtained after alkaline hydrolysis was washed two times with 2 ml methanol. Thereafter, 2 ml 3 M HCl in methanol was added and the mixture was incubated at 110°C under nitrogen atmosphere in a tightly capped test tube for 24 h. Thereafter, the incubation mixture was extracted 3 times with 1.5 ml hexane. The combined extracts were dried under N₂ at 37°C, methylated and the amount of methylesters was quantified with the use of capillary gas chromatography.

In a subset of experiments the procedure to extract lipids from cardiac tissue (the various chloroform/methanol steps mentioned above) was repeated once to test whether lipid extraction was complete prior to alkaline and acid hydrolysis of the tissue residue.

Isolation of cardiac myocytes

For the isolation of cardiomyocytes, the hearts were placed in ice-cold buffer A (containing in mM: 115 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, 10 HEPES, 11 glucose, pH 7.4). The aorta was mounted on a cannula (diameter 2.0 mm) and hearts were perfused at 37°C for 5 minutes with buffer A at a flow-rate of 7 ml·min⁻¹ retrogradely according to Langendorff to remove blood from the coronary circulation. Thereafter, recirculating perfusion was started using 40 ml buffer A supplemented with 0.7% (w/v) BSA, 0.075% (w/v) collagenase and 15 mM butanedione monoxime. After 1

minute, flow rate was increased to $8 \text{ ml}\cdot\text{min}^{-1}$ and then increased linearly to $14 \text{ ml}\cdot\text{min}^{-1}$ at 15 minutes after the start of the recirculating perfusion. Flow was allowed to continue at this level for 1 minute. At time points 2 and 12 minutes $40 \mu\text{l}$ 0.1 mM CaCl_2 was added to the perfusion buffer. At the end of the perfusion period, the heart was removed from the cannula, transferred to a Petri dish and carefully opened using forceps. The opened heart was incubated with 10 ml perfusate and 10 ml medium B (medium A supplemented with 0.2 mM Ca^{2+} and 2% BSA) for 10 minutes in a shaking waterbath (37°C , 180 rev./min) (HT Infors, model Aquatron, Bottmingen, Switzerland). The heart was subsequently transferred to a Petri dish and was further dissected with forceps. After dissection, the suspension was transferred to an Erlenmeyer flask and placed in a shaking waterbath (37°C , 180 rev./min) for 5 minutes while the Ca^{2+} concentration was increased with steps of $200 \mu\text{M}$ to 1 mM . Next, cell suspension was filtered through a 0.2 mm nylon mesh and cardiomyocytes were pelleted by centrifugation (2 min , $17 g$). The cell pellet was resuspended in medium C (medium A supplemented with 1 mM Ca^{2+} and 2% BSA) and centrifuged (2 min , $23 g$), and this procedure was repeated once. The cell pellet was resuspended in 20 ml medium C and transferred to a 50 ml conical tube. Cardiomyocytes were allowed to rest for 1.5 hours under mild rotation. Prior to the start of the metabolic studies, cells were washed and resuspended in medium C.

At the end of the metabolic studies, viability of the cardiomyocytes was assessed by determining the percentage of rod-shaped cells. Furthermore, cellular wet weight was obtained by centrifugation in a microcentrifuge and subsequent removal of the supernatant from samples taken at the end of the performed experiments. All solutions used during the experiment were continuously gassed ($\text{O}_2\text{:CO}_2$, $95\%:5\%$).

Incubation of isolated cardiomyocytes

Prior to the start of the experiments, the cardiomyocytes were washed three times with PBS. Subsequently, cells were incubated for 0, 0.5, 1 or 2 hours with $100 \mu\text{Ci}$ ^3H -labelled palmitate (43 Ci/mmol) or $125 \mu\text{Ci}$ ^3H -labelled myristate (49 Ci/mmol), each dissolved in $10 \mu\text{l}$ ethanol and transferred to 1 ml DMEM per flask. Cells were incubated for 2 hours with $100 \mu\text{Ci}$ (2.3 nmol) ^3H -labelled palmitate in the presence of zero or 150 nmol non-radiolabelled palmitate per incubation. These conditions were supplemented with addition of acetoacetate (AcAc, 3 mM), 3- β -hydroxybutyrate (3HB, 3 mM) or a combination of both (2 mM AcAc and 4 mM 3HB). At the end of the

incubation, cells were washed three times with ice-cold PBS. Cells were lysed using 1 ml harvesting buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (w/v), and 10 μ l protease inhibitor cocktail) for 30 minutes on ice.

Statistical analysis

Differences between values obtained in control and diabetic cardiac tissue were analysed with student *t* test for statistical significance. $P < 0.05$ was accepted as a statistically significant difference.

RESULTS AND DISCUSSION

Fatty acyl content of control and diabetic rat heart

The bulk of fatty acyl moieties in control and diabetic cardiac tissue are present in the membrane phospholipid fraction (table 5.1). An appreciable proportion of fatty acyl chains are also present in the neutral lipid fraction (triacylglycerols) of the heart. The combined data show that circa 99.6% of all fatty acyl moieties are esterified to a glycerol backbone. In the control heart minor part of the fatty acyl chains is present in the non-esterified form.

The latter observation corroborates earlier findings^{2,3} and underscores the notion that the cardiac cell is capable of maintaining the content of FA as low as possible. From a teleological point of view, it can be argued that a low intracellular FA content is advantageous for the cardiac myocyte because: i) it prevents FA from a deleterious action on cardiac structures when present in elevated concentrations¹ and ii) it creates a steep gradient for FA delivered from the blood compartment to cardiac muscle cells. This gradient most likely acts as main driving force for the net uptake of circulating FA by the heart *in situ*^{1,13}.

In the diabetic heart, the content of FA was approximately 4.5 fold higher than in control hearts (table 5.1). This observation is in line with previous findings^{11, 14} and indicates that fatty acid handling is disturbed in the diabetic myocardium. Moreover, the amount of triacylglycerols (TG) in diabetic hearts was found to be twice the content in control hearts. The cardiac content of phospholipids (PL) did not differ between control and diabetic hearts.

Table 5.1 Content of fatty acyl moieties in various lipid pools and acylated proteins in control and cardiac tissue.

	<i>Fatty acyl chains(nmol·g⁻¹ dw)</i>		<i>Percentage of total fatty</i>	
	control	diabetic	control	diabetic
Fatty acids (non-esterified)	346 ± 110	1,559 ± 329 *	0.22	0.93
Triacylglycerols	27,960 ± 17,400	44,710 ± 19,320 *	18.4	26.4
Phospholipids	122,980 ± 31,760	122,100 ± 34,100	80.9	72.2
Acylated proteins				
-amide linked	46 ± 5	44 ± 5	0.03	0.03
-thio-esterified	614 ± 59	748 ± 72 *	0.40	0.44

*, significantly different from corresponding value in control hearts ($n=12$), diabetic hearts ($n=10$).

In table 5.2 the relative fatty acyl composition of the various lipid classes and acylated proteins is shown. In the control heart the most common fatty acyl moieties in the (non-esterified) FA pool are palmitic, stearic, oleic and linoleic acids. In the diabetic heart, the relative composition of fatty acyl chains in the cellular FA pools shows some significant differences. For instance, in the FA pool the relative presence of stearic, oleic and linoleic acid are significantly increased when compared to control. However, arachidonic and docosahexaenoic acid showed a significant decrease.

Content of acylated proteins in control and diabetic rat heart

Table 5.1 shows that, although measurable amounts of fatty acyl chains were covalently bound to proteins (on the order of 600 to 700 nmol·g⁻¹ dry weight of tissue), this fatty acyl pool represents only circa 0.43% of the total amount of fatty acyl chains present in normoxic cardiac tissue. The alkaline and acid treatment of the protein residue showed that the bulk of fatty acyl moieties bound to cardiac proteins are thio-esterified. Only a minor part (circa 7%) of the total fatty acyl pool in acylated proteins is covalently connected to proteins via an amide linkage. As expected the vast majority of fatty acyl chains attached via an amide linkage to cardiac proteins were identified as myristic acyl chains. Therefore, data in table 5.1 refer to the amount of myristoyl chains covalently attached to proteins.

In contrast, the thio-acylated proteins contained substantial amounts of palmitic, stearic and arachidonic acyl chains. Oleic, linoleic and docosahexaenoic acyl moieties contributed to a lesser extent (table 5.2).

Table 5.2 Relative fatty acyl composition of non-esterified FA and thio-esterified proteins in control and diabetic rat cardiac tissue

Fatty acyl chain	FA		Thio-esterified	
	Control	Diabetic	Control	Diabetic
myristic acid	2.5 ± 1.2	1.9 ± 0.6	0.1 ± 0.1	0.3 ± 0.2
palmitic acid	22.4 ± 2.2	23.4 ± 1.4	23.5 ± 0.9	23.8 ± 1.7
palmitoleic acid	1.9 ± 1.4	1.2 ± 0.3	0.3 ± 0.3	n.d. *
stearic acid	14.5 ± 1.5	16.2 ± 1.5 *	31.8 ± 1.6	32.3 ± 1.6
oleic acid	14.8 ± 2.4	17.4 ± 2.1 *	12.8 ± 0.5	12.2 ± 0.9
linoleic acid	25.3 ± 4.1	29.1 ± 2.9 *	9.6 ± 0.9	10.1 ± 1.5
arachidonic acid	5.1 ± 1.5	2.0 ± 0.8 *	18.5 ± 0.9	16.9 ± 1.0 *
docosahexaenoic acid	8.8 ± 3.3	6.1 ± 1.8 *	2.7 ± 0.5	3.3 ± 0.8

Data (mean ± S.D.) are expressed as % of total amount of fatty acyl moieties in the FA pool and thio-esterified protein fraction, respectively. n.d.=not detectable. * indicates a significant difference between control ($n=12$) and diabetic ($n=10$) cardiac tissue.

In the diabetic heart, the content of amide linked fatty acyl chains did not differ from that in control hearts (table 5.1). However, the content of thio-esterified fatty acyl chains was significantly enhanced in the diabetic myocardium by 22%. The relative fatty acyl composition of thio-esterified proteins showed that only the percentages of palmitoleic and arachidonic acid were altered. When the pattern of the relative fatty acyl composition of the thio-esterified proteins was compared with that of the FA pool, in the diabetic and control heart, it is striking that the composition of the thio-esterified proteins is not similar to the relative fatty acyl pattern of this lipid pool and that it does not follow alterations observed in the cardiac FA pool under diabetic conditions.

Because the acyl chains in acylated proteins represent only a relatively small proportion of the total cellular fatty acyl pool, it was tested whether the acylated protein pool was contaminated with traces of other lipid classes. To this end, the extraction procedure applied to obtain FA, triacylglycerols and phospholipids was repeated once prior to alkaline and acid treatment of the tissue residue. This additional extraction procedure did neither result in a measurable decrease in the amount of fatty acyl chains in the acylated protein pool nor did it affect the relative fatty acyl composition of acylated proteins (thio-esterified or amide linked) (data not shown).

Protein palmitoylation in isolated adult cardiomyocytes derived from control and diabetic rats.

In isolated myocytes derived from control and diabetic rat hearts, the rate of ^3H -palmitate (2.3 nmol per incubation) was significantly higher than in control cells (table 5.3). Addition of ketone bodies did not affect the rate of palmitoylation. When control as well as diabetic cardiomyocytes were incubated with 152.3 nmol palmitate (2.3 nmol ^3H -labelled and 150 nmol non-labelled palmitate) it was found that under high FA conditions the amount of fatty acyl moieties covalently bound to proteins was significantly increased.

The presence of ketone bodies in the incubation medium did not significantly alter the incorporation of ^3H -palmitate in the protein fraction of control cardiomyocytes at high *i.e.* 152.3 nmol palmitate. However, in diabetic cardiomyocytes the presence of ketone bodies significantly altered the incorporation of fatty acyl moieties. In all cases (AcAc, 3HB or the combination) palmitoylation was increased.

Significance of acylated proteins in cardiac tissue

In spite of the fact that only 0.43% of all fatty acyl moieties present in the heart is covalently bound to proteins, this particular fatty acyl pool might play a significant role in the biological function of a number of cardiac proteins. Assuming that the heart contains 800 mg protein per gram dry weight of tissue and that the average molecular mass of cardiac proteins is 40 kDa, the ratio of number of fatty acyl chains, covalently bound to cardiac proteins, to the total number of cardiac proteins amounts to 3:100. Moreover, studies on other tissues and cell types have shown that some proteins, crucial for normal cellular functioning, such as G-protein α subunits, sodium channel α subunits, various membrane receptors and CD36, are present in the acylated form ¹⁵⁻¹⁸. It should be emphasised, however, that the identity and function of acylated proteins in cardiac tissue is unknown, but is subject of future studies.

Table 5.3 Protein palmitoylation in isolated cardiomyocytes derived from control and diabetic rats incubated with 2.3 or 152 nmol palmitate in the presence of ketone bodies.

<i>2.3 nmol palmitate-incubation⁻¹</i>				
	(-)	AcAc	3HB	combination
Control	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Diabetic	0.05 ± 0.01*	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
<i>152.3 nmol palmitate-incubation⁻¹</i>				
	(-)	AcAc	3HB	combination
Control	0.07 ± 0.02 #	0.05 ± 0.01 #	0.07 ± 0.01 #	0.09 ± 0.02 #
Diabetic	0.11 ± 0.03 §*	0.22 ± 0.04 §*	0.27 ± 0.07 §*	0.21 ± 0.05 §*

Data are expressed as nmol palmitoyl moiety·mg protein⁻¹ (n=3) * indicates significantly different from corresponding incubations of control cardiomyocytes. § indicates significantly different from corresponding cardiomyocytes derived from diabetic rats, incubated with 2.3 nmol palmitate; # indicates significantly different from corresponding control cardiomyocytes incubated with 2.3 nmol palmitate. (-) means in the absence of ketone bodies; AcAc; acetoacetate (3 mM), 3HB; 3-β-hydroxybutyrate (3 mM); combination refers to a mixture of 3HB (4 mM) and AcAc (2mM).

It is of interest to note that the majority of protein-linked fatty acyl moieties are attached to cardiac proteins via a thio-ester bond, whereas a relatively small proportion is covalently bound via an amide linkage. Amide linkage is accomplished co-translationally by linking the myristoyl chain fatty acid moiety to the α-aminogroup of an N-terminal glycine residue through the catalytic action of protein N-myristoyl transferase¹⁹. Others have shown that thio-esterification in cellular proteins occurs post-translationally via a covalent binding of the carboxyl head group of the fatty acid to the sulphur atom of the cysteine residue in the protein molecule¹⁵. In myocardial tissue, thio-esterified fatty acyl chains are not only derived from palmitic acid but also from other long-chain saturated and unsaturated FA. This finding indicates that in the heart, like in other organs and cell types, thio-esterification shows a relatively relaxed fatty acyl chain specificity¹⁶.

Novel is the finding that the content of thio-esterified proteins is enhanced in the diabetic heart. This increase may be caused by the increased intracellular availability of FA¹¹ and/or their CoA-derivatives²⁰.

The consequences of enhanced protein thio-esterification on myocardial cells are incompletely understood. This issue can only be resolved if the identity and function of the acylated proteins in cardiac structures is known in full detail. At present one may speculate that the increased levels of acylated proteins may contribute to the altered substrate handling in the diabetic heart and ultimately to the decline in function of the affected organ.

It is of interest to note that in contrast to thio-esterified proteins the cardiac content of myristoylated proteins is not altered in the diabetic state. This finding implies that co-translational acylation of proteins is not affected in diabetic conditions. In contrast, post-translational modification, and in particular thio-esterification appears to be sensitive to alterations in the heart evoked by the diabetic state.

The present findings also show that the rate of protein palmitoylation is intrinsically higher in diabetic cardiomyocytes than in control. Moreover, both increased levels of FA and enhanced availability of ketone bodies significantly further stimulate the rate of palmitoyl incorporation in the protein pool of the diabetic cell. Since in the diabetic animal the *in vivo* heart is exposed to both elevated FA and ketone body levels, these metabolic alterations provide a feasible explanation for the increase in amount of acylated proteins in the intact diabetic heart.

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Chapter 6

Ketone bodies disturb fatty acid handling in isolated cardiomyocytes derived from control and diabetic rats

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Abstract

According to the current paradigm, the diabetic heart shows an increased oxidation rate of long-chain fatty acids (FA). This notion is mainly based on the outcome of studies on isolated hearts. Taking into account that the plasma concentration of competing substrates, such as ketone bodies, is substantially increased during diabetes, we have explored the effect of these substrates on cardiac fatty acid handling.

Experiments were performed on cardiomyocytes isolated from both control and streptozotocin-induced diabetic rats. Cardiomyocytes were incubated with either normal (80 μM) or elevated palmitate concentrations (160 μM) in the absence or presence of ketone bodies, either acetoacetate (AcAc), 3- β -hydroxybutyrate (3HB), or a combination of both.

When diabetic cardiomyocytes were incubated under normal conditions (80 μM palmitate, no ketone bodies), initial FA uptake rate was found to be identical to that of control cardiomyocytes. However, when diabetic cardiomyocytes were exposed to elevated palmitate concentrations (160 μM palmitate), initial uptake rate was significantly higher than in control cardiomyocytes incubated under normal conditions (64.0 ± 13.8 versus 35.2 ± 4.8 nmol·g wet weight⁻¹·3 min⁻¹). Neither 3 mM AcAc nor 3 mM 3HB affected the initial uptake rate of palmitate in control and diabetic cardiomyocytes.

Oxidation in palmitate was significantly higher in diabetic cardiomyocytes than in control cells when both were incubated under normal conditions (80 μM palmitate no ketone bodies), *i.e.* 92.1 ± 25.9 and 58.9 ± 23.6 nmol·g wet weight⁻¹·30 min⁻¹, respectively. When diabetic cardiomyocytes were also exposed to 3 mM AcAc (in the presence of 160 μM palmitate), palmitate oxidation rate dropped to 53.2 ± 18.5 nmol·g wet weight⁻¹·30 min⁻¹. In contrast, the addition of 3 mM 3HB did not affect palmitate oxidation.

Varying the concentration of AcAc in the incubation medium revealed that palmitate oxidation in diabetic and control cardiomyocytes is highly sensitive to AcAc. Half maximum inhibition of palmitate oxidation in both cell types was reached at 0.3–0.5 mM AcAc.

When diabetic cardiomyocytes were incubated under diabetic conditions (160 μM palmitate, 3 mM AcAc) significant amounts of unmetabolized palmitate accumulated inside the cells. This effect was not observed in control cardiomyocytes.

The collected findings indicate that due to the presence of ketone bodies FA oxidation in diabetic cardiomyocytes is not increased, despite the

elevated supply of extracellular FA. This result may explain the discrepancy observed in FA oxidation rates between isolated hearts and hearts studied *in vivo*. Moreover, the increase in palmitate uptake and concomitant inhibition of palmitate oxidation in diabetic cardiomyocytes, incubated under diabetic conditions (160 μ M palmitate, 3 mM AcAc), most likely explains the accumulation of FA in the diabetic heart.

Introduction

Diabetes mellitus (DM) is a clinical syndrome characterized by hyperglycaemia caused by a relative or absolute deficiency of insulin or by resistance to the action of insulin at the cellular level. The diabetic condition leads to a significant elevation of blood glucose and plasma lipids if left untreated^{1,2}. Insulin deficiency also results in increased hepatic synthesis of ketone bodies, leading to elevated plasma levels of acetoacetate (AcAc) and 3- β -hydroxybutyrate (3HB)³, which in turn can evoke serious pathophysiological complications, such as diabetic ketoacidosis.

On the basis of studies on isolated hearts it was concluded that cardiac long-chain fatty acid (FA) oxidation is increased in the diabetic state⁴⁻⁶. These findings are however at variance with observations on hearts *in situ* of human diabetic patients⁷. This suggests that FA oxidation by the diabetic heart is governed by factors present in the *in situ* situation, but absent when isolated rat hearts are studied.

Under normal conditions cardiac energy demand is mainly covered by the oxidation of FA and glucose⁸. Lactate and ketone bodies are also able to serve as oxidizable substrates⁹. It is tempting to state that the discrepancy between isolated hearts and hearts of human diabetic patients with respect to cardiac FA utilization is caused by the availability of alternative substrates, such as ketone bodies.

In the present study we have tested the hypothesis that ketone bodies, AcAc and 3HB, affect both uptake and intracellular handling of FA in diabetic cardiomyocytes. To this end, cardiomyocytes were isolated from diabetic rats, 4 weeks after streptozotocin (STZ) treatment. The cells were exposed to AcAc, 3HB, or a combination of both. FA uptake, oxidation, and esterification were studied in the presence of normal (physiological) or increased (diabetic) extracellular FA concentrations.

Methods

Treatment of animals

Male Lewis rats were fed *ad libitum*, had free access to water, and were kept under a light-dark cycle of 12 hours¹⁰. The Institutional Animal Care and Use Committee, Maastricht University, approved the experiments.

Animals (200-250 g body weight) were anaesthetized with ketamine and xylazine (Eurovet, Bladel, the Netherlands), 37.5 and 7.5 mg·kg⁻¹, respectively, via intraperitoneal injection. Subsequently, the animals were treated with 70 mg·kg⁻¹ streptozotocin (STZ) in disodium citrate (100 mM, pH 4.5) via intravenous injection in the tail vein to induce type 1 diabetes, and received a subcutaneous injection of 2.5 ml 20% (w/v) glucose on both lateral sides. Controls received vehicle only. STZ-treated animals were given daily a 5% glucose solution to drink. Four weeks after STZ treatment, rats were anaesthetized with a subcutaneous injection of 48 mg sodium pentobarbital (CEVA Sante Animale B.V., Maassluis, the Netherlands) and 1000 i.u. of heparin. Thereafter, hearts were surgically removed from the thorax. In a subset of experiments (*n*=6) the anaesthetized animals did not receive heparin. After thoracotomy, blood was collected from the left ventricle with a citrate rinsed syringe. Blood glucose was measured with Euroflash™ (Lifescan, Milpitas, CA, USA) and plasma FA by gas chromatography¹¹.

Isolation of cardiomyocytes

Cardiomyocyte isolation procedure was partially adapted from that described by Luiken and colleagues¹². The surgically removed hearts were placed in ice-cold buffer A (containing in mM: 115 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, 10 HEPES, 11 glucose, pH 7.4). The aorta was mounted on a cannula (diameter 2.0 mm) and hearts were perfused at 37°C for 5 minutes with buffer A at a flow-rate of 7 ml·min⁻¹ retrogradely as described by Langendorff¹³ to remove blood from the coronary circulation. Thereafter, recirculating perfusion was started using 40 ml of buffer A supplemented with 0.7% (w/v) BSA, 0.075% (w/v) collagenase and 15 mM butanedione monoxime. After 1 minute, flow rate was increased to 8 ml·min⁻¹ and then increased linearly to 14 ml·min⁻¹ at 15 minutes after the start of the recirculating perfusion. Flow was allowed to continue at this level for 1 minute. At time points 2 and 12 minutes 40 µl 0.1 mM CaCl₂ was added to the perfusion buffer. At the end of the perfusion period, the heart was removed from the cannula, transferred to a Petri dish and carefully

opened using forceps. The opened heart was incubated with 10 ml perfusate and 10 ml medium B (medium A supplemented with 0.2 mM Ca^{2+} and 2% BSA) for 10 minutes in a shaking waterbath (37°C, 180 rev./min) (HT Infors, model Aquatron, Bottmingen, Switzerland). The heart was subsequently transferred to a Petri dish and was further dissected with forceps. After dissection, the suspension was transferred to an Erlenmeyer flask and placed in a shaking waterbath (37°C, 180 rev./min) for 5 minutes while the Ca^{2+} concentration was increased with steps of 200 μM to 1 mM. Next, cell suspension was filtered through a 0.2 mm nylon mesh and cardiomyocytes were pelleted by centrifugation (2 min, 17 g). The cell pellet was resuspended in medium C (medium A supplemented with 1 mM Ca^{2+} and 2% BSA) and centrifuged (2 min, 23 g), and this procedure was repeated once. The cell pellet was resuspended in 20 ml medium C and transferred to a 50 ml conical tube. Cardiomyocytes were allowed to rest for 1.5 hours under mild rotation. Prior to the start of the metabolic studies, cells were washed and resuspended in medium C.

At the end of the metabolic studies, viability of the cardiomyocytes was assessed by determining the percentage of rod-shaped cells. Furthermore, cellular wet weight was obtained by centrifugation in a micro centrifuge and subsequent removal of the supernatant from samples taken at the end of the performed experiments. All solutions used during the experiment were continuously gassed ($\text{O}_2:\text{CO}_2$, 95%:5%).

Preparation of palmitate:BSA complex

To prepare a stock solution of palmitate-BSA complex, 100 μCi [$1\text{-}^{14}\text{C}$]palmitate and either 1.8 or 3.6 mM palmitate in 10 ml 100% ethanol, were mixed with water containing KOH (1.5 times the amount of palmitate on molar basis). After evaporation of ethanol at 45°C under nitrogen, the saponified palmitate was added to 40 ml medium A supplemented with 2.5% BSA. Prior to incubation, the stock solution was diluted 4.5 fold with medium A supplemented with 1.0 mM CaCl_2 .

Palmitate uptake rate

Rate of palmitate uptake was determined during 3 minutes as described previously ¹⁴. In short, a 2.0 ml suspension of freshly isolated cardiomyocytes was incubated in a capped glass vial ($\text{O}_2:\text{CO}_2$, 95%:5%) in the absence or presence of ketone bodies, in a shaking waterbath (37°C, 180 rev./min)

(HT infors, model Aquatron, Bottmingen, Switzerland). After thermal equilibration, 0.5 ml of labelled palmitate (80 or 160 μM final concentration) was added and incubation proceeded for 3 minutes. Next, 2.0 ml of the incubation mixture was transferred to a tube containing 8.0 ml ice-cold stop solution. Correction for extracellular radioactivity was performed according to Luiken and co-workers¹². Uptake experiments were performed in triplicate for each condition tested.

Palmitate oxidation rate

The rate of palmitate oxidation was determined in the presence or absence of ketone bodies during 30 minutes as described previously¹⁴. The reaction was started by addition of [$1\text{-}^{14}\text{C}$]palmitate to the capped glass vial. After termination of the reaction, production of $^{14}\text{CO}_2$ was measured after base trapping in NaOH. The obtained $^{14}\text{CO}_2$ values were corrected for $^{14}\text{CO}_2$ trapped in vials supplemented with [$1\text{-}^{14}\text{C}$]palmitate but in the absence of cardiomyocytes in the incubation medium. Thereafter, this value was normalized on mg wet weight of cardiomyocytes and corrected for the percentage of non rod-shaped cells. Oxidation experiments were performed in triplicate for each condition tested.

Incorporation of palmitate into intracellular lipids

A 2.0 ml suspension of cardiomyocytes was incubated in a capped glass vial in the absence or presence of ketone bodies, in an Aquatron shaking waterbath (37°C, 180 rev./min). Reaction was started by addition of [$1\text{-}^{14}\text{C}$]palmitate. After 30 minutes reaction was stopped according to Luiken and colleagues¹². Cells were pelleted by centrifugation (2 min, 60 g, 4°C) and washed twice with 5.0 ml ice-cold stop solution. The pellet was solubilized in buffer containing (in mM), 50 Tris-HCl pH 7.6, 150 NaCl, 5 EDTA, and 1% (w/v) Triton X-100. Lipids were extracted with chloroform/methanol and separated with thin layer chromatography¹¹. The lipid spots corresponding to triacylglycerol (TG), phospholipids (PL), and FA were scraped from the plate and the amount of radioactivity was determined with a liquid scintillation counter. Incorporation experiments were performed in duplicate for each condition tested.

Choice of substrate concentrations

Final extracellular concentrations of 80 μM and 160 μM [$1\text{-}^{14}\text{C}$]palmitate were used. They correspond with the interstitial concentrations of FA in healthy and diabetic rats, respectively, taking into account that the interstitial FA concentration is approximately 60% of the arterial FA level¹⁵. These FA conditions were combined with varying concentrations of AcAc, 3HB, or a combination of both ketone bodies. In general, in STZ-treated rats plasma concentration of ketone bodies is on the order of 3 to 6 mM^{16, 17} with a ratio of 3HB to AcAc of 2:1¹⁶. Since AcAc was added to the incubation medium as lithium salt, the effect of 3 mM Li^+ (LiCl) was tested on FA uptake, oxidation, and deposition of label in the various lipid pools. No effect of Li^+ on FA handling was found (data not shown).

Materials

[$1\text{-}^{14}\text{C}$]Palmitic acid was obtained from NEN Life Science Products (Boston, MA, USA). Collagenase type 2 (LS04176) from Worthington (Lakewood, NJ, USA), phloretin, Bovine Serum Albumin fraction V (A4503), streptozotocin, LiCl , acetoacetic acid (lithium salt), DL- β -hydroxybutyric acid, palmitic acid, and 2,3-butanedione monoxime from Sigma (St. Louis, MO, USA).

Statistics

Results were obtained from at least four different cardiomyocyte isolations and are presented as means $\pm$ SD. Analysis of experiments within one group was performed with Two-tailed Student's t tests. Comparison between groups was performed with the one-way analysis of variance (ANOVA). In case the F ratio obtained indicated that significant differences between groups were present, a two-tailed Student's t test for unpaired data was carried out, applying Bonferroni's adjustment for multiple comparison¹⁸. For all analyses, the level of significance was set at 0.05.

Results

General characteristics of STZ-induced diabetic rats

Body weight of the diabetic rats (301 ± 49 g) was significantly lower than that of controls (394 ± 18 g). Diabetic plasma FA levels were significantly higher than control (265 ± 88 versus 134 ± 59 μM). Diabetic blood glucose concentrations were significantly higher than control, 20.4 ± 6.5 and 9.1 ± 1.0 mM, respectively.

Table 6.1 Initial palmitate uptake rate in control and diabetic cardiomyocytes

[Palmitate] (μM)	Ketone bodies	Initial uptake (nmol·g wet weight ⁻¹ ·3 min ⁻¹)	
		control	diabetic
80	-	35.2 ± 4.8	40.1 ± 7.6
	3 mM AcAc	33.4 ± 3.9	39.3 ± 9.0
	3 mM 3HB	36.0 ± 8.0	41.2 ± 9.4
	6 mM combination	36.0 ± 5.0	41.4 ± 12.8
160	-	$54.8 \pm 5.0^*$	$64.0 \pm 13.8^*$
	3 mM AcAc	$55.3 \pm 8.4^*$	$60.2 \pm 14.0^*$
	3 mM 3HB	$60.7 \pm 7.6^*$	$71.5 \pm 14.2^*$
	6 mM combination	$64.0 \pm 10.1^*$	$70.1 \pm 27.5^*$

Data are expressed as means $\pm$ SD ($n=5$). Asterisk indicates significantly different from the value of the corresponding incubation with 80 μM palmitate ($p<0.05$). Statistical analysis revealed that the higher mean values in diabetic cardiomyocytes as compared to corresponding controls did not reach the level of significance. (-) means in the absence of ketone bodies; AcAc refers to acetoacetate; 3HB to 3- β -hydroxybutyrate; 6 mM combination refers to a mixture of 3HB (4 mM) and AcAc (2 mM). The final incubation medium contained (in mM) NaCl (115), KCl (2.6), KH_2PO_4 (1.2), MgSO_4 (1.2), NaHCO_3 (10), HEPES (10), glucose (11), BSA (0.3), pH 7.4, palmitate and ketone body concentration as indicated.

Initial palmitate uptake rate

There was no difference in the initial uptake rate of palmitate between control and diabetic cardiomyocytes at either normal (80 μM) or elevated (160 μM) palmitate concentrations (table 6.1). However, increasing the palmitate concentration from 80 to 160 μM significantly enhanced its uptake. The percentage of increase did not differ between control and diabetic cardiomyocytes, being on the average 56% and 59%, respectively. Ketone bodies did not affect the initial uptake rate of FA in either control or diabetic cardiomyocytes, irrespective of the palmitate concentration.

Figure 6.1 Influence of ketone bodies on the rate of FA oxidation

Cardiomyocytes derived from control and diabetic rats were incubated with [$1\text{-}^{14}\text{C}$]palmitate for 30 minutes. See for further details legends to table 6.1. AcAc to acetoacetate (3 mM) ; 3HB to 3- β -hydroxybutyrate (3 mM) ; 6 mM combination means a mixture of 3HB (4 mM) and AcAc (2 mM). Data are expressed as mean $\pm$ SD ($n=6$). $p < 0.05$ * versus corresponding (-); # control versus corresponding diabetic; + normal FA versus corresponding high FA.

Palmitate oxidation rate

The rate of palmitate oxidation at 80 μM palmitate, in the absence of ketone bodies, is about 56% higher in diabetic cardiomyocytes than in those of control ($p < 0.05$) (figure 6.1). Increasing the palmitate concentration from 80 to 160 μM resulted in a 70% increase in oxidation rate in control cardiomyocytes ($p < 0.05$). The percentage increase in diabetic cardiomyocytes was comparable (68%). Addition of 6mM ketone bodies (3HB/AcAc, 2:1) to control cardiomyocytes significantly decreased the oxidation rate, both at normal and elevated palmitate concentrations, by 51 and 58%, respectively. Comparable inhibitory effects were seen in diabetic cardiomyocytes. Figure 6.1 also shows the differential effect of 3HB and AcAc. When cardiomyocytes were exposed to 3HB (3mM), the inhibitory effect was insignificant under all conditions analysed. In contrast, exposure to 3mM AcAc substantially inhibited palmitate oxidation to a comparable extent in control and diabetic cardiomyocytes, at both palmitate concentrations.

Influence of varying concentrations of ketone bodies on FA oxidation

Figure 6.2 shows that palmitate oxidation can be suppressed to approximately 30% of its maximal rate by AcAc. This is true for cardiomyocytes derived from control and diabetic animals. The maximal percentage of inhibition was found to be independent from the two palmitate concentrations applied (figure 6.2). Half-maximum inhibition is reached at 0.3-0.5 mM AcAc and maximum inhibition at 2 mM AcAc and higher. AcAc is therefore a highly potent inhibitor of cardiomyocyte FA oxidation, and the sensitivity to AcAc hardly differs between control and diabetic cells. The findings in figure 6.2 also indicate that it is mainly AcAc that causes the inhibitory effect of the mixture of AcAc and 3HB, supporting the results shown in figure 6.1.

Incorporation of labelled palmitate in various intracellular lipid pools

In control cardiomyocytes, incubated under normal conditions (80 μM palmitate, no ketone bodies) incorporation of labelled palmitate in the cellular PL, TG and FA pool amounted to 0.27 ± 0.14 , 1.46 ± 0.80 and 0.07 ± 0.05 nmol·g wet weight⁻¹·30 min⁻¹, respectively.

Figure 6.2 Influence of varying concentrations of ketone bodies on the rate of FA oxidation

Control and diabetic cardiomyocytes were incubated with either 80 or 160 μM palmitate, both with varying concentrations of ketone bodies, for 30 minutes. See for further details legends to **table 1**. Black circles = AcAc; White circles = 3HB/AcAc (2:1). Data are expressed as mean $\pm$ SD ($n=4$).

In diabetic cardiomyocytes, incubated under diabetic conditions (160 μM palmitate, 3 mM AcAc) substantial higher amounts of labelled palmitate were incorporated in the PL, TG and FA pool, *i.e.* 0.69 ± 0.32 , 5.15 ± 3.02 and 0.19 ± 0.18 nmol·g wet weight⁻¹·30 min⁻¹. However, due to a relatively high degree of variation between the individual experiments, the increase in the deposition of palmitate into the various lipid pools in the diabetic cells did not reach the level of significance. AcAc, was set at 1.0. To evaluate the effect of varying the ambient palmitate concentration and the addition of AcAc to

the incubation medium, the values obtained in either control or diabetic cells incubated in the presence of 80 μM palmitate, in the absence of This procedure enabled us to use the cells as their own control. Raising the concentration from 80 to 160 μM tended to increase the incorporation of palmitate in the PL and TG pool of control cells (figure 6.3). The increased deposition of label in the PL, TG and FA pool of diabetic cardiomyocytes was highly significant when the extracellular palmitate concentration was increased from 80 to 160 μM ($p<0.05$).

Figure 6.3 Deposition of radiolabelled palmitate in various lipid pools in control and diabetic cardiomyocytes

After incubation for 30 minutes with radiolabelled palmitate, lipids were extracted and subjected to TLC. Data are presented relative to the values obtained in control and diabetic cardiomyocytes, respectively, incubated in the presence of 80 μM palmitate without ketone bodies. See for further details legends to **table 6.1**. Asterisk indicates significantly different from the corresponding incubation with 80 μM palmitate, without ketone bodies. Data are expressed as mean $\pm$ SD ($n=4$).

Exposure of cardiomyocytes, either derived from control or diabetic hearts, to 3 mM AcAc at 80 μ M palmitate did not affect the incorporation of palmitate in the PL and TG pool. The FA pool in diabetic cardiomyocytes incubated in the presence of 80 μ M palmitate showed a significant expansion when exposed to 3 mM AcAc. This effect was not seen in control cardiac muscle cells. When the concentration of palmitate was increased from 80 to 160 μ M in the incubation medium of control or diabetic cells, exposed to 3 mM AcAc, the deposition of labelled palmitate tended to increase (control) or significantly increased (diabetic cells) in the PL and TG pool. Since this pattern was also observed in cells incubated in the absence of AcAc, increased deposition of labelled FA in the esterified lipid pools is mainly caused by increased availability of FA rather than by a modulating effect of AcAc. The same conclusion holds for the FA pool in control cardiomyocytes. In contrast, in diabetic cardiomyocytes, bulk accumulation of labelled palmitate in the FA pool seems to be mainly caused by the presence of AcAc in the incubation medium.

Discussion

The present observations indicate that ketone bodies, in particular AcAc, affect FA handling in the cardiac muscle cell. This finding is of interest because it allows one to fully appreciate the changes in cardiac FA utilization in the diabetic heart, since the circulating concentration of ketone bodies is substantially increased in this pathophysiological condition. AcAc causes a mismatch between FA uptake (not affected by AcAc, but increased due to enhanced supply) and FA oxidation (significantly inhibited by AcAc) when diabetic cardiomyocytes, derived from STZ-treated rats and incubated under diabetic conditions, *i.e.* 160 μ M palmitate, 3 mM AcAc, were compared with control cardiomyocytes, incubated under normal conditions, *i.e.* 80 μ M palmitate, no ketone bodies. This mismatch most likely explains the accumulation of lipids, FA and TG, in the diabetic heart *in situ*. Moreover, the present findings are challenging the current paradigm that FA oxidation is enhanced in the diabetic heart, since this notion is based on experiments performed on isolated hearts perfused with media devoid of ketone bodies.

General background

Earlier findings in hearts isolated from STZ-treated rats and immediately freeze-clamped for tissue lipid analysis indicated a 4.5 fold accumulation of FA in the diabetic heart, while blood FA levels were enhanced only two-fold¹¹. Accumulation of FA in cardiac tissue, also reported by Heyliger and colleagues¹⁹, strongly suggests a mismatch between the uptake of extracellular FA and their intracellular utilization. A common feature in patients suffering from diabetes and in experimental diabetic animals is an increased circulatory level of ketone bodies, AcAc and 3HB²⁰. This increase is most striking in diabetic ketoacidosis, a major complication of type 1 diabetes. Elevated levels of ketone bodies have been also observed in poorly controlled type 2 diabetes, chronic cardiac failure and during the consumption of high fat diet²¹⁻²⁴. Pioneering studies of Randle and colleagues have shown that ketone bodies serve as alternative substrates for energy conversion in cardiac muscle²⁰. Considering the enhanced availability of ketone bodies in the diabetic state, it is likely that the presence of those substances interfere with myocardial lipid metabolism.

To fulfil cardiac energy requirements two main nutrients, FA and glucose, are continuously supplied to the heart, extracted from the extracellular compartment and oxidized in the mitochondrial matrix to regenerate the myocytal ATP pool. Under normal circumstances, oxidation of plasma-borne FA contributes up to 60-70% of energy conversion in the healthy heart⁸. In the diabetic heart fuel selection is significantly changed, but the direction and magnitude of the alterations in cardiac FA consumption appear to differ greatly between studies dealing with this subject matter⁸. These considerations prompted us to investigate the effect of ketone bodies on FA handling by cardiac muscle cells derived from STZ-treated rats.

Initial palmitate uptake rate

The present findings clearly indicate that the rate of palmitate uptake by isolated cardiomyocytes is not changed in the diabetic state when the cells were exposed to normal extracellular palmitate concentrations, *i.e.* 80 μ M. Elevation of the concentration to 160 μ M (diabetic conditions) caused a significant and comparable increase in the initial rate of palmitate uptake both in diabetic and control cardiomyocytes, indicating that, under the present conditions, trans-sarcolemmal FA transport is not altered in diabetic cells. This result might be surprising since earlier studies showed that STZ-treatment resulted in an increase in the myocardial content of proteins

known to facilitate FA uptake, including FAT/CD36^{25, 26}. A possible explanation is that the increased amount of FAT/CD36 is stored in the cytoplasmic compartment rather than incorporated into the plasma membrane.

The fact that neither AcAc nor 3HB, present in the incubation medium, altered the uptake rate of palmitate in cardiomyocytes indicates that the mechanisms underlying the unidirectional influx of FA across the sarcolemma are insensitive to the presence of ketone bodies in the interstitial compartment. Since diabetic cardiomyocytes *in vivo* are exposed to both elevated FA levels and enhanced concentrations of ketone bodies the conclusion can be drawn that diabetic cardiomyocytes take up higher amounts of FA from their surroundings than control, merely because the extracellular FA availability is increased (figure 6.4).

Figure 6.4 Initial uptake and oxidation of palmitate by control and diabetic cardiomyocytes

This figure is a compilation of data shown in **table 6.1** and **figure 6.1**. Control cardiomyocytes were incubated for either 3 minutes (initial uptake) or 30 minutes (oxidation) under normal conditions, *i.e.* 80 μM palmitate, in the absence of acetoacetate (AcAc); diabetic cardiomyocytes were incubated during similar time intervals under diabetic conditions, *i.e.* 160 μM palmitate in the presence of 3 mM AcAc. Data are expressed as mean ± SD ($n=5-6$). Asterisk indicates a statistically significant difference between control and diabetic cells.

Palmitate oxidation rate

The rate of palmitate oxidation was found to be significantly higher in cardiomyocytes isolated from diabetic hearts than in cardiomyocytes from control hearts when incubated under comparable, normal conditions (80 μM palmitate, no ketone bodies). These findings support some earlier observations in isolated rat hearts, namely that the rate of FA oxidation is increased in hearts obtained from diabetic animals ^{4, 6}. Increasing the extracellular palmitate concentration from 80 to 160 μM enhanced the FA oxidation rate about 70% both in control and diabetic cardiomyocytes. Comparing control cells at normal ambient FA levels with diabetic cardiomyocytes subjected to diabetic FA concentrations revealed that the rate of palmitate oxidation is approximately 3 times higher in the latter (figure 6.1). However, this picture substantially changes when ketone bodies were added to the incubation medium to mimic the diabetic conditions as much as possible. Co-incubation of diabetic cardiac muscle cells with 160 μM palmitate and 3 mM AcAc inhibited palmitate oxidation by approximately 65%. The resulting oxidation rate ($53.2 \pm 18.5 \text{ nmol-g wet weight}^{-1}\cdot 30 \text{ min}^{-1}$) was not significantly different from that measured in control cells, incubated in control conditions ($58.9 \pm 23.6 \text{ nmol-g wet weight}^{-1}\cdot 30 \text{ min}^{-1}$; 80 μM palmitate, no ketone bodies) (figure 6.4). It is noteworthy that the inhibitory effect of ketone bodies on myocyte FA oxidation is almost exclusively caused by AcAc, since the effect of the reduced counterpart, 3HB, was found to be negligible. The lack of effect of 3HB is remarkable, considering that both 3HB and AcAc can be used as oxidizable substrate by the heart ²⁰. At present no conclusive explanation can be offered for this intriguing observation, although differences in redox state in the cardiomyocytes exposed to either AcAc or 3HB should be considered. In this respect, earlier findings of Isales and colleagues are worth mentioning, as they also observed differential effects of 3HB and AcAc, namely on the regulation of growth factors in brain endothelial cells ²⁷.

Detailed analysis of the inhibitory effect of AcAc on cardiomyocyte palmitate oxidation revealed that, both in control and diabetic cells, AcAc is a potent inhibitor of palmitate oxidation. The maximal degree of inhibition in both cell types was on the order of 70%, irrespective of the concentration of palmitate in the extracellular fluid. Since ketone bodies are permeant to cells via the H^+ monocarboxylate co-transporter ²⁸⁻³⁰, transport limitations of ketone bodies through this protein-mediated process could therefore contribute to the inability to depress FA oxidation any further.

Half-maximal inhibition was obtained at about 0.3-0.5 mM AcAc, indicating that, on the one hand, FA oxidation by cardiomyocytes is highly

sensitive to AcAc in the surrounding medium since this relatively low concentration of AcAc is readily reached under a variety of conditions ³¹ and, on the other, that the sensitivity to AcAc did not differ between diabetic and healthy cardiomyocytes. The latter indicates that cardiomyocytes in diabetic hearts do not adjust to chronically elevated levels of ketone bodies by altering their sensitivity towards the inhibitory action of these compounds. This kind of adjustment might have been expected since previous observations by Grinblat and colleagues ³² and Kante and co-workers ³³ showed that the expression and activity of two enzymes involved in ketone body handling, namely 3- β -hydroxybutyrate dehydrogenase and 3-oxoacid-CoA transferase, are significantly decreased in the diabetic heart as compared to controls. Obviously, not merely the intracellular conversion but also other, as yet unidentified properties may be involved in the inhibitory action of ketone bodies on cardiac FA oxidation.

Incorporation of palmitate in cellular lipid pools

In general, the amount of labelled palmitate sequestered in the esterified lipid pool (TG and PL) depended on the extracellular FA concentration. Increasing the supply of palmitate resulted in a higher degree of esterification, both in control and diabetic cardiomyocytes. AcAc exerted no significant effect on the rate of palmitate esterification under these conditions. The observation that the TG content is enhanced in the diabetic heart *in situ* ³⁴ is supported by the present findings. It is noteworthy that the increased supply of extracellular FA, rather than the presence of ketone bodies, is instrumental in the enhanced deposition of palmitate into the intracellular TG pool (figure 6.3). Previous studies also indicated that ketone bodies are able to inhibit lipolysis ³⁵, which may contribute to the net expansion of the TG pool in the diabetic heart *in situ*. The present findings, however, do not support this notion, since (additive) effects of AcAc on the incorporation of palmitate into the intracellular TG pool were absent.

The present study clearly shows that in diabetic cardiomyocytes AcAc increases the intracellular level of non-esterified palmitate, both at the extracellular concentration of 80 and 160 μ M palmitate (figure 6.3). It is noteworthy that the 4-fold increase in deposition of labelled palmitate in the cellular FA pool matches very well with the 4.5-fold increase in the FA content of diabetic hearts *in situ* ¹¹. Collectively, the present findings strongly suggest that accumulation of FA in cardiac tissue is caused by a combination of two separate changes in the diabetic cardiomyocyte: the initial uptake rate of FA by diabetic cardiomyocytes is enhanced, due to the increased

supply of extracellular palmitate, on the one hand and the inhibitory action of ketone bodies, in particular AcAc, on the intracellular rate of FA oxidation, on the other. This notion is illustrated in figure 6.4, summarizing data shown in table 6.1 and figure 6.1. It should be emphasized, however, that the present data are obtained in resting cardiac muscle cells. It is of interest to explore how at enhanced workload (*e.g.* the beating heart) circulating ketone bodies interact with cardiac FA handling.

Possible implications of the present findings

One may speculate on the pathophysiological consequences of chronically elevated FA levels inside the cardiomyocytes of the diabetic heart. Pioneering studies of Feuvray and colleagues have shown that accumulation of lipotoxic FA and their intermediates are associated with mechanical dysfunction and cell damage in diabetic hearts subjected to ischemia ³⁶. Furthermore, other studies indicated that FA at elevated concentrations exert cytotoxic effects, including aberrations in signal transduction cascades ³⁷⁻³⁹. More recent observations by Van der Lee and colleagues ¹⁰ and others ⁴⁰⁻⁴² have convincingly shown that chronic exposure of cardiomyocytes to FA modulate the expression of a panel of cardiac enzymes involved in carbohydrate and fatty acid handling. The down-regulation of hexokinase II and the up-regulation of long-chain acyl-CoA dehydrogenase, especially, may explain the decline in glucose utilization and enhanced FA oxidation commonly observed in isolated hearts obtained from STZ-treated diabetic rats ¹⁰.

The present findings, however, challenge the current paradigm that FA oxidation is enhanced in the intact diabetic heart ^{43, 44}. The latter notion is based on experimental findings showing a significant increase in the oxidation rate of radiolabelled palmitate by hearts isolated from STZ-treated rats ⁴⁵. Results of other studies, however, do not support these findings. The rate of FA oxidation was found to be decreased in hearts from spontaneously diabetic rats ⁴⁴, and unchanged in hearts obtained from insulin-resistant obese rats ⁴⁶. Moreover, the utilization of FA by the hearts *in vivo* of diabetic swine was found to be not significantly different from control ⁴⁵, while the uptake and oxidation of iodine-labelled FA analogues by hearts of patients with impaired glucose tolerance was decreased ⁷. Finally, cardiac FA uptake and oxidative utilization of patients suffering from type 1 and type 2 did not significantly differ from healthy controls ⁷.

Since the contrasting findings are most pronounced between isolated hearts (enhanced FA utilization) and intact hearts *in situ* (no changes or even a decline in FA uptake and/or oxidation) the regulatory role of other factors

present in blood plasma, but not in buffers used in perfusion experiments on isolated hearts should be considered. These factors may be, among others, elevated blood concentration of ketone bodies in the intact experimental animal or subjects, suffering from diabetes.

The present findings might also have ramifications to other conditions associated with enhanced plasma ketone body levels, such as starvation, high fat diets, and cardiac failure. Under these conditions cardiac FA handling may be compromised by elevated exposure of the heart to circulating ketone bodies.

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Chapter 7

Metabolic and genetic adaptations in the streptozotocin-treated rat heart

An explorative study

Abstract

It is known that patients with diabetes mellitus have a higher rate of mortality than the general population. This higher mortality has been attributed, among others, to the development of diabetic cardiomyopathies. The precise mechanisms underlying the development of this diseased state are unclear. As diabetes is mainly a metabolic disorder, one can assume that alterations in myocardial metabolic properties contribute or cause the development of diabetic cardiomyopathy. To obtain more insight into metabolic remodelling of the diabetic heart, we examined the available substrates, hormone levels, cofactor content, enzyme activity, and mRNA levels under diabetic conditions. Studies were performed on intact hearts, isolated adult and neonatal cardiomyocytes.

Introduction

In essence diabetes mellitus can be classified as a disorder characterized by abnormal insulin secretion and/or resistance to the action of insulin. In the order of 10% of the diabetic patient population exhibit a profound lack of insulin (type-1 diabetes, insulin dependent diabetes mellitus), while the remaining patients suffer from varying degrees in the lack of action of insulin at the target organ (type-2 diabetes, non-insulin dependent diabetes mellitus). A major cause of death among patients with diabetes is malfunctioning of the heart caused by diabetes-induced cardiomyopathies.

The heart can be considered as an omnivorous organ, whereas circulating long chain fatty acids (FA) are the most prominent source of energy under normal conditions. However, in the diabetic state the concentration of substrates present in blood plasma significantly alters. The most prominent changes are the increases in glucose and FA. Besides these major changes, the concentration of substrates such as the triacylglycerols (TG) content and ketone body levels are also altered.

The changes in substrate availability may cause a shift in metabolic flux, which is likely to be involved in the adaptation of the heart to changes in its environment. The current paradigm considering energy metabolism in the diabetic heart, states that glucose oxidation is decreased and that FA oxidation is increased as compared to normal ^{1,2}. However, it still remains a matter of debate whether the paradigm is actually valid ³.

Considering the chronic nature of diabetes mellitus, it is likely that this pathophysiological state induces a response that over time will lead to a variety of metabolic and/or genetic adaptations or maladaptations. This chapter will try to elucidate, in an explorative manner, the various metabolic

and genetic changes occurring in the heart during diabetes and underlying mechanisms at various levels. First, the effect of chronic diabetes (4-5 weeks after streptozotocin treatment) on the cardiac substrate content and levels of coenzyme A and carnitine (both important cofactors in cardiac energy conversion) was monitored. Second, the activity of a set of enzymes playing a key role in cardiac energy conversion (pyruvate dehydrogenase and carnitine palmitoyl transferase) was assessed in diabetic rat myocardial tissue. Third, to explore metabolic adaptation at the genetic level, mRNA content of a variety of transport proteins and enzymes involved in cardiac glucose and FA utilization was measured. Special attention was paid to uncoupling protein 3 (UCP3), which is suggested to protect the mitochondrion against FA overload ⁴.

In order to obtain more insight into the potential effect of enhanced supply of blood-borne FA and ketone bodies ("diabetic condition") to the cardiac muscle cells experiments were performed on isolated cultured neonatal cardiomyocytes incubated under normal and diabetic conditions. The impact of chronic exposure of cultured neonatal cardiomyocytes to FA and/or ketone bodies on the mRNA levels of a set of metabolic genes was subsequently monitored. Moreover, the effect of exposure of FA and/or ketone bodies on the promoter activity of carnitine palmitoyl transferase I (CPT1) was measured in transfected neonatal cardiomyocytes. In the latter experiments, distinction was made between acetoacetate (AcAc) and 3- β -hydroxybutyrate (3HB), the most prominent ketone bodies in diabetic blood plasma.

Material en methods

Determination of substrates in cardiac tissue and blood plasma

To assess the content of FA and TG in diabetic rat heart, aliquots of muscle tissue ranging from 100 to 300 mg wet weight were pulverized in an aluminum mortar with a stainless steel pestle, both cooled in liquid nitrogen.

The tissue powder was transferred to glass test tubes cooled in liquid nitrogen. The test tubes containing pulverized tissue were placed at -21°C in a mixture of melting ice and NaCl. The tissue powder was wetted with 2 ml ice-cold methanol. The content of the test tubes was allowed to warm up to room temperature and weighed. According to the extraction procedure of Folch and co-workers ⁵ 4 ml chloroform was subsequently added. Butylated hydroxytoluene (0.01%) was present in both methanol and chloroform to prevent auto-oxidation of lipids. Thereafter, heptadecanoic acid (8 nmol) was added to correct for losses of FA during the extraction and assay procedure

(recovery standard). To assess the content of FA, TG and PL in blood plasma, 200 μ l of blood plasma was extracted with a similar protocol.

The above mentioned lipid extracts were evaporated and subjected to one-dimensional thin-layer chromatography (TLC). The spots corresponding with FA, TG and PL were scraped from the plate and transmethylated according to Morrison and Smith ⁶. The identification and quantification of the fatty acyl moieties in the various lipid classes were achieved by capillary gas chromatography with the use of a standard solution of methyl esters of fatty acids ⁷.

The glycogen content was determined in aliquots of freeze dried cardiac tissue, as previously described by Van der Vusse and colleagues ⁸. Blood glucose was measured with the use of an automated glucose analyser (Euroflash™, Lifescan, Milpitas CA, USA).

The content of CoA and carnitine and their derivatives in cardiac tissue

Cardiac tissue was homogenized in 3% perchloric acid (v:v) and centrifuged for 2 minutes at 10,000 x *g*. Long-chain acylcarnitines were determined in the resulting pellets after alkaline hydrolysis, and free in the perchloric acid supernatant by a radioenzymatic method originally described by Cederblad & Lindstedt ⁹ and modified by Brass and Hoppel ¹⁰.

Homogenized cardiac tissue was extracted using a Bligh-Dyer technique, and long-chain acyl-CoA esters are harvested in the methanolic aqueous phase. Complex lipids and PL were removed in the chloroform-rich organic Bligh-Dyer second phase, and long-chain acyl-CoA compounds were further purified from the methanolic aqueous Bligh-Dyer first phase on C18 extraction columns after removal of the methanol. The acyl-CoA esters were then quantitated by RP-HPLC ¹¹.

Malonyl-CoA content in cardiac tissue was assayed radioenzymatically in neutralized perchloric acid filtrates by the method of McGarry and co-workers ¹², as described previously by Saha and colleagues ¹³.

Enzyme activity and UCP3 content

Enzyme activities were measured in homogenates prepared according to the following standard technique. Aliquots of frozen tissue were thawed in SET buffer, containing (mM), sucrose (250), EDTA (2) and TRIS (10) (pH 7.4), and subsequently homogenized with the use of an Ultra Turrax. Thereafter, the circa 10% homogenate (w/v) was sonicated for 1 min (4 x 15 s, with 15 s

rests) at 0°C with the use of an Ultrasonic disintegrator. The sonicated homogenates were centrifuged at 1200 x *g* and 4°C for 10 minutes, and the supernatants were stored at -80°C for further analysis.

The activities of carnitine palmitoyl transferase I and II were assessed employing a radiochemical technique as previously described by Scholte and colleagues¹⁴.

To monitor pyruvate dehydrogenase (PDH) activity in cardiac samples, the following homogenization procedure was followed. Aliquots of frozen heart tissue were ground to a fine powder under liquid nitrogen. Each sample (circa 200 mg tissue) was homogenized in an appropriate buffer (see below). The sample was then frozen in liquid nitrogen, allowed to thaw and re-homogenized. This freeze-thaw cycle was repeated once more. The sample was subsequently centrifuged in a cooled micro-centrifuge for 8 minutes. The supernatant was used in the PDH assay. To measure total PDH activity, tissue was homogenized in buffer containing (mM): HEPES (75), dichloroacetate (5), MgCl₂ (5), ADP (1), dithiotreitol (1), leupeptin (0.05) and Triton-X (1%, v/v), pH 7.0. The buffer used to monitor the active form of PDH consisted of (mM): HEPES (25), KH₂PO₄ (25), KF (25), dichloroacetate (1), EDTA (3), ADP (1), dithiotreitol (1), leupeptin (0.05) and Triton-X (1%, v/v), pH 7.0.

The activity of PDH was assayed in the presence of a pyruvate generating system, consisting of lactate, NAD⁺ and lactate dehydrogenase. To this end aliquots of the homogenates were incubated at 30° C in an incubation buffer containing in mM: HEPES (50), MgCl₂ (1), EGTA (0.08), dithiotreitol (1), rotenone (0.004), NAD⁺ (1.67), thiamine pyrophosphate (0.2), lactate (16.7) and lactate dehydrogenase (2 U/l), pH 7.2. The production of NADH was continuously followed at 340 nm for 5 minutes. PDH activity was calculated from the linear portion of the curve, *i.e.*, 0-30 s after the start of the reaction by addition of an aliquot of the tissue supernatant sample to the incubation buffer.

The cardiac tissue content of UCP3 was measured according to Hesselink and co-workers¹⁵. In short, immunoblots of homogenized cardiac tissue were performed with the UCP3 antibody which was raised against amino acids 147-166 of human UCP3. The single protein band at 34 kDa, representing UCP3, was quantified.

mRNA content in cardiac tissue and neonatal cardiomyocytes

Total RNA was isolated from cardiac tissue or neonatal cardiomyocytes with TRIzol reagent (GIBCO). RNA (5 g) was size-fractionated on a denaturing gel (1% agarose, 6.6% formaldehyde, 1x MOPS), transferred to a nylon

membrane (Hybond-N, Amersham, Slough, UK) by capillary transfer and fixed using standard techniques. Following prehybridization, the filters were probed with glucose transport protein 4 (GLUT4), hexokinase II (HKII), pyruvate dehydrogenase kinase 4 (PDK4), muscle type carnitine palmitoyl transferase (mCPT1), fatty acid translocase (FAT), fatty acid binding protein (FABP), acyl-CoA synthetase (ACS), or long-chain acyl-CoA dehydrogenase (LCAD) according to Van der Lee and colleagues¹⁶. The cDNA probes were labeled with [α -³²P]dCTP (3000 Ci/mmol; Amersham) by random-priming (Radprime, Life Technologies) to a specific activity of $> 0.5 \times 10^9$ cpm·g DNA⁻¹. To correct for possible differences in transfer and loading, the filters were also hybridized with a ³²P-labelled ribosomal 18S probe. Following hybridization, filters were washed with the appropriate stringency to remove non-specific binding. The filters were exposed to phosphor imaging screens and subsequently scanned with a Phosphor-imager (Molecular Dynamics, Sunnyvale CA, USA), and quantified using ImageQuant software (Molecular Dynamics).

Promoter-reporter mCPT-assay in neonatal cardiomyocytes

For transient transfection, neonatal cardiomyocytes were seeded on 6-well plates at a density of 2.5×10^5 cells in 2 ml medium per well. Cells were transfected 16 h before addition of experimental medium (DMEM/M199 4:1, 0.25 mM L-carnitine, 0.25 mU/ml insulin and 0.15 mM bovine serum albumin) using the transfection reagent FUGENE 6 (Roche, Indianapolis IN, USA). Cells were transfected with 0.5 μ g of promoter/reporter vector, *i.e.* 1.3 kb fragment of the human mCPT-1 promoter generated by high fidelity PCR (Expand, Roche) of genomic DNA (corresponding to position 61203-62543, Genbank Accession number U62317) and cloned into the pGL3 luciferase vector (mCPT1-luc). The CMV- β -galactosidase containing vector pON249 (0.25 μ g) was co-transfected to determine transfection efficiency. The total amount of plasmid DNA per well was always kept constant by adding empty pSG5 vector (Promega, Madison, WI, USA).

Cells were harvested 24 hours after addition of the experimental medium and immediately processed for the determination of reporter activity. Luciferase activity was determined using a commercial firefly luciferase assay according to the suppliers' instructions (Steady Glo™, Promega) in white 96-well plates (Nalge Nunc International, Naperville, IL, USA) and FluorS imager (Bio-Rad) for measuring luminescence. β -galactosidase activity was determined spectrophotometrically (Titertek Multiscan Plus MKII, Thermo LabSystems, Helsinki, Finland) as described previously¹⁷.

Statistical analysis

Analysis of experiments within one group was performed with a two-tailed Student's *t* test. Comparison between groups was performed with the one-way analysis of variance (ANOVA). In case the *F* ratio obtained indicated that significant differences between groups were present, a two-tailed Student's *t* test for unpaired data was carried out. For all analyses, the level of significance was set at 0.05.

Figure 7.1 Schematic representation of cardiac energy converting pathways

3HB, 3- β -Hydroxybutyrate; AcAc, acetoacetate; CoA, Coenzyme A; GLUT, glucose transport protein; HKII, hexokinase II; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; FAT, fatty acid translocase; FABP, fatty acid-binding protein; ACBP, acyl-CoA binding protein; ACS, acyl-CoA synthetase; CPT1/2, carnitine palmitoyl transferase 1/2, TCA, tricarboxylic acid cycle; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Results and discussion

As expected and previously shown by others^{18,19}, plasma glucose concentration dramatically increases in the streptozotocin-treated animals, due to the destruction of the β -cells of the pancreas and the subsequent lack of circulating insulin (table 7.1). Plasma long-chain FA significantly increased in the diabetic animals. This increase is most likely caused by a decline in the capacity to store FA in adipose tissue due to the lack of insulin. The latter

hormone is, among others, able to inhibit hormone sensitive lipase, and hence, to enhance the sequestration of FA in adipocytes.

Table 7.1 Substrate and hormone concentration in blood plasma of streptozotocin-treated rats and age-matched controls

	Control	Diabetic
Substrates		
Glucose (mM)	9.1 ± 1.0	20.4 ± 6.5 *
Fatty acid (μM)	134 ± 59	265 ± 88 *
Triacylglycerols (mM)	1.7 ± 0.4	9.1 ± 1.9 *
Phospholipids (mM)	2.6 ± 0.7	5.0 ± 1.1 *
Acetoacetate (μM)	15	996 § *
3-β-hydroxybutyrate (μM)	16	2278 § *
Hormones		
Insulin (ng·ml ⁻¹)	10.5 ± 2.3	0.6 ± 0.1 # *
Glucagon (pg·ml ⁻¹)	62 ± 10	57 ± 13 #

§ data from Charlton et al.; # data from Nannipieri et al. *. * indicating significant difference ($p < 0.05$). $n=8$ for both control and diabetic group

The increases in plasma triacylglycerol (TG) and phospholipids (PL) (table 7.1) point towards a severe disturbed lipoprotein metabolism in streptozotocin-treated rats. Lack of lipoprotein lipase at the endothelial glycocalix, due to the virtual absence of insulin, and enhanced lipoprotein synthesis in liver as a consequence of increased supply of blood-borne FA to this organ, most likely contributes to the significant increase in circulating esterified lipids in streptozotocin-treated rats

The unchanged level of plasma glucagon and the decline in insulin strongly promotes the synthesis of ketone bodies in the liver cells²⁰. This effect is strengthened by an increased supply of FA, being the substrate for the synthesis of acetoacetate and 3-β-hydroxybutyrate in the mitochondrial compartment of the hepatocyte²⁰.

The alterations in circulating substrate concentrations are also reflected in changes in cardiac tissue substrate content. Glycogen content more than doubled in the diabetic heart (table 7.2) indicating a mismatch between glucose uptake and glucose degradation. Since glucose uptake is most likely impaired due to the lack of insulin, cardiac glucose degradation must be severely impaired in order to explain the accumulation of glycogen. In this respect the findings shown in table 7.4 are of interest. In the diabetic heart,

the total, maximal activity of pyruvate dehydrogenase (PDH) is significantly depressed, most likely reflecting a down-regulation of the expression of this enzyme.

Moreover, the percentage of the enzyme in its active form dropped from 44% (control) to 4% (diabetic). The latter finding indicates that the activity of PDH, catalysing the conversion of pyruvate into acetylCoA (figure 7.1) is also impaired at the post-translational level, most likely due to increased phosphorylation of the enzyme complex by pyruvate dehydrogenase kinase^{21,22}.

Table 7.2 Substrate content in cardiac tissue of normal and diabetic rats

	Control	Diabetic
<i>Substrates</i>		
Glycogen ($\mu\text{mol}\cdot\text{g dw}^{-1}$)	119 ± 51	$278 \pm 55^*$
Fatty acids ($\text{nmol}\cdot\text{g dw}^{-1}$)	338 ± 83	$1631 \pm 315^*$
Triacylglycerols ($\mu\text{mol}\cdot\text{g dw}^{-1}$)	19.6 ± 5.0	$36.4 \pm 5.3^*$
Phospholipids ($\mu\text{mol}\cdot\text{g dw}^{-1}$)	152 ± 14	152 ± 9

* significantly different from control; $n=8$ for both control and diabetic group

It is tempting to state that enhanced phosphorylation is caused by increased PDK activity, most likely due to enhanced expression of the latter enzyme, since cardiac mRNA levels of PDK4 showed a significant, more than 7-fold increase in the streptozotocin-treated heart (table 7.6). The decline in mRNA content of GLUT4 and HKII (table 7.6) also suggest that uptake and glycolytic conversion of glucose into pyruvate is hampered in the streptozotocin-treated heart. It is intriguing to notice that although these alterations in key enzymes of glucose metabolism, favoring decreased uptake and glycolytic conversion of glucose, uptake of glucose still surpasses the amount of glucose consumed as indicated by the increase in the glycogen content.

As shown before in *chapter 3* and by others²³, cardiac FA content significantly increases in the diabetic heart. The same holds for the TG pool, containing glycerol-esterified fatty acyl moieties. Both alterations indicate a mismatch between uptake and oxidation of FA in the streptozotocin treated heart. There is no doubt that the increased supply of blood-borne FA will add to increased uptake of FA by the cardiac muscle cell. This notion is supported by findings presented in *chapter 6* showing that the uptake rate of FA by isolated cardiomyocytes is significantly enhanced when the extracellular concentration of FA is increased.

In spite of the current paradigm that FA oxidation is augmented in the diabetic heart ², uptake rate of FA must surpass utilization rate to explain accumulation of FA and TG. In *chapter 6* this paradigm was challenged by showing that the elevated availability of ketone bodies such as acetoacetate, effectively inhibits cardiomyocyte FA oxidation without affecting cellular FA uptake rate, thereby creating a mismatch between uptake and utilization and providing a feasible explanation for lipid accumulation in the streptozotocin-treated rat heart.

Disturbed fatty acid utilization is also characterized by the changes in the tissue content of cofactors, such as CoA and carnitine, required for mitochondrial uptake and oxidation of long-chain fatty acyl moieties (figure 7.1). In this respect it is of interest to note that both the total carnitine and the free carnitine pool are depressed in the diabetic heart (table 7.3) ²⁴.

Table 7.3 Content of cofactors involved in energy metabolism of normal and diabetic rat heart

	Control	Diabetic
<i>Cofactor</i>		
Total carnitine (nmol·g ww ⁻¹)	812 ± 171	385 ± 191 *
Free carnitine (nmol·g ww ⁻¹)	295 ± 139	n.d. *
Free CoA (nmol·g dw ⁻¹)	414 ± 69	262 ± 74 *
Acetyl-CoA (nmol·g dw ⁻¹)	143 ± 86	151 ± 79
Malonyl-CoA (nmol·g dw ⁻¹)	19 ± 5	14 ± 5

n.d.: not detectable; * indicates significantly different from control (P<0.05)

Since carnitine is required for transport of FA across the mitochondrial inner membrane a decline in the pool size of this cofactor might not be in favour of enhanced FA oxidation by the diabetic heart. However, experiments performed with isolated cardiomyocytes derived from control and diabetic rats seem contraindicative to the importance or contribution of the total and free carnitine pool to the disturbed energy metabolism observed in diabetes. Cardiomyocytes isolated from diabetic rats which have a significant decreased total and free carnitine pool, show increased fatty acid oxidation in the absence of alternate substrates such as ketone bodies as compared to control (*chapter 6*). This observed increase in fatty acid oxidation in diabetic cardiomyocytes questions the importance and the precise role of the carnitine system in fuel selection and oxidation. It is of interest to note that

the findings in isolated cardiomyocytes support the observations in intact, isolated hearts of diabetic rats. When these hearts were perfused with FA containing buffer, *i.e.* in the absence of competing substrates, FA oxidation was found to be higher than in control hearts ^{1,2}. However trials with human patients which received carnitine supplementation showed that FA oxidation as well as glucose disposal is increased ^{25,26}. This might indicate that there is a role for carnitine in the disturbed energy metabolizing pathway as seen during diabetes. Another observation concerning carnitine, was made by Aoshima and colleagues ²⁷ who showed that not only the carnitine pool size is altered, but also the distribution of long-chain acylcarnitine is distorted, with an increased level of long-chain acylcarnitine in the mitochondrial matrix. The authors concluded that this disturbance might be partially responsible to the impaired energy metabolism observed in diabetes, leading to the accumulation of FA and TG.

The decline in tissue of free coenzyme A content in the diabetic rat heart (table 7.3) may also point to a disturbed FA handling. Unfortunately, no information is present about the intracellular distribution of the decreased CoA content. In case the intramitochondrial CoA concentration is also decreased, this change may contribute to impaired FA oxidation in the mitochondrial matrix.

It should be emphasized that the concentration of malonyl-CoA is not significantly changed in the streptozotocin-treated rat heart. Malonyl-CoA has been assigned a prominent role in controlling the oxidation rate of FA in cardiac muscle cells ²⁸. A decline in malonyl-CoA is thought to lessen the inhibition of carnitine palmitoyl transferase I. However, due to the lack of change in tissue malonyl-CoA content, a significant role in governing cardiac FA oxidation under the present conditions does not seem feasible.

Table 7.4 Enzyme activity in cardiac tissue of normal and diabetic rats

	Control	Diabetic
Carnitine palmitoyl transferase 1 (mU·g ww ⁻¹)	60.8 ± 21.7	42 ± 21.9
Carnitine palmitoyl transferase 2 (mU·g ww ⁻¹)	165 ± 97	164 ± 80
Total PDH activity (mU·g ww ⁻¹)	4.69 ± 0.68	2.84 ± 1.26 *
Active form PDH (mU·g ww ⁻¹)	2.17 ± 0.78	0.12 ± 0.22 *
Percentage (active/total)	44.3	4.2 *
PDK4 (AU·min ⁻¹)	0.67 ± 0.10	1.71 ± 0.09 § *

PDH-pyruvate dehydrogenase; PDK4-pyruvate dehydrogenase kinase 4. AU-arbitrary units

* indicates significantly different from control ($p < 0.05$); § data from Wu and colleagues ²².

Table 7.4 shows that the activity of CPT1, although not significantly altered, shows a trend to decline. As CPT1 is involved in the transport of fatty acyl moieties across the mitochondrial membrane, it is likely that the decreased activity of CPT1, if any, is repressing the increased FA oxidation due increased substrate availability. In this respect, it should be noted that the activity of CPT2, an enzyme which is also involved in the transport of FA across the mitochondrial membrane, is not decreased.

Finally, cardiac FA oxidation may be inhibited by the elevated concentration of competing substrates such as ketone bodies. This notion is strongly supported by findings presented in *chapter 6*.

One of the possible points of regulation by altered availability of substrates is the expression of metabolic genes, since previous studies have shown that for instance FA influences the expression of glucose and FA handling genes¹⁶. Data in table 7.6 clearly shows that the expression levels of two enzymes, mCPT1 and ACS are not altered in the diabetic heart compared to control. This finding corroborates with data on the enzymatic activity of CPT1 (table 7.4).

Taking into account that chronic exposure of isolated cardiomyocytes to FA (reference 16 and figure 7.2) significantly elevates the mRNA levels of FA handling genes; the present findings suggest a complex mechanism, underlying the regulation of gene expression in the diabetic heart. The fact that expression of mCPT1 and ACS is not altered despite the substantial accumulation of FA in the diabetic heart may point towards, among others, to i)- profound differences in adult and neonatal cardiomyocytes, ii)-alterations in the intrinsic mechanisms underlying gene expression in the diabetic state and/or iii)- the presence of other compounds mitigating the stimulatory effects of FA. Likely candidates are ketone bodies, known to be significantly increased under diabetic conditions.

To investigate the role of ketone bodies on gene expression and their effect on FA modulated gene expression, neonatal cardiomyocytes were incubated in the presence or absence of ketone bodies, and a number of genes in the fatty acid metabolizing pathway were measured (figure 7.2). It shows that as previously found¹⁶, FA is capable to induce the expression of genes encoding for FAT, FABP, ACS and LCAD in neonatal cardiomyocytes. AcAc or 3HB or a combination of both did not have any effect on gene expression. When neonatal cardiomyocytes are incubated in the presence of AcAc or 3HB combined with FA, the FA induced upregulation of genes encoding for the above mentioned FA handling proteins was not altered.

When transfected neonatal cardiomyocytes are incubated with a combination of FA and AcAc, there is no significant change as compared to

up-regulation observed in incubations with FA alone. However, incubations with FA in the presence of 3HB or a combination of ketone bodies showed a significant decline in promoter activity, which could explain the trend in decreased enzyme activity of CPT1 as observed in the intact diabetic heart, exposed to increased levels of circulating ketone bodies (table 7.4).

Figure 7.2 Effect of exposure to FA and ketone bodies of neonatal cardiomyocytes on mRNA levels

FAT (fatty acid translocase); FABP (fatty acid binding protein); ACS (acyl-CoA synthetase) and LCAD (long-chain acylCoA dehydrogenase). AcAc (acetoacetate, 3mM), 3HB (3- β -hydroxybutyrate, 3 mM), combi (2 mM AcAc + 4 mM 3HB). * significantly different from control (glucose only) ($p < 0.05$) $n = 3$ for all conditions. N.D. Not Determined

The extent of inhibition is however insufficient to fully explain the total lack of increase of the mRNA of CPT1 in the diabetic heart, indicating that other factors and/or mechanisms, the nature of which are unknown at present, are playing a role in the regulation of gene expression in the diabetic heart. The same conclusion most likely holds for the other FA handling genes. As outlined by Young and colleagues in a recent review on this subject matter glucose may fulfil this inhibitory role via repression of PPAR activation²⁹.

In contrast, the decline in the expression of glucose handling genes such as GLUT4 and HKII in the diabetic heart follows the pattern observed in studies on neonatal cardiomyocytes chronically exposed to increased levels of FA¹⁶. These findings suggest that the substantially elevated FA content in the diabetic heart negatively affects the expression rate of genes encoding for glucose handling proteins and enzymes.

Figure 7.3 Effect of exposure to FA and ketone bodies of cultured neonatal cardiomyocytes transfected with the mCPT1-luciferase promoter-reporter construct.

Control (glucose only) was set at 1.0, other values relative to glucose only. See for details, legends to **figure 7.2**. #significantly different from control ($p < 0.05$) * significantly different from FA only ($p < 0.05$)

It is of interest to note that in the diabetic heart both mRNA levels of UCP3 (A. Gilde, unpublished results and others^{30,31}) and protein levels (table 7.5) are significantly enhanced. The increase in protein levels (16-fold) substantially surpasses the increase in mRNA (4-fold). This increase in tissue UCP3 is most likely due to the chronic exposure of the diabetic cardiac muscle cells to elevated intracellular FA levels. This notion is supported by observations of Gilde and co-workers (unpublished observation) showing that neonatal cardiomyocytes incubated in the presence of FA significantly up-regulate the expression of UCP3.

Table 7.5 UCP3 content in cardiac tissue of normal and diabetic rats

	Control	Diabetic
UCP3 protein (AU)	6.7 ± 3.4	107 ± 25 *

AU; arbitrary units; * indicates significantly different from control ($p < 0.05$); $n = 6$

This strongly suggests a causal relationship between intracellular FA levels and the expression rate of UCP3. As pointed out earlier elevated intracellular FA levels may jeopardize intramitochondrial function if the intramitochondrial content of FA surpasses safety limits⁴. It is hypothesized that the main function of UCP3 is to transport FA molecules from the mitochondrial matrix into the cytoplasm to prevent FA-induced damage, rather than to uncouple respiratory chain activity from oxygen consumption, rendering the diabetic cardiomyocyte less efficient from an energetic point of view.

Table 7.6 mRNA of transport proteins and enzymes in cardiac tissue of normal and diabetic rats

	Control	Diabetic
GLUT4	1.0 ± 0.2	0.4 ± 0.05 *
Hexokinase II	1.0 ± 0.3	0.4 ± 1.0
PDK4	1.0 ± 0.2	7.1 ± 3.1 *
mCPT1	1.0 ± 0.1	1.2 ± 0.2
ACS	1.0 ± 0.2	1.1 ± 0.4

Mean of control levels was set at 1.0; *, significantly different from control ($p < 0.05$), $n = 5$

In summary, it has become clear that the diabetic heart *in situ* adapts at various levels to the altered circumstances to which it is exposed. Although these adaptations allow the heart to cope with the changes it has to endure, these same adaptations eventually may lead to the development of cardiomyopathies. There is however a lack in understanding at which point or how these adaptations become maladaptations and thus contributes to the diseased state of the diabetic heart. More research concerning genetic and metabolic alterations is therefore necessary to unravel the mechanisms behind the development of diabetic cardiomyopathies.

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Chapter 8

General Discussion

Introduction

For the production of energy, the heart is able to use a wide variety of substrates, including glucose, fatty acids and ketone bodies. Under certain pathophysiological conditions, like diabetes mellitus (DM), utilization of these substrates is altered. The disturbed balance between the uptake and utilization of these substrates and the altered preference for substrates, may eventually contribute to the development of diabetic cardiomyopathy¹⁻⁴.

Cardiac energy metabolism has been the focus of research for a considerable time. During the early and mid nineteen's, experiments were performed on isolated hearts, on basis of which it was concluded that long-chain fatty acid (FA) oxidation is increased in the diabetic state⁵⁻⁷. Turpeinen and colleagues, however, showed that the increased FA oxidation observed in isolated hearts were in contrast with observations in human diabetic patients⁸. They observed that cardiac FA oxidation of the commonly used FA tracer ¹²³I-heptadecanoic acid was not different between diabetic patients, both insulin dependent DM (IDDM; type-1) and non-insulin dependent DM (NIDDM; type-2), and healthy controls. The same study by Turpeinen and colleagues showed a significant decrease in cardiac ¹²³I-heptadecanoic acid oxidation in subjects with impaired glucose tolerance, which even stresses the differences in findings between isolated hearts from experimental animals and hearts *in situ* of human diabetic patients. The collected observations suggest that alterations of FA oxidation by the diabetic heart depend on the severity of the underlying disease, and furthermore that FA oxidation is governed by factors present in the *in situ* situation, but absent under experimental conditions.

The studies described in this thesis are aimed to explore major alterations in cardiac FA handling under diabetic conditions, with special reference to enhanced circulating levels of FA and ketone bodies and the consequences thereof on protein acylation. Moreover, a second goal of this thesis is to explore the discrepancy between observation in isolated hearts and results obtained from patients. Therefore, the role of the altered substrate availability, as seen in diabetes, on FA uptake and utilization, along with possible implications for the tissue content of cofactors and enzymes, involved in FA handling was investigated. Furthermore, the studies described in this thesis will try to add to the limited knowledge concerning the impact of the increased FA availability on the expression of metabolic genes under diabetic conditions and the regulatory role, if any, of ketone bodies therein.

Alterations in substrate availability under diabetic conditions

Notwithstanding the discussion about the underlying causes that are ultimately responsible for the development of diabetic cardiomyopathies, it is agreed upon that the levels of FA and glucose during diabetes mellitus are increased in plasma as compared to substrate levels in healthy subjects. There is, however, still a great deal of debate about the consequences of the substrate alterations in plasma on energy metabolism and functioning of the cardiomyocyte.

The increases in plasma of FA and glucose during diabetes could be responsible for alterations in their uptake and utilization by sheer mass effects alone. The uptake and thus eventually the utilization of glucose is severely hampered by the lack of insulin or the lack of action of insulin, which is marking for diabetes, type-1 and type-2, respectively. Although the transport of glucose is limited due to the inability of GLUT4 to translocate to the plasmamembrane⁹ and thus to facilitate glucose uptake, glucose is still able to enter the cardiomyocyte via other glucose transporters such as, for instance, GLUT1⁹. Despite the decline in the overall availability of transport proteins and, hence, transport of glucose across the plasmalemma, the uptake of glucose still surpasses the utilization, as the glycogen content in the diabetic cardiomyocyte is increased (*chapter 7*). Evidence is accumulating that the flux through the glycolytic pathway and subsequent oxidation of pyruvate in the mitochondrial matrix is severely depressed in the diabetic heart (*chapter 7*, Kuo and colleagues¹⁰). Besides down regulation of key enzymes in the glycolytic pathway and of pyruvate dehydrogenase, post-translational modification of the latter enzyme by enhanced activity of pyruvate dehydrogenase kinase is a likely cause of the severe decline in cardiac glucose degradation. Enhanced supply of blood-borne glucose and accumulation of glucose-derivatives in the cardiac cells may lead to glucotoxicity¹¹.

As indicated above, the general paradigm states that FA oxidation in the diabetic heart is increased as compared to control, although this is in contrast with observations made in human subjects. The study described in *chapter 6* showed intriguing observations explaining the contradictions observed between FA oxidation in isolated hearts versus cardiac FA oxidation in patients. This study made clear that the availability of an alternate substrate, namely acetoacetate (AcAc), one of the ketone bodies, was capable to suppress FA oxidation in cardiomyocytes derived from both healthy and diabetic rat hearts. In the absence of AcAc, the diabetic

cardiomyocyte showed an increased FA oxidation when compared to control, which fits in the current paradigm concerning energy metabolism in the diabetic heart. It is worthy to mention that the concentration of AcAc needed for half maximum inhibition is between the 0.3-0.5 mM. This is a concentration which is reached far upfront the physiological signs of diabetic ketoacidosis and which can furthermore be reached under numerous other physiological or pathophysiological conditions, such as, fasting, pregnancy, alcoholic ketoacidosis and salicylate poisoning ¹².

Lipid accumulation in the diabetic heart and lipotoxicity

Lipotoxicity describes the negative effects of an excess of lipids which are detrimental for proper functioning of the organism or cell. Lipotoxicity is not only considered as an acute effect, by for instance causing apoptosis ¹³, but also as an important process in the gradual decline of functioning occurring in diabetic cardiomyopathy.

The prolonged availability of a surplus of lipids in plasma as seen during diabetes leads to an increase in cardiac content of FA and TG. The increased intracellular FA level might be the primary cause of the altered fatty acyl composition of the plasma-membrane of the cardiomyocyte. The fatty acyl composition of the membrane is responsible for the properties of the phospholipid bilayer such as curvature and charge. In turn, membrane curvature can modulate the activity of membrane bound proteins ¹⁴. The altered membrane properties can therefore be seen as primary lipotoxicity, while impaired functioning of membrane bound proteins can be considered as secondary lipotoxicity. Furthermore, the increase of intracellular FA is associated with several direct toxic effects which act on myofibrillar function, apoptosis and generation of reactive oxygen species ¹⁵⁻¹⁸. The alterations in lipid metabolism could lead to the activation of signalling pathways related to myocyte dysfunction. Each of these points in interaction might contribute to occurrence of lipotoxicity and, hence, ultimately the development of diabetic cardiomyopathy.

An example of a recent discovery which may contribute to secondary lipotoxicity is an observation by Chao and co-workers who showed that the interaction of acyl-CoA binding protein (ACBP) is also influenced by membrane charge and curvature ¹⁹. The functioning of ACBP might be hampered by the alterations in the interaction of the protein with the plasma-membrane. Considering that ACBP is associated with the targeting of acyl-CoA to the various processes in which this fatty acyl derivative is

used, it is likely that the disturbed interaction contributes to the lipotoxic effects observed in diabetes.

As earlier stated, it is clear that the balance between FA uptake and FA utilization is changed during diabetes mellitus, as can be appreciated from the enlarged TG pool observed in the diabetic heart ²⁰. Besides the increased TG pool, the surplus of FA has other effects, such as, an increased cardiac pool size of unesterified FA (*chapter 3*), an increase in the diacylglycerol (DG) content ²¹, accumulation of acyl-CoA and an increase of acyl-carnitines ^{22,23}. Each of these disturbances could contribute to the lipotoxicity observed with increased FA plasma levels since toxic effects of acyl-CoA and acyl-carnitines have been described ²⁴.

Fatty acids and signal transduction

In addition to the role of FA in lipotoxicity, FA can also play a modulatory role by altering gene expression and influencing signalling transduction pathways. It is likely that FA are capable to alter signal transduction pathways in a different fashion than via influencing gene expression, namely via protein acylation. It is known that protein acylation is a versatile modification playing a role in, for instance, protein-protein interactions, conformational changes, protein localization and protein activity ²⁵⁻²⁹.

There are two forms of protein acylation, namely myristoylation and S-acylation (palmitoylation) ³⁰. Myristoylation is a co-translational modification of which the enzymology is well known ³¹⁻³³. S-acylation is a post-translational modification and the enzymatic process underlying this modification is less clear. Among the proteins which become covalently modified by FA, many of them are involved in signalling cascades, such as G-proteins and G-protein coupled receptors ³⁴⁻³⁶. Since it has been shown that during diabetes signalling cascades are hampered, it is likely that altered protein acylation plays a significant role in this phenomenon.

There are two observations that might support the notion that there is a role of protein acylation in diabetes. First, King and colleagues showed that the activity of N-myristoyl transferase, the enzyme responsible for the myristoylation of proteins, is lower in the liver of the obese Zucker rat than in control ³⁷. These authors suggested that inside the cell N-myristoyl transferase exists in 2 populations, with the active form residing in the membrane. This may imply that translocation of N-myristoyl transferase from the cytosol to the membrane is under direct influence of insulin and, thus, that the lack of insulin hampers this translocation. It is however

doubtful that myristoylation is also impaired in the diabetic heart since the total amount of cardiac myristoylated proteins did not change under diabetic conditions (*chapter 3*). Second, the observation made in *chapter 5*, which showed that the amount of proteins which become S-acylated in diabetic rat myocardium is significantly enhanced as compared to control underlines the notion that increased FA availability may affect signalling pathways. This chapter also shows that the enhanced availability of ketone bodies may play a role in increased palmitoylation of proteins under diabetic conditions. It is of interest to note that although all fatty acyl moieties, including myristic acid, are increased during diabetes, the pool size of myristoylated proteins does not increase.

Previous studies have shown that chronic exposure of cardiomyocytes to FA is able to modulate, via PPAR, cardiac gene expression of a panel of cardiac enzymes/proteins involved in carbohydrate and fatty acid handling³⁸⁻⁴¹. The elevation of plasma FA levels in diabetes is thought to result in activation of PPAR α and this, in turn, might induce the expression of PPAR α regulated genes, such as, UCP3, MCAD, LCAD, mCPT1, MCD and FAT⁴¹⁻⁴⁶.

However, the findings of mRNA expression in experiments with isolated neonatal cardiac myocytes derived from neonatal rats and of adult diabetic cardiac tissue are, partly, in contradiction with each other. Studies with neonatal cardiomyocytes show that chronic exposure to FA substantially induces mRNA expression of metabolic genes, *e.g.* FAT, FABP, ACS, LCAD and mCPT1⁴². However, in diabetic cardiac tissue the mRNA content of mCPT and ACS was not altered despite the chronic exposure of the heart to FA during diabetes. Although the underlying mechanism is not entirely clear, this phenomenon can be partially explained by the availability of alternative substrates which are capable to down-regulate gene expression to a certain extent. *Chapter 7* showed that when neonatal cardiomyocytes are chronically exposed to FA in the presence of 3- β -hydroxybutyrate (3HB), the expression of mCPT1 is decreased when compared to incubations in the absence of 3HB. The extent of inhibition is, however, insufficient to fully explain the total lack of increase of the mRNA of mCPT1 in the diabetic heart, indicating that other factors and/or mechanisms, the nature of which are unknown at present, are playing a role in the regulation of gene expression in the diabetic heart. In this respect, it is worthy to note that Young and colleagues recently hypothesized that glucose or glucose-derivatives are able to prevent the up-regulation of FA handling genes⁴.

It is intriguing to observe that there is a striking difference between the effects of the two most common ketone bodies, AcAc and 3HB. AcAc is able

to inhibit FA oxidation in isolated adult cardiomyocytes at physiological levels (0.3-0.5 mM) while 3HB does not influence FA oxidation at all. Conversely, 3HB is capable to down-regulate mCPT1 to a certain extent, while AcAc does not seem to possess this property. Considering the resemblance between AcAc and 3HB and the possibility of conversion of AcAc to 3HB and vice versa, these observations might indicate the existence of a complex and subtle mechanism capable of influencing metabolism at both the level of substrate utilization and gene expression.

Intervention and treatment

It should be taken into account that the process underlying diabetic cardiomyopathy is multifactorial and that the development takes place gradually in time. Therefore, treatment of this major cause of death of the diabetic patient must be based on this notion.

One should keep in mind that the preferred point of intervention should be as early as possible in the chain of events, to minimize the side-effects of the applied therapy. Although significant differences between type-1 and type-2 diabetes exist, they have in common that plasma glucose and FA levels are disturbed. Therefore, the preferred initial pathway concerning treatment is identical between these two different forms of diabetes. In both cases one should strive to lower both plasma glucose and FA levels in order to normalize substrate availability to control values.

Considering the paradigm stating that FA oxidation is increased during diabetes and, hence, that the overshoot of FA oxidation is responsible for the detrimental effects observed in diabetes, newly developed anti-diabetica are aimed at lowering FA oxidation in the diabetic heart. However, the results from *chapter 6* and observations in diabetic patients strongly suggest that cardiac FA oxidation is not increased and thus, that lowering FA oxidation might further enhance the lipotoxicity as observed in diabetic patients. In this light, one should consider to stimulate FA oxidation or the total oxidation capacity in general, to prevent the development of diabetic cardiomyopathy due to lipotoxicity, rather than to block FA oxidation in the diabetic heart.

In patients with type-1 diabetes normalization can be partially achieved via insulin treatment⁴⁷. Although patients with type-2 diabetes show a decline in response to insulin, it might be possible to partially restore the insulin sensitivity in these patients via medication, thereby also achieving (partial) normalization of plasma glucose and FA.

Recently, it has been advocated that the cellular uptake of FA might be a suitable target for pharmaceutical interventions. Since FA uptake is at least partially protein mediated, manipulation of the proteins involved in FA uptake might be a potential therapeutic target. A known inhibitor of fatty acid translocase (FAT), a protein associated with bulk transport of FA, is sulpho-N-succimidyl-oleate (SSO). Limiting uptake via the use of this kind of inhibitors might, however, have some major drawbacks which should be taken into account. As FAT is considered a multifunctional protein, homologous to CD36 which is present in macrophages and erythrocytes, one should be aware that inhibiting FAT with SSO would affect all these cell types, and thus hamper their proper function as well. Furthermore, if FA uptake is diminished by blocking FAT/CD36, blood plasma FA levels most likely increase which may enhance the risk of lipotoxicity in the vascular wall and in organs such as the liver.

In order to lower the plasma concentration of glucose and lipids, one may intervene in the uptake and utilization of other substrates such as ketone bodies. The intervention in the uptake of ketone bodies is a new lead which comes forth from the observations made in *chapter 6*, which indicated that fatty acid oxidation in diabetic cardiomyocytes under “diabetic” conditions (160 μ M palmitate, 3 mM AcAc) is comparable to FA oxidation in control cardiomyocytes under “normal” conditions (80 μ M palmitate, 0 mM AcAc). Monocarboxylate transporters (MCT) are involved in the transport of short-chain aliphatic monocarboxylates, such as ketone bodies⁴⁸. The existence of a high affinity monocarboxylate transporter, MCT2, present in cardiomyocytes, opens up the possibility for attenuating ketone body uptake. By limiting cardiac ketone body uptake, the cardiomyocytes would be forced to switch to other substrates to sustain the constant need for ATP generation, thereby possibly increasing FA and glucose oxidation, and thus preventing intracellular accumulation of FA, TG and glycogen.

If the above mentioned measures are not sufficient to prevent lipotoxicity or the development of diabetic cardiomyopathy one could consider intervening lower in the chain leading to these events.

For instance, total and free carnitine contents in the diabetic myocardium are significantly decreased. In essence, L-carnitine plays a crucial role in lipid and carbohydrate metabolism and is required for proper functioning of heart and skeletal muscle. The carnitine system is of importance to regulate the localized, limited pools of CoA derivatives^{49,50}. Short-chain acylcarnitines are generated by the action of carnitine acetyl-CoA transferase⁵¹. This reaction liberates free CoA which is an important

substrate for different steps in mitochondrial energy metabolism such as pyruvate dehydrogenase and fatty acid oxidation.

The carnitine content in diabetic cardiac tissue, which is lower than in control hearts, is most likely responsible for the decreased availability of free CoA and the disruption of the ratio between free CoA and acyl-CoA. It is hypothesized that increasing the availability of free carnitine should lead to rise in free CoA, by restoring the ratio between free CoA and acyl-CoA, and potentially counteracting deleterious lipotoxic effects of acyl-CoA ⁵¹. In addition, a decline in carnitine content might be associated with a limited ability to oxidize FA. However, *chapter 6* shows that myocytes derived from diabetic hearts with decreased carnitine content (see *chapter 7*) have a significantly higher FA oxidation rate than control cardiomyocytes when incubated in the absence of competing substrates. This observation is contra-intuitive and underlines the complexity of how FA oxidation is regulated in the cardiac muscle cells. The collected data indicate that besides carnitine other factors are playing an important role in governing the flux rate of FA in diabetic hearts. Notwithstanding these considerations, attempts have been made to mitigate lipotoxicity under diabetic conditions through enhancing the availability of carnitine in the heart.

Although L-carnitine is mainly synthesized by the liver, from where it is transported to heart and skeletal muscle, it can also be taken up from food. Dietary L-carnitine supplementation could therefore be a means by which the free carnitine pool can be restored. Although the beneficial effects of carnitine supplementation are recognized ⁵², general acceptance of this treatment has been hampered by the lack of knowledge of both the metabolic disturbances caused by carnitine deficiency and of the underlying cellular mechanism. However, Coker and colleagues strongly recommended, on the basis of a clinical study concerning carnitine metabolism in diabetes mellitus, carnitine supplementation to diabetic patients ⁵³.

At the same level of entry, there is the CPT system that can be used as therapeutic target. The β -oxidation of activated fatty acids occurs within the mitochondrial matrix and is catalyzed by the sequential action of four enzymes (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl thiolase). The carnitine dependent transport precedes the β -oxidative chain shortening of these activated fatty acids. This transport system consists of three proteins namely, carnitine palmitoyl transferase I (CPT 1), carnitine acyl-transferase (CAT) and carnitine palmitoyl transferase II (CPT 2), each with a different localization inside the mitochondria. The first step is the conversion of fatty acyl-CoA to acyl-carnitine, catalyzed by CPT1 which is localized in the mitochondrial

outer-membrane. In general, CPT1 mediated transport is recognized as the rate-limiting step in the overall transport of acyl-carnitines across the mitochondrial membrane, and thus crucial in regulating myocardial fatty acid oxidation.

Two isoforms of CPT1 exist, the l(iver) and m(uscle) isoforms. Both isoforms are present in the heart, although the m-type dominates. Malonyl-CoA is associated with allosteric inhibition of CPT1, resulting in reduced fatty acid oxidation⁵⁴. Situations, in which the intracellular malonyl-CoA concentration is increased, are associated with decreased FA oxidation⁵⁵⁻⁵⁷. The two isoforms have different kinetic and regulatory properties and differ in tissue distribution^{54,58}. The l-type displays a higher affinity for carnitine and lower affinity for malonyl-CoA in contrast to the m-type. By finding means to alter the ratio between the l-type and the m-type of CPT1, differential overall sensitivity to malonyl-CoA could be achieved. This would be a mean by which FA oxidation in the cardiomyocyte can be tuned to regain the balance between FA oxidation and glucose utilization.

In the recent past, the use of an inhibitor of the carnitine palmitoyl transferase system has been advocated as therapeutic measure under diabetic conditions. Treatment with etomoxir, a well known irreversible CPT1 inhibitor, is based on the paradigm that increased FA oxidation is detrimental to the heart and aggravates the unfavourable conditions created by diabetes mellitus. The decline in FA oxidation should force the muscles to increase glucose oxidation and, hence, to mitigate the unfavourable effects of disturbed carbohydrate homeostasis on the body. In studies with isolated cardiomyocytes, etomoxir was able to inhibit - oxidation of palmitate virtually completely in combination with a decreased palmitate uptake of approximately 50% (H. Niessen and co-workers, unpublished results; Luiken and colleagues⁵⁹). In clinical trials concerning patients with diabetes or chronic congestive heart failure, which lasted three months or less, showed that inhibition of CPT1 led to an increase of the SERCA2 protein and the α -myosin heavy chain. Furthermore, it was shown that treatment with etomoxir gave an improvement of clinical end points, such as, improved clinical status and central haemodynamics⁶⁰. Although inhibition of CPT1 seems beneficial, the *in vivo* use of etomoxir showed to be detrimental in a variety of other experimental settings. Administration of etomoxir to rats led to marked liver hypertrophy and to a lesser extent to hypertrophy of the heart⁶¹⁻⁶³. Furthermore, it was shown that prolonged inhibition of CPT1 with etomoxir caused intramyocellular lipid accumulation and insulin resistance in rats⁶⁴. Finally, in a recent study Cabrero and colleagues showed that etomoxir led to increased cellular oxidative stress

and NF- κ B activity in hearts of the etomoxir treated mice ⁶⁵. The evidence mentioned above indicates that CPT1 inhibition might not be an appropriate target for the treatment of diabetes due to the observed hepatic and myocardial toxicities observed in animals.

To intervene at an even lower level in the chain, at the point of signal transduction pathways, would not be without risk since it is difficult to predict the outcome due to cross-talk between the different signalling pathways. Nevertheless, it would still be of interest to explore the possibilities for attenuating signal transduction pathways via, for instance, modulation of protein acylation. However, until recently no protein could be purified which could be held responsible for S-acylation. The recent discovery of a polytopic membrane protein, Akr1p, containing a cysteine rich domain, as a palmitoyl transferase opens a variety of new and intriguing possibilities ⁶⁶. Although the protein was discovered in yeast, it is the first time that a protein was purified to near homogeneity. Earlier attempts to identify a protein with palmitoyl transferase activity were not successful and led only to partially purified membrane fractions with enzymatic activity ^{67,68}. The Akr1p protein can now be used as a tool to identify possible eukaryotic candidates, sharing homology, with the same characteristics as Akr1p. This would be the first step in unravelling the enzymatic pathways underlying protein palmitoylation, and could lead to new areas of therapeutic interventions.

Intervening distal in the chain of events, plasma lipid and glucose lowering can also be achieved via treatment with PPAR γ agonists. Originally, it has been suggested that this can be established via altered conformational stability leading to distinctive gene expression profiles, reduced adipogenic cellular effects, and potentially improved *in vivo* biological responses. Therefore, PPAR γ modulators may lead to preferred therapies for diabetes and obesity ^{69,70}. However Larsen and co-workers questioned the long term use of PPAR γ agonists, due to a major drawback of the chronic application of thiazolidinediones, a group of PPAR γ agonists, namely the substantial increase in body fat mass ⁷¹. Considering the fact that excess body fat plays a role in the development of diabetes, long term treatment of diabetes with this medication may be detrimental to the diabetic patient. Alternatively, the recent use of PPAR β/δ agonists in rhesus monkeys showed a significant decrease in triacylglycerols, combined with an increase in HDL cholesterol, thereby increasing the reverse cholesterol transport, which might be beneficial in altering the atherogenic particle composition ^{72,73}.

The results suggest that PPAR β/δ agonists may be a more effective drug to treat diabetic patients than PPAR γ agonists.

Future perspectives

To fully comprehend energy metabolism in the diabetic heart, it is necessary to unravel the contribution of the different substrates in a variety of circumstances to energy homeostasis. The results in *chapter 6*, indicating that ketone bodies are potent inhibitors of FA oxidation, even at subclinical concentrations, are of great interest. In this respect, it is of the utmost importance to unravel the causes and the consequences of relatively small increases in the level of circulating ketone bodies on FA oxidation. In order to prevent an early onset of diabetic cardiomyopathy and lipotoxicity, it is advisable to monitor plasma ketone body content in diabetic patients.

As the development of diabetes and, thus, the induction of diabetic cardiomyopathy and lipotoxicity take place in time, it is of interest to perform studies aiming to clarify the entire spectrum of changes occurring in the transition of the normal heart to the diabetic heart. To study which genes are involved in the transition, it is advisable to use the micro-array technique using cardiac specific mRNA chips. This would enable the possibility to detect genes which become either up-regulated or down-regulated. The same regime should be applied to the identification of proteins which might play a role in this transition. A technique that can be used, which has sufficient throughput to identify a large number of proteins, is high-resolution two-dimensional gel electrophoresis followed by peptide mass fingerprinting⁷⁴.

Even now, there is a lack of knowledge concerning the precise pathways responsible for the alterations in gene expression observed during diabetes. Therefore, more research needs to be done to identify the mechanisms by which gene expression is regulated in the diabetic heart and to identify sensors used to achieve this regulation.

At the level of lipotoxicity, the need for knowledge is even more stressing. To describe the precise nature and cause of lipotoxicity and the relationship with diabetes in the course of its development needs to be clarified. Therefore, the pathophysiological consequences of the increased lipid burden in diabetic subjects have to be investigated. New insights in lipotoxicity will hopefully lead to novel treatments, such as the development of compounds which ideally would prevent, but at least should be able to attenuate the lipotoxic effects in cardiac tissue.

Considering the limited knowledge of the role of protein acylation and signal transduction in diabetes, there is need for more research. The results

in *chapter 4*, indicated that protein palmitoylation might play a role in diabetes, as the thio-esterified fatty acyl moieties are significantly increased in diabetic myocardium. To gain more insight in the exact role of protein palmitoylation, it would be necessary to identify proteins which become covalently modified with fatty acyl moieties, including the quantity of the identified proteins. This would require 2-D gel-electrophoresis in combination with mass-spectrometry, with appropriate resolution and sensitivity to identify and characterize the modified proteins ⁷⁵.

Finally, there is a great need for detailed studies in diabetic cardiomyopathies in the clinical setting to extrapolate the findings in experimental animals, isolated hearts and isolated cardiomyocytes to the diabetic patient. The collected data will lead to an increased understanding of the process underlying diabetes and will eventually lead to better possibilities for treating patients suffering, among other, from diabetic cardiomyopathy.

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Summary

In the heart the main supply of energy is the oxidation of long-chain fatty acids (FA), which contributes for up to 60% of the cardiac energy need under normal physiological conditions. Besides FA, the heart is capable to use substrates such as lactate, pyruvate, ketone bodies and glucose to fulfil its energy requirements.

During several pathophysiological states, such as diabetes, the relative contribution of the substrates the heart can utilize for energy conversion is altered. The current paradigm concerning diabetes mellitus, a condition characterized primarily by lack of insulin or insulin action, states that during diabetes the contribution of FA to the total cardiac energy need are increased at the expense of glucose utilization.

The research described in this thesis is based on two separate but intertwined paths of investigation. The first path is aimed at gaining insight in the process of protein acylation, which means the covalent attachment of FA derivatives to intracellular proteins. The second research line of this thesis is to elucidate various aspects of FA handling in the diabetic heart, such as the extent of FA oxidation and esterification in this organ. The two paths converge in investigations of alterations in cardiac FA handling under diabetic conditions and the impact thereof on protein acylation.

In the present thesis a multitude of approaches is used which may not only lead to new insights in the role of protein acylation in the diabetic heart but also to knowledge challenging the current paradigm concerning cardiac FA utilization in diabetic conditions.

Chapter 1 contains the introduction to the thesis. The role of FA in maintaining energy homeostasis is briefly discussed, with special reference to other roles of FA besides serving as energy substrate. This includes the transport of FA in combination with the various sites of action to which FA can be channelled. Furthermore, attention is paid to the incorporation of FA into various lipid pools such as phospholipids (PL) and triacylglycerols (TG), as well as the covalent attachment of FA derivatives to proteins. Finally, the research aims of this thesis are described in this chapter.

Chapter 2 gives an extensive overview in the critical steps concerning the handling and utilization of blood-borne FA up until the entering into mitochondria where the FA is oxidized for the formation of ATP. In addition, to gain more insight in the role of protein acylation, the two forms of protein acylation are described in detail. The processes underlying S-acylation and

myristoylation and the consequences of these modifications are discussed. It illustrates the potential role of protein acylation as a mean to influence, for instance, signal transduction pathways and enzyme activity.

In *chapter 3* the use of capillary gas chromatography in cardiac research is described. This method is a sensitive and reliable analytical technique which can provide information on the total amount of FA and the relative fatty acyl composition of FA and the esterified lipid pools, TG and PL, in cardiac tissue and blood plasma. It was found that in blood plasma of diabetic rats both total amounts of FA and PL are doubled, while the TG content was 5.4 fold increased. The total quantities of FA, TG and PL showed a different pattern in cardiac tissue, with a 4.8 fold increase of FA, a 2 fold increase of TG, while the PL pool remained stable. Furthermore, this chapter revealed significant differences in lipid profiles in cardiac tissue as well as blood plasma between control and diabetic rats, supporting earlier observations that in addition to carbohydrate handling, lipid metabolism is also affected during diabetes.

In *chapter 4* the development of a model system is described which was used to study the features of S-acylation and myristoylation in more detail. For this purpose the H9c2 cell line, originating from embryonic rat heart tissue, was used. The distribution and the incorporation of radioactive labelled palmitate and myristate into the various intracellular pools, such as lipids and proteins, were monitored. The outcome of this study showed that more than 99% of the labelled FA taken up by the cell was present in the combined lipid pools, while 0.3 percent of less became covalently attached to proteins. In addition, the effect of increased availability of FA on the amount of acylated proteins as well as the pattern of covalently modified proteins was investigated. It was observed that the rate of protein acylation increased when the amount of extracellular FA was enhanced. Applying autoradiography to visualize radioactively labelled proteins demonstrated that the increased amount of exogenous palmitate commonly led to a decreased visibility of most protein bands, with the most striking exception of a 22 kDa protein band of a yet unknown protein. Furthermore, two known inhibitors of protein acylation, cerulenin and tunicamycin, were used to explore whether this protein modification could be inhibited in the present experimental setting. Both cerulenin and tunicamycin were able to inhibit protein acylation in the H9c2 cells and the mechanism achieving this is most likely different between the two inhibitors.

Chapter 5 indicates that capillary gas chromatography can also be used to determine the absolute amount and the relative composition of the fatty acyl chains which are covalently bound to proteins in cardiac tissue derived

from control and diabetic rats. This study showed that there are major differences between S-acylation and myristoylation in response to diabetes. Although the substrate required for both processes, *i.e.* FA, is increased, only the amount of S-acylated proteins was significantly enhanced while the amount of myristoylated proteins remained equal to that in control cardiac tissue. Furthermore, this chapter illustrates that ketone bodies, a substrate which can be oxidized by the heart and the plasma concentration of which is increased under diabetic circumstances, are capable to significantly elevate S-acylation of proteins in cardiac myocytes derived from diabetic rats. This suggests that the presence of ketone bodies in the diabetic animal is a possible explanation for the significantly increased amount of S-acylated proteins in diabetic cardiac tissue.

In *chapter 6* the effect of ketone bodies on several aspects of cardiac fatty acid handling was investigated. To this end, isolated cardiac myocytes derived from control and diabetic rats were used in a variety of experimental conditions mimicking as much as possible the *in situ* circumstances in control and diabetic animals. It was shown that the ketone bodies, acetoacetate (AcAc) and 3- β -hydroxybutyrate (3HB), were not able to influence initial uptake rate either in control or diabetic cardiomyocytes. However, experiments studying FA oxidation showed that AcAc significantly inhibited FA oxidation in both control and diabetic cardiomyocytes, whereas 3HB did not exert any influence on FA oxidation. In addition, diabetic cardiomyocytes incubated under diabetic conditions showed that exposure to AcAc was responsible for an increase in unmetabolized FA inside the cells. Taken together, these findings indicate that ketone bodies are playing an important role in compromising cardiac FA handling under diabetic conditions.

Chapter 7 deals with an explorative study which concerns the metabolic and genetic changes occurring in the heart of a diabetic rat. At the metabolic level, alterations in substrate and co-factor content were measured. An increase in both blood plasma as well as cardiac tissue of almost all substrates was observed, while at the intracellular co-factor level the free carnitine, total carnitine as well as the free CoA pool showed a significant decline. Furthermore, the activity of enzymes playing a key role in cardiac energy conversion was determined, with as most striking results a decline in the pyruvate dehydrogenase activity which is most likely a consequence of the enhanced activity of pyruvate dehydrogenase kinase. At the genetic level, mRNA of proteins and enzymes involved in cardiac glucose and FA utilization was determined, with special attention to uncoupling protein 3. A 4-fold increase in mRNA content of UCP3 associated with a 16-fold increase of this

member of the uncoupling protein family at the protein level was observed. In addition, the effect of ketone bodies on the mRNA levels on a number of metabolic genes was examined. Finally, the effect of FA and/or ketone bodies, either AcAc or 3HB, on the activity of carnitine palmitoyl-transferase 1 (CPT1) was measured with the use of transfected neonatal cardiomyocytes. It was found that 3HB was able to suppress promoter activity of CPT1 in contrast to AcAc, thereby elucidating another level of regulation and complexity of cardiac gene expression. Taken together, this chapter illustrates that the heart adapts in a multiple fashion to the altered circumstances to which it is exposed during diabetes mellitus.

The final chapter of this thesis, *chapter 8*, discusses the major findings and gives suggestions for future research. The impact of the disturbed FA handling on a variety of features, such as signal transduction and lipotoxicity, as seen in diabetes are discussed. The present findings suggest that measures aiming to stimulate cardiac FA oxidation rather than to inhibit cardiac FA utilization should be considered as novel therapeutic interventions to mitigate the lipotoxic effects of FA accumulation in the diabetic heart.

Samenvatting

De voornaamste bron van energie in het hart is de oxidatie van lang-ketenige vetzuren, welke onder fysiologische omstandigheden voor ongeveer 60% bijdragen aan de energie behoefte van het hart. Om in de energie behoefte te voorzien is het hart, naast vetzuren, ook in staat om andere substraten, zoals lactaat, pyruvaat, ketonlichamen en glucose te oxideren.

Verschillende pathofysiologische omstandigheden, zoals diabetes, veroorzaken een verschuiving in de relatieve bijdrage van de diverse substraten aan de energie omzetting in het hart. Het huidige paradigma stelt dat gedurende diabetes mellitus, een ziektebeeld dat gekarakteriseerd wordt door een gebrek aan insuline of de werking van insuline, de bijdrage van vetzuren aan de totale energie behoefte van het hart is verhoogd ten koste van glucose.

Het onderzoek dat beschreven wordt in dit proefschrift is gebaseerd op twee verschillende maar in elkaar verweven onderzoekslijnen. Het eerste deel van het proefschrift is gericht op het verkrijgen van inzicht in het proces van eiwit-acylering, een proces dat gekenmerkt wordt door een covalente binding van vetzuurderivaten aan intracellulaire eiwitten. Het tweede deel van dit proefschrift belicht de verschillende aspecten van vetzuurgebruik, onder andere vetzuuroxidatie en -esterificatie in het diabete hart. Beide aspecten worden verenigd in onderzoek naar de veranderingen in vetzuurgebruik en de impact daarvan op het eiwit acyleringsproces onder diabete omstandigheden.

In dit proefschrift zijn diverse benaderingen gebruikt die niet alleen tot nieuwe inzichten leiden in de mogelijke betekenis van eiwit-acylering in het diabete hart, maar ook vraagtekens plaatsen bij het huidige paradigma betreffende het vetzuurgebruik in het diabete hart.

Hoofdstuk 1 bevat de inleiding van dit proefschrift. De rol van vetzuren in het handhaven van de lipidenhomeostase wordt kort besproken, met speciale aandacht voor andere aspecten van vetzuren dan alleen het dienen als energie-leverend substraat. Dit betreft het transport van vetzuren in combinatie met de verschillende plaatsen waar vetzuren hun biologische werking kunnen uitoefenen. Verder is aandacht besteed aan de inbouw van vetzuren in diverse intracellulaire lipiden zoals fosfolipiden (PL) en triacylglycerolen (TG), alsmede de covalente binding van vetzuurderivaten aan eiwitten. Tot slot wordt in dit hoofdstuk het onderzoeksdoel van dit proefschrift besproken.

Hoofdstuk 2 geeft een uitgebreid overzicht van belangrijke stappen in het gebruik van vetzuren die afkomstig zijn uit bloedplasma tot en met de opname in mitochondria waar vetzuren geoxideerd worden voor de vorming van ATP. Om meer inzicht te verkrijgen in de rol van eiwit-acylering worden de twee verschillende vormen van acylering in detail besproken. De processen die ten grondslag liggen aan S-acylering en myristoylering en de consequenties van deze eiwitmodificatie worden toegelicht. Dit dient ter illustratie van de potentiële rol van eiwit-acylering als middel om bijvoorbeeld signaaltransductie-ketens te beïnvloeden.

In *hoofdstuk 3* wordt beschreven hoe met behulp van capillaire gas chromatografie vetzuren bepaald kunnen worden. Dit is een gevoelige en betrouwbare analytische methode die informatie kan verschaffen over de totale hoeveelheid vetzuren en de relatieve samenstelling van vetzuren en veresterde lipiden, TG en PL, in hartweefsel en in bloedplasma. In bloedplasma van diabete ratten is gevonden dat de totale hoeveelheid van vetzuren en PL verdubbeld is, terwijl de hoeveelheid TG ongeveer 5 maal verhoogd is. De totale hoeveelheden van vetzuren, TG en PL in hart weefsel laten een verschillend patroon zien: een 4.8-voudige verhoging van vetzuren, een 2.0-voudige verhoging van TG, terwijl de hoeveelheid fosfolipiden niet veranderde. Tevens laat dit hoofdstuk significante verschillen in de vetzuur profielen zien in hartweefsel alsmede bloedplasma tussen controle en diabete ratten. Dit resultaat ondersteunt eerdere bevindingen dat naast het koolhydraatmetabolisme, ook het vetzuurmetabolisme is aangedaan onder diabete omstandigheden.

In *hoofdstuk 4* wordt de ontwikkeling van een modelsysteem beschreven dat gebruikt is om de eigenschappen van S-acylering en myristoylering in detail te bestuderen. Voor dit doel is de H9c2 cellijn gebruikt, welke afkomstig is van embryonaal rattenhartweefsel. De distributie en de uiteindelijke inbouw van radioactief gelabeld palmitine zuur en myristine zuur in de verschillende weefsel fracties, zoals lipiden en eiwitten, zijn bepaald. De resultaten van deze studie laten zien dat meer dan 99% van de hoeveelheid opgenomen radioactief gemerkte vetzuren aanwezig zijn in de verschillende lipiden fracties, terwijl 0.3% of minder covalent gebonden worden aan eiwitten. Tevens werd het effect van het verhogen van de beschikbare hoeveelheid vetzuur op de hoeveelheid geacyleerde eiwitten als ook het patroon van de covalent-gemodificeerde eiwitten onderzocht. Het bleek dat de snelheid van eiwit-acylering toegenomen is bij een verhoogd extracellulair vetzuuraanbod. De toepassing van autoradiografie om de radioactief gemerkte eiwitten zichtbaar te maken toonde aan dat een verhoging van het vetzuur aanbod leidt tot een afname van de meerderheid

van de zichtbare eiwit-banden. Een uitzondering hierop vormt een 22 kDa band van een tot nu toe onbekend eiwit. Tevens werden twee inhibitoren van eiwit-acylering, namelijk cerulenine en tunicamycine, toegepast in dit experimentele model. Zowel cerulenine als tunicamycine waren in staat om eiwitacylering tot op zeker hoogte te remmen in de H9c2 cellijn. Tevens bleek dat het mechanisme waarmee eiwit-acylering geremd werd verschillend was tussen deze twee inhibitoren.

Hoofdstuk 5 laat zien dat capillaire gaschromatografie ook gebruikt kan worden om de absolute hoeveelheid en de relatieve samenstelling van de vetzuurketens die covalent gebonden zijn aan eiwitten te bepalen. Deze studie toonde aan dat er grote verschillen bestaan tussen S-acylering en myristoylering van eiwitten tussen diabete en controle harten. Ondanks het feit dat het substraat dat voor beide processen nodig is onder diabete omstandigheden is verhoogd, is alleen de hoeveelheid S-geacyleerde eiwitten significant toegenomen, terwijl de hoeveelheid gemyristoyleerde eiwitten niet toeneemt. Verder laat dit hoofdstuk zien dat keton-lichamen, een substraat dat verhoogd in bloedplasma aanwezig is en geoxideerd kan worden door de hartspeer, in staat zijn om de mate van S-acylering van eiwitten significant te doen toenemen in hartspeerzellen verkregen uit diabete ratten. Dit suggereert dat de toegenomen aanwezigheid van keton-lichamen in het diabete dier een mogelijke verklaring is voor de significant verhoogde aanwezigheid van S-geacyleerde eiwitten in diabete hart weefsel.

In *hoofdstuk 6* is de rol van keton-lichamen op diverse aspecten van vetzuurgebruik onderzocht. Hiervoor zijn geïsoleerde cardiomyocyten van controle en diabete ratten geïncubeerd onder experimentele condities die de *in situ* situatie zoveel mogelijk nabootsten. Hieruit bleek dat de keton-lichamen, acetoacetaat (AcAc) en 3- β -hydroxybutyraat (3HB), niet in staat waren om de initiële opname van vetzuren in controle en diabete cardiomyocyten te beïnvloeden. Experimenten met betrekking tot vetzuuroxidatie laten echter zien dat AcAc in staat is om de vetzuuroxidatie significant te remmen in controle alsmede diabete hartspeerzellen, terwijl 3HB hiertoe niet in staat bleek te zijn. Daarbij komt dat diabete hartspeerzellen geïncubeerd onder diabete omstandigheden lieten zien dat blootstelling aan AcAc verantwoordelijk is voor een toename van niet omgezet vetzuur in de cellen. Samenvattend, deze bevindingen tonen aan dat keton-lichamen een belangrijke rol spelen in de veranderde vetzuurhuishouding in het hart onder diabete omstandigheden.

Hoofdstuk 7 beschrijft een verkennende studie betreffende de metabole en genetische veranderingen die optreden in het hart van de diabete rat. Op het metabole niveau zijn de veranderingen in de hoeveelheden substraat en

co-factoren gemeten. In zowel bloedplasma als hartweefsel waren bijna alle substraat niveau's verhoogd, terwijl het intracellulaire cofactor gehalte van vrij carnitine, totaal carnitine en de vrij CoA een significante afname liet zien. Bovendien werd de activiteit bepaald van enzymen die een sleutelrol spelen bij de energieomzetting in het hart, met als meest opvallende resultaat een afname in de pyruvaat dehydrogenase activiteit, waarschijnlijk als gevolg van de toegenomen activiteit van het pyruvaat dehydrogenase kinase. Op genetisch niveau werden de hoeveelheden mRNA bepaald van eiwitten en enzymen die betrokken zijn bij glucose- en vetzuurgebruik in het hart, met speciale aandacht voor uncoupling protein 3. Een 4-voudige toename in het mRNA gehalte van dit eiwit ging gepaard met een 16-voudige toename op eiwitniveau. Voorts werd het effect van keton-lichamen op de hoeveelheden mRNA van een aantal metabole genen bestudeerd. Tenslotte werd de invloed van vetzuren en/of keton-lichamen (hetzij AcAc of 3HB) op de activiteit van carnitine palmitoyl-transferase 1 (CPT1) gemeten door gebruik te maken van getransfecteerde neonatale cardiomyocyten. In tegenstelling tot AcAc bleek 3HB wel in staat om de CPT1 promoteractiviteit te onderdrukken, hetgeen de complexiteit van de regulatie van de expressie van metabole genen in het hart benadrukt. Samengevat illustreert dit hoofdstuk dat de hartspier zich op velerlei manieren aanpast aan de gewijzigde omstandigheden zoals die zich voordoen bij diabetes mellitus.

In het laatste hoofdstuk van dit proefschrift, *hoofdstuk 8*, worden de belangrijkste bevindingen bediscussieerd en suggesties aangereikt voor toekomstig onderzoek. De mogelijke consequenties van het verstoorde vetzuurgebruik in het diabete hart voor processen zoals signaaltransductie en lipotoxiciteit, zoals aangetroffen bij diabetes, worden besproken. De huidige bevindingen suggereren dat ingrijpen met als doel het stimuleren van de vetzuuroxidatie in het hart in plaats van het remmen ervan beschouwd dient te worden als een potentiële therapeutische interventie om de lipotoxische effecten van vetzuurophoping in het diabete hart te verminderen.

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Curriculum Vitae

Danny Hasselbaink werd op 31 maart 1973 geboren te Rotterdam. Na het afronden van de MAVO en HAVO behaalde hij in 1993 zijn VWO diploma op het Kalsbeek college te woerden. In datzelfde jaar startte hij de studie Biomedische Wetenschappen aan de Universiteit Leiden. Het wetenschappelijk onderzoek begon met het lopen van de hoofdvakstages bij de vakgroep farmacochemie onder begeleiding van Dr. D. P. T. Steenvoorden, en bij TNO preventie en gezondheid onder supervisie van Dr. B. J. M. van Vlijmen en Dr. B van de Water. Na zijn afstuderen in 1998, startte hij op 1 januari 1999 als assistent in opleiding bij de afdeling fysiologie aan de universiteit van Maastricht. Het onderzoek dat werd verricht staat beschreven in dit proefschrift. Momenteel is hij bezig zich te oriënteren op de arbeidsmarkt.

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In 1998 ben ik vanuit het noorden, omdat vanuit maastricht nu eenmaal alles in het noorden en boven de rivieren is, naar beneden afgezakt om in maastricht te beginnen aan mijn promotieonderzoek, wat uiteindelijk geleid heeft tot dit boekje. Dit werk heb ik de afgelopen jaren niet alleen gedaan. Er zijn namelijk een groot aantal mensen die daar aan bij hebben gedragen.

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Danny

abbreviations

3HB	3- β -hydroxybutyrate
AcAc	acetoacetate
ABP	albumin binding protein
ACBP	acyl-CoA binding protein
ACS	acyl-CoA synthetase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAT	carnitine acyltransferase
CPT	carnitine palmitoyl transferase
DG	diacylglycerols
FA	long-chain fatty acids
FA-CoA	fatty acyl coenzyme A
FABPc	cytoplasmic fatty acid binding protein
FABPpm	plasmalemmal fatty acid binding protein
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
GLUT4	glucose transporter 4
HKII	hexokinase II
HPLC	high performance liquid chromatography
LCAD	long-chain acyl-CoA dehydrogenase
LPL	lipoprotein lipase
MA	myristate
PA	palmitate
PDH	pyruvate dehydrogenase
PDK4	pyruvate dehydrogenase kinase 4
PL	phospholipids
PPAR	peroxisome proliferators activated receptors
SSO	sulfo-N-succinimidyl oleate
STZ	streptozotocin
TCA	tricarboxylic cycle
TLC	thin layer chromatography
TG	triacylglycerols
UCP3	uncoupling protein 3
VLDL	very low density lipoprotein