Cells of adult rat heart : isolation, characterization and some aspects of fatty acid metabolism

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CELLS OF ADULT RAT HEART

Isolation, characterization and some aspects
of fatty acid metabolism

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CELLS OF ADULT RAT HEART

Isolation, characterization and
some aspects of fatty acid metabolism

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
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volgens het besluit van het college van Dekanen, in het openbaar te
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of andere manier heeft bijgedragen
aan het tot standkomen van dit
proefschrift.

Aan mijn ouders

Aan Peter

LIST OF ABBREVIATIONS

AA	: arachidonic acid
BSA	: bovine serum albumin
CEC	: cardiac endothelial cells
CFLC	: cardiac fibroblast-like cells
CMC	: cardiomyocytes
CoA	: coenzyme A
DiI-Ac-LDL	: 1,1'-dioctadecyl-1-3,3,3',3'-tertramethyl-indocarbocyanine perchlorate-Acetylated Low Density Lipoprotein
FA	: fatty acids
EDTA	: ethylenediaminetetraacetate
FABP	: fatty acid binding protein
FACS	: fluor activated cellsorter
FCS	: fetal calf serum
HBSS	: Hanks' balanced salt solution
HEPES	: 2-(4-(2-hydroxy ethyl)-1-piper-aziny)-ethanesulfonic acid
HETE	: hydroxyeicosatetraenoic acid
HHT	: 12-hydroxyheptadecatrienoic acid
HPLC	: high performance liquid chromatography
HUVEC	: human umbilical vein endothelial cells
ICAM	: intercellular adhesion molecule
IFN	: interferon
LT	: leukotriene
MHC	: major histocompatibility complex
MKR	: modified Krebs Ringer buffer
PBS	: phosphate buffered saline
PG	: prostaglandin
PL	: phospholipids
RECA	: rat endothelial cell antibody
RHEC	: rat heart endothelial cell line
TG	: triacylglycerols
TLC	: thin liquid chromatography
Tx	: thromboxane
VWF	: Von Willebrand Factor

CONTENTS

Abbreviations	6
Chapter 1 Introduction	
1.1 Functional morphology of the heart	10
1.2 Lipid metabolism under normoxic and hypoxic conditions	12
1.3 Aim of the study and outline of the thesis	14
1.4 References	15
Chapter 2 Isolation and cultivation of rat heart cells	
2.1 Introduction	20
2.2 Initial isolation methods	20
2.3 Final isolation procedure	22
2.4 Characterization of cells	25
2.5 Summary	29
2.6 References	29
Chapter 3 Continuous endothelial cell lines obtained from rat heart	
3.1 Introduction	32
3.2 Methods	32
3.3 Results and discussion	36
3.4 Summary	41
3.5 References	41
Chapter 4 Fatty acid oxidation capacity and fatty acid-binding protein content of different cell types isolated from rat heart	
4.1 Introduction	44
4.2 Methods	44
4.3 Results	45
4.4 Discussion	47
4.5 Summary	48
4.6 References	49
Chapter 5 Production of arachidonic acid metabolites in adult rat cardiac myocytes, endothelial cells, and fibroblast-like cells	
5.1 Introduction	52
5.2 Methods	53
5.3 Results	58
5.4 Discussion	64
5.5 Summary	68
5.6 References	69

Chapter 6 Arachidonic acid incorporation in cardiomyocytes, endothelial cells and fibroblast-like cells isolated from rat heart	
6.1 Introduction	74
6.2 Methods	75
6.3 Results	78
6.4 Discussion	82
6.5 Summary	85
6.6 References	85
Chapter 7 General discussion and conclusions	
7.1 Discussion and conclusions	88
7.2 References	91
Summary	93
Samenvatting	95
Publications	97
Curriculum Vitae	99

CHAPTER 1

INTRODUCTION

1.1 FUNCTIONAL MORPHOLOGY OF THE HEART

During evolution from one to multicellular organisms a circulatory system developed to transport nutrients and waste products to and from the various parts of the body, respectively. In order to keep this system in a operational state, a contractile organ capable of periodically propelling blood through the circulatory system developed as well.

Since this organ, the heart, is never in rest, chemical energy is continuously converted into mechanical energy. The contractile function of the heart is regulated by an intricate system of signal transduction, involving transmembrane fluxes of cations, such as Na^+ , K^+ and Ca^{2+} . Chemical energy is provided by oxidative degradation of fatty acids, glucose and lactate supplied to cardiac cells from the blood in the cardiac capillary network.

The pump function of the heart and the circulation of blood was first described by Harvey (1578-1657, ref. 11). In cardiovascular physiology, experiments with *in situ* hearts and intact isolated hearts, first by Langendorff (17) and later on by many other investigators, have improved our understanding of fundamental metabolic processes. The obligatory role of oxygen in cardiac energy conversion and of Ca^{2+} in cardiac contraction became evident. Even nowadays the isolated intact working heart is an important tool in cardiovascular research. More than in the past, it is appreciated that the complex cellular organization of the heart requires a different experimental approach to elucidate fundamental questions related to cardiac function. Studies on intact hearts provide overall information on cellular performance not discerning the contribution of different cell types. Since each cell type has unique properties investigations on isolated cells and cell lines are required. The last 20 years isolation and culturing of cells has become a well accepted technique that provides a means to study basic physiological properties of individual cells, as far as their enzyme content, receptors, transduction systems, and gene regulation are concerned. In turn these experimental findings have lead to concepts with potentiation for therapeutic interventions. However, it should be kept in mind that in addition to the use of isolated cells, experiments on whole organs are necessary, because cells may behave differently *in vitro* and *in vivo*, while interactions between various cell types may occur when located in their natural environment.

The cellular organization of the mammalian heart is perfectly adapted to its function. About 75% of the heart mass is made up of muscle cells, i.e., cardiomyocytes with contractile properties. The remaining part of the heart consists of blood vessels and interstitial space. In the latter compartment connective tissue cells, conductive tissue cells and macrophages are present.

One gram of cardiac tissue contains on the average $2 \cdot 10^7$ cardiomyocytes. Ventricular myocytes are about 25-50 μm in diameter and 100-150 μm in length (1, 2, 9, 12). The cell membrane or sarcolemma invaginates into the interior of the cell to form an extensive tubular network.

Figure 1.1. A: Schematic representation of the ultrastructure of cardiomyocytes surrounded by collagen and capillaries. From; *An introduction to cardiovascular physiology*. Ed. JR Levick, Butterworth & Co London, 1991, with permission of the publishers. B: schematic representation of the collagen matrix of the normal left ventricle. From; *The heart, physiology and metabolism*. Ed. LH Opt, Raven Press New York, 1991, with permission of the publisher.

The cells are for more than 80% binucleated (6, 16) and are filled with myofibrils and many mitochondria, lipid droplets and glycogen granules. The myofibrils are composed of actin and myosin filaments and are connected to the fascia adherent of the intercalated discs, i.e., specific areas of the sarcolemma between neighbouring cells (fig. 1.1a). The sarcolemma is covered with a layer of glycoproteins, the "glycocalyx". Myocytes are connected to other myocytes and their adjacent capillaries by struts of collagen (a fibrous material). Groups of myocytes form a myofiber that is held together by a collagen network. Further strands of collagen connect myofibers to each other (fig. 1.1b).

The cells building up the vessel wall are different in size and properties. Endothelial cells cover the inner surface of the blood vessels and form a

continuous monolayer. They comprehend 2-3% of cardiac cell mass and 90% of the endothelial cells are of capillary origin (1, 2, 12). Endothelial cells have a length of about 25 μm , a width of about 10 μm and a thickness of 0.2-0.5 μm and are thus much smaller than cardiomyocytes (29). However, in the adult heart they are more abundantly present than cardiomyocytes (about $2 \cdot 10^8$ endothelial cells/gram wet weight (3, 28, 36). In situ the endothelial cells are elongated with the long axis aligned with the blood stream. Beside the nucleus, which extends into the vascular lumen, mitochondria, endoplasmatic reticulum and Golgi-complex have been identified. In the cytoplasm several intermediate filaments can be seen. The "glycocalyx", surrounding the endothelial cell membrane, forms at the abluminal side a basal lamina that contains heparan and chondroitin sulfates, collagen, proteoglycans and other glycoproteins. Endothelial cells have an important barrier function and can synthesize and secrete biologically active compounds into the vascular compartment and/or the subendothelial matrix (13, 29). The endothelial cells are surrounded by pericytes or smooth muscle cells, depending on their position in the vascular tree. Pericytes cover (in part) capillary endothelial cells and are together with the endothelial cells surrounded by the basal lamina (1, 21). Smooth muscle cells lie round arterioles, venules, arteries, and veins. The pericytes and smooth muscle cells are contractile cells and, hence, contribute to the regulation of blood flow through the coronary system. Beside the vascular cells and cardiomyocytes a variety of other cell types is found in the interstitial space of the heart. The presence of fibroblasts, nerve cells, macrophages, and mast cells has been established in the heart (1). The function and metabolic behavior of these cells vary substantially.

1.2 LIPID METABOLISM UNDER NORMOXIC AND HYPOXIC CONDITIONS

To fulfil its energy demands, the heart continuously extracts oxidizable substrates from the intravascular compartment. With respect to the nature of the substrates, the heart can be considered as an 'omnivorous' organ (34). Fatty acids, lactate and glucose are major fuels, but pyruvate, ketone bodies and amino acids are also potential sources of energy (22, 38). Under normal conditions the cardiomyocytes use predominantly fatty acids as fuel (24). The endothelial cells, however, prefer glucose and glutamine above fatty acids as energy source (18, 31). Fatty acids are not only important substrates for energy metabolism, but are also components of phospholipids, which are major constituents of cell membranes. They are important determinants of cellular integrity. Furthermore some polyunsaturated long-chain fatty acids are substrates for the synthesis of biologically active compounds, the eicosanoids.

Cardiac fatty acid metabolism is characterized by a complex of processes. Various storage forms are present and numerous lipid converting enzymes and transport routes are involved (for an extensive review see ref. 38).

In blood, fatty acids are present as unesterified molecules complexed to albumin, or esterified in molecules as mono, di and triacylglycerols, phospholipids and cholesteryl esters. The triacylglycerols form the core of very low density lipoproteins or chylomicron particles. The fatty acyl moieties are hydrolyzed from the triacylglycerol molecules on the luminal side of the endothelium by the enzyme lipoprotein lipase, or released from the fatty acid-albumin complex by a yet not completely elucidated process in which the endothelial membrane is involved (fig. 1.2). From the vascular space the fatty acids have to pass several barriers before they reach the mitochondria of the cardiomyocytes where they are oxidized. First there is the endothelial lining. It has been hypothesized that transport of fatty acids occurs by lateral diffusion within the plasmalemma leaflets of endothelial cells (26, 27). More likely, fatty acids cross the endothelial luminal and abluminal membranes and diffuse through the cytoplasm (4).

Figure 1.2. Schematic representation of uptake, transport, and activation of fatty acids (FA) in myocardial tissue. Numbers refer to enzymes involved: 1) acyl-CoA synthetase, 2) carnitine acyltransferase I, 3) carnitine acylcarnitine translocase, 4) carnitine acyltransferase II. FABP, fatty acid-binding protein. From; Van der Vusse et al. (ref. 38), with permission of the American Physiological Society.

The existence of carrier molecules for facilitation of transport of the hydrophobic fatty acids through the aqueous cytoplasm of endothelial cells is not clear yet. Subsequently the interstitial space has to be passed. This is most likely mediated by albumin. The translocation of the fatty acids from the interstitial space through the sarcolemma into the sarcoplasm could be explained by protein-mediated transfer (30, 33) or passive diffusion (25), but the actual mechanism is incompletely understood. In the cardiomyocyte the intracellular translocation of fatty acids is facilitated by a tissue-specific cytoplasmic fatty acid-binding protein (FABP) (10, 14, 32).

Under normoxic conditions the fatty acids then are transported to the mitochondria and oxidized to produce energy. Parts of the fatty acids are temporarily stored in triacylglycerols, and a relative small quantity is incorporated in phospholipids and other complex lipids. The phospholipids are constituents of membranes and form a bilayer. The polar headgroups of the phospholipids form the outer surfaces of the two leaflets of the bilayer. The hydrophobic fatty acyl chains of phospholipids are buried in the lipophilic core of the lipid bilayer. A relatively small part, 5-10%, of the cardiac phospholipids is located in the sarcolemma, the majority is localized in intracellular membranes (38). Phospholipids are in a continuous turnover process to reconstitute, among others, chemically modified phospholipid molecules and to adjust the biological properties of membranes to changes in cellular conditions. An important commonly present polyunsaturated fatty acyl moiety of membrane phospholipids is arachidonic acid. This fatty acid serves also as precursor for the synthesis of eicosanoids.

During reduced coronary flow (ischemia) to the heart, supply of substrates and removal of waste products is hampered. The oxidation of fatty acids becomes depressed and the heart anaerobically metabolizes carbohydrates (19, 23). Prolonged ischemia results in increased turnover of the cardiac triacylglycerol pool and disturbed resynthesis, and degradation of membrane phospholipids. Hereby various fatty acyl-containing intermediates accumulate (7, 20, 37). Some of these intermediates seem to have a deleterious effect on the myocardium. Restoration of flow (reperfusion) only partly results in normalization of fatty acid metabolism, depending on the duration of ischemia (5, 35). Experimental findings indicate that the production and release of eicosanoids is enhanced in the post-ischemic heart (8, 15).

Fatty acid homeostasis in cardiac tissue has been extensively investigated during the past two decades. Although many basic questions regarding the metabolism of fatty acids under normal and ischemic conditions have been clarified, information about differences in fatty acid handling between the different cell types present in the heart is relatively scarce. This information is needed to understand the specific role of each cell type in overall cardiac fatty acid homeostasis and to elucidate the interplay between cardiac cells with respect to uptake, transport and enzymatic conversion of fatty acyl moieties.

1.3 AIM OF THE STUDY AND OUTLINE OF THE THESIS

The main purpose of the present study was to investigate a variety of aspects of fatty acid metabolism in cells isolated from cardiac tissue.

The first aim was to develop and implement techniques to isolate cardiomyocytes, endothelial cells and fibroblasts from adult rat hearts. These cell types were

primarily selected since they are thought to play an important role in overall cardiac fatty acid homeostasis.

In chapter 2, a detailed description is given of the techniques currently used in our laboratory to isolate myocytes, endothelial cells and fibroblast-like cells from the heart. Cardiomyocytes were used for metabolic studies within 24 hours after isolation. Endothelial cells and fibroblast-like cells were cultured to increase the number of cells, since appreciable amounts of material are needed for biochemical/physiological studies. Efforts were made to immortalize endothelial cell cultures to obtain stable cell lines. Two successful attempts are described in chapter 3.

Fatty acid homeostasis is characterized by a multitude of transport routes and metabolic conversions. Intracellular transport is thought to be mediated by cytoplasmic FABP. In chapter 4 the cellular content of heart-type FABP, as assessed in cardiomyocytes, endothelial cells and fibroblast-like cells is described. The FABP content is related to the maximal capacity to oxidize fatty acids.

Cardiac tissue has the ability to produce a variety of eicosanoids, compounds with specific biological activity, from endogenous and exogenous substrates. Since the maximal capacity to synthesize eicosanoids in various cardiac cell types is largely unknown, the conversion of exogenous arachidonic acid into eicosanoids by isolated cardiomyocytes, and cultured endothelial cells and fibroblast-like cells was investigated. The results of these studies are presented in chapter 5.

In cardiac tissue arachidonic acid is mainly present in membrane phospholipids. In order to get more insight into the kinetics of incorporation of exogenous arachidonic acid in the esterified lipid pool of various cardiac cell types, the deposition of labeled arachidonic acid in isolated cardiomyocytes and cultured endothelial cells and fibroblast-like cells was studied under normoxic conditions. The fate of exogenously administered arachidonic acid was monitored by biochemical and autoradiographical techniques (chapter 6). Moreover, the total fatty acid content in the unesterified fatty acid pool, and in triacylglycerols and phospholipids was estimated with the use of gaschromatographical techniques (chapter 6).

In chapter 7, a general discussion on the isolation and culturing of cardiac cells is presented. Moreover, the results obtained on cellular fatty acid handling are discussed in a broader perspective.

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CHAPTER 2

ISOLATION AND CULTIVATION OF RAT HEART CELLS

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2.1 INTRODUCTION

To elucidate fundamental questions related to cardiac function one has to understand the physiology of the various cell types present in the heart. Cardiomyocytes and vascular cells, especially the endothelial cells, but also fibroblasts, play a role in the cardiac metabolism. So, the use of isolated and cultivated cells and cell lines provides a tool to study the individual features of cardiac cells.

For the isolation of cells a variety of methods have been described depending on the organ they have to be harvested from. The isolation of cardiomyocytes mostly starts with a perfusion of the isolated (intact) heart according to Langendorff with a solution containing a proteolytic enzyme and is followed by mincing of the tissue in smaller pieces and a second digestion with enzymes (4, 8, 15,). A large variety of enzymes has shown to be successful in cardiomyocyte isolation, but mostly collagenase is used. This enzyme cleaves the cellular connections with the extracellular matrix. This treatment should be as short as possible to prevent damage to the myocytes themselves. The concentration of calcium in the perfusion medium was found to be critical in the procedure. It has to be low otherwise the Ca^{2+} -dependent desmosome structures at the intercalated discs between neighboring myocytes do not dissociate. The Ca^{2+} concentration should not be too low to preserve the structure and function of the sarcolemma and its associated glycoproteins. Besides the proteolytic enzymes need calcium for activation (4, 7, 8, 15). During isolation the cardiomyocytes are sensitive to abrupt temperature changes and mechanical stress and have to be treated carefully.

For the isolation of endothelial cells several techniques have been described. From larger vessels endothelial cells can be dissociated by enzymatic digestion (5, 9, 13) or are allowed to grow out of a vessel by the explant technique (2, 12). Endothelial cells from smaller arteries and veins and from microvessels are collected after perfusion with proteolytic enzymes (3, 14,), or after enzymatic digestion of the (minced) organ (16, 17).

2.2 INITIAL ISOLATION METHODS

Several methods have been explored in our laboratory to obtain a pure fraction of rat heart endothelial cells. First we followed the procedure as described by Nees et al. (14). Hereby hearts were first retrogradely perfused with a Krebs-Henseleit buffer (KHB) containing: 127 mM NaCl, 4.6 mM KCl, 25 mM NaHCO_3 , 1.1 mM MgSO_4 , 1.2 mM KH_2PO_4 , 2 mM Na-pyruvate, 8.3 mM glucose, 2.5 mM CaCl_2 , 1% phenolred and pH 7.4. After 5 min the perfusion medium was changed to Collins A buffer (43 mM K_2HPO_4 , 15 mM KH_2PO_4 , 15 mM KCl, 10 mM NaHCO_3 ; pH 7.4) containing several enzymes (0.1% collagenase, 0.1% DNase and 0.1% papaine). Then the flow was switched off and the enzyme-solution, present in the coronary vessels, was allowed to dissociate the endothelial cells. Hereafter

the coronary vessels were perfused with KHB and the perfusate was collected. This solution was centrifuged and the pelleted cells were purified with a 70% percoll gradient. The superficial fraction with a density of about 1.04 was collected, washed and plated on two 3.5 cm diameter culture dishes. The harvest from six rats before centrifugation on the percoll gradient was on the average 3.10^5 cells. After a week of culturing the cells had divided and grown to confluency. Microscopic evaluation of the percentage positive cells, after three hours incubation with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate-Acetylated Low Density Lipoprotein DiI-Ac-LDL (18) revealed that only 40-80% of the cells were of endothelial origin. Attempts to purify the culture after labeling of the endothelial cells with the fluorescent DiI-Ac-LDL and using a fluor activated cellsorter (FACS) were only partly successful. The risk of contamination was high and the yield was low. For qualitative analyses of endothelial cells this method was suitable. However for biochemical experiments this procedure was too complicated and too low in yield.

A second method for isolation of cardiac endothelial cells was performed according to the method described by Simionescu et al. (17) and modified by Piper (16). Hereby cardiomyocytes and endothelial cells are isolated from the same preparation. In this method rat hearts are first perfused according to the Langendorff technique with a modified Krebs Ringer buffer (MKR) containing: 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 10 mM NaHCO_3 , 10 mM 2-(4-(2-Hydroxy Ethyl)-1-Piper-aziny)-Ethanesulfonic acid (HEPES), 11 mM glucose, 4 mM taurine; pH was set at 7.4 and the buffer solution was gassed with 95% O_2 plus 5% CO_2 . After 10 min the perfusion medium was changed to MKR with 0.06% collagenase and 25 μM CaCl_2 . After 30 min recirculation with this solution at 37°C, the heart was cut into small pieces and digested into single cells during a second incubation in MKR with 0.06% collagenase, 25 μM CaCl_2 and 1% bovine serum albumin (BSA) V (essentielle fatty acid free). The solution was filtered and centrifuged at 25 g for 3 min. The pellet consisted of cardiomyocytes, and the supernatant of endothelial and other cardiac cells. After a third incubation with 0.1% trypsin the latter mixed cell solution was plated on tissue culture dishes. Although the yield of cardiomyocytes was good, the endothelial cell preparation was not pure. After a week cultivation, on the average 15-50% endothelial cells were present. Efforts to purify the preparation with the FACS, after labeling with DiI-Ac-LDL, failed because the percentage of endothelial cells was too low to prevent contamination with non-endothelial cells. Rerun of the sorted endothelial positive fraction revealed that still 10-20% non-endothelial cells were present. These DiI-Ac-LDL-negative cells overgrew the endothelial cells when placed in culture.

The method with which we finally succeeded to obtain a pure culture of endothelial cells is a modification and combination of the protocols as described by Diglio et al. (3) and Piper et al. (15) and is written hereafter (see also fig. 2.1).

2.3 FINAL ISOLATION PROCEDURE

Animals

Hearts were removed from male adult Wistar or Lewis rats of 12 weeks old under ether anesthesia. The hearts were subsequently placed in ice-cold MKR. After cannulation of the aorta, the heart was perfused with MKR at 37°C according to the Langendorff technique with a constant coronary flow of 10 ml/min for 10 min (washing period).

Figure 2.1. Schematic representation of the procedure for the isolation and cultivation of adult rat heart cells. CEC, cardiac endothelial cells, CMC, cardiomyocytes, CFLC, cardiac fibroblast-like cells.

Isolation of cells

Cells were isolated from the hearts in sterile conditions. After the washing period (see above) the heart was recirculated with 50 ml MKR containing 25 μM CaCl_2 and 0.06% collagenase for 30 min. Cardiac endothelial cells (CEC) were collected from the recirculation medium according to Diglio et al. (13). The heart was removed from the apparatus and blood vessels, atria and fibrous tissue were trimmed from the heart. Thereafter the ventricles were chopped and incubated in 20 ml MKR containing 25 μM CaCl_2 , 0.06% collagenase and 1% BSA V (essentially fatty acid free) under gently pipetting the suspension for 30 min (25). The solution was filtered through a 200 μm nylon sieve. Cells were either centrifuged at 25 G for 90 s or allowed to settle down at room temperature during 10 min. The pellet consisted predominantly of cardiomyocytes (CMC). This fraction was purified by washing before plating. The supernatant contained a mixed cell population consisting of fibroblasts, cardiomyocytes and smooth muscle, endothelial and blood cells. This supernatant was used for cultivation of cardiac fibroblast-like cells (CFLC).

The CMC pellet was washed three times in MKR with increasing concentrations of Ca^{2+} (0.2, 0.5 and 1 mM, respectively). CMC were collected by gravity at room temperature. The final pellet contained 2 to 5.10⁶, 40-70% rod shaped CMC. This pellet was resuspended in CMC culture medium (M199, 2 mM L-glutamine, 13 mM NaHCO_3 , 10 mM HEPES, 100 U/ml penicilline, 100 $\mu\text{g}/\text{ml}$ streptomycine, 4% fetal calf serum (FCS) from Boehringer and plated on tissue culture dishes. The dishes were precoated overnight with CMC culture medium. After 4 hours of culture at 37°C in a humidified 95% air plus 5% CO_2 stove, the rod shaped CMC were attached to the dishes and, then or overnight used in the experiments.

Cultivation of endothelial and fibroblast-like cells

The preparations containing either CEC (recirculation medium) or mixed cells (supernatant of the collected CMC) were centrifuged twice in CEC culture medium (40% M199, 40% RPMI1640, 20% FCS, 2 mM L-glutamine, 13 mM NaHCO_3 , 10 mM HEPES, 50 $\mu\text{g}/\text{ml}$ gentamicine, 20 U/ml heparine and approximately 40 $\mu\text{g}/\text{ml}$ bovine brain extract, prepared according to Maciag et al. (11)) supplemented with 2.5 $\mu\text{g}/\text{ml}$ fungizone. The cells were resuspended in culture medium, plated on fibronectine (100 $\mu\text{g}/\text{ml}$) precoated Falcon dishes and placed in a humidified 95% air plus 5% CO_2 stove at 37°C. Primary cultures were provided with fresh culture medium every day, passaged cultures every 2-3 days. When almost confluent, CEC were further purified by removing non-endothelial cells under the microscope according to Diglio et al. (13). In primary cultures of the mixed cell preparations 50-75% of the cells had a fibroblast-like (CFLC) appearance. Confluent CEC and CFLC were treated with phosphate buffered saline (PBS; 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM, pH 7.4), containing 0.67 mM ethylenediaminetetraacetate (EDTA) and 1.25 mg/ml trypsin to detach the cells, and subcultivated in CEC culture medium.

Characterization of cells

The isolated myocytes were microscopically checked for cell morphology and trypan blue exclusion.

The primary cultures of CFLC and CEC were tested for the uptake of Dil-Ac-LDL (18). Cell cultures in Costar 96 well plates were incubated with 10 μ g Dil-Ac-LDL/ml culture medium at 37°C for four hours. The medium was renewed and the cells were studied with a Zeiss fluorescence microscope equipped with the barrier filters BP 530-560, FT 580 and LP 590.

For immunofluorescence microscopy cell solutions were plated on Costar 96 well plates and grown to confluency. Cell monolayers were fixed in 3% paraformaldehyde and treated with 0.05% Nonidet P40 to permeabilize the membranes as described by Laurila et al. (10). After washing three times in PBS, the cells were incubated with rabbit anti human-Von Willebrand Factor (VWF) (1) diluted 1:100 in PBS containing 1% BSA and 0.1% NaN₃ in a humidified atmosphere at 37°C for 30 min. Another well with cells was incubated with RECA-1 (a rat endothelial cell antibody, ref. 5) diluted 1:500 in PBS containing 1% BSA plus 0.1% NaN₃ at 37°C for 30 min. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti rabbit-IgGs or FITC-conjugated rabbit anti mouse-IgGs. The cells were washed and mounted in glycine buffered glycerol (pH 8.6) for fluorescence microscopic evaluation with barrier filters BP 450-490, FT 510 and LP 515.

Electron microscopy

For transmission electron microscopy cells were grown on fibronectin precoated, thermanox tissue culture coverslips and fixed in a PBS solution containing 2% glutaraldehyde and 50 mM L-lysine or 2% tannine. Thereafter a 60 min 2.5% glutaraldehyde fixation was performed. The cells were postfixated with 1% osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated and embedded in epon. Ultrathin sections were briefly counterstained with lead citrate and examined with the electron microscope (EM 400, Philips).

Materials

The culture media RPMI1640, M199, fungizone and the trypsin solution were purchased from Gibco (Life Technologies Ltd., Paisley, Scotland UK); FCS batch 60045402 from Boehringer Mannheim GmbH (Mannheim, FRG). or batch 40G3700P from Gibco, or batch 701064 from Sera-Lab Ltd. (Crawley Down, UK) or batch 0135827 from Flow Laboratories Ltd. (Irvine, Scotland UK). Collagenase (CLS) batch 67047M was obtained from Cooper Biomedical Worthington (Malvern, PA USA) and BSA V (essentially fatty acid free), L-glutamine, L-lysine and DNase were obtained from Sigma Chemical Co. (St Louis, MO USA). BSA V from Boehringer Mannheim GmbH and heparin sodium from Serva GmbH (Heidelberg FRG).

The Falcon tissue culture dishes were obtained from Becton Dickinson Ltd. (Plymouth, UK) and microtiter plates from Costar Corp. (Cambridge, MA USA). FITC conjugated antibodies were purchased from Dakopatts (Copenhagen, Denmark) and Dil-Ac-LDL from Biomedical Technologies Inc. (Stoughton, MA USA). RECA1 was a gift from Dr. A.M. Duijvestijn (Department of Immunology, University of Limburg, The Netherlands). Fibronectin and rabbit polyclonal anti-Von Willebrand protein IgG were obtained from the Central Laboratory of the Netherlands Red Cross Transfusion Service (CLB, Amsterdam, The Netherlands).

Thermanox tissue culture coverslips were obtained from Miles Laboratories Inc. (Naperville, IL USA). Tannine was obtained from Mallinckrodt Inc. (Paris, TX USA), osmium tetroxide from Johnson Matthey (Herts, UK) and glutaraldehyde and uranyl acetate from Merck (Darmstadt, FRG).

2.4 CHARACTERIZATION OF CELLS

Morphology and immunocytochemistry

From adult rat hearts three different types of cells were isolated: cardiomyocytes (CMC), cardiac endothelial cells (CEC) and cells that resembled fibroblasts in morphology and growth pattern and therefore were designated cardiac fibroblast-like cells (CFLC).

The cardiomyocytes, isolated from an adult rat heart have special features. They do not divide and keep their typical elongated morphology in culture for a certain time, but after a couple of days they can dedifferentiate and change from rod shaped to rounded cells when kept in medium with FCS. The isolated CMC were attached to dishes and were kept in culture overnight after isolation. Microscopic evaluation showed that over 95% of the cells did not take up trypan blue and were rod shaped (fig. 2.2a). They were mechanically at rest. Electron microscopically the CMC had a normal appearance; they contained myosin and actin filaments, densely stained glycogen particles and mitochondria with matrix granula (fig. 2.3a).

The CEC preparation consisted of positively identified endothelial cells (see below). Whenever during the first culture non-typically growing cells were observed microscopically, these particular cells were removed from the plate as described by Diglio et al. (3). The remaining cells were allowed to grow to confluency. The CEC showed an elongated shape and formed a monolayer at confluency (fig. 2.2d). The CEC internalized Dil-Ac-LDL and stained positively with antibodies against Von Willebrand Factor and with an endothelial cell specific rat monoclonal antibody (RECA-1) (fig. 2.3).

Figure 2.2. Phase contrast light micrographs of cardiomyocytes and cultured endothelial and fibroblast-like cells from adult rat heart.

A: primary culture of cardiac endothelial cells.
B: confluent endothelial cells passage 5.

C: primary culture of a mixed cell preparation with fibroblast-like cells surrounding a clone of endothelial cells.

D: Passaged fibroblast-like cells in a post-confluent state.

E: isolated cardiomyocytes.

Bar represents 0.1 mm.

The primary CFLC preparation was not pure. Beside fibroblast-like cells several other cell types were present, among which endothelial cells. The latter cell type could easily be recognized due to its typical growth pattern (fig. 2.2b). On the order of 15-25% of the primary CFLC culture consisted of endothelial cells. The proportion of endothelial cells rapidly decreased during subcultivation. They were most likely overgrown by the fibroblast-like cells. After passage two, endothelial cells could no longer be seen in the CFLC preparation. The CFLC were larger than the CEC, formed a monolayer if confluent, while in the post-confluent state they showed a tendency to overgrow each other and had a more spindle-like appearance (fig. 2.2c). The CFLC reacted negatively to Von Willebrand Factor and endothelial cell antibody staining, and did not internalize Dil-Ac-LDL.

At the electron microscopic level the CEC and CFLC had a normal appearance, the CEC differed from the CFLC in the number of filaments and vesicles. CFLC contained more filaments and less pinocytotic vesicles than CEC (figs., 2.3b and 2.3c).

Figure 2.3. Transmission electron micrographs of cells isolated from adult rat heart. *A:* section of a cardiomyocyte fixed with 50 mM lysine and 2% glutaraldehyde, bar represents 1 μ m. *B:* fibroblast-like cell fixed with 50 mM lysine and 2% glutaraldehyde showing typical filaments in cytoplasm, bar represents 10 μ m. *C:* endothelial cell fixed with 2% tannine and 2% glutaraldehyde resulting in a solid fixation of membranes and showing a multitude of vesicles in the vicinity of cell membrane. Bar represents 10 μ m.

To identify the nature of CFLC, these cells were immunological tested for the presence of actin, desmin and vimentin (see table 2.1). They stained positively to vimentin and negatively to actin and desmin and behave in this way like fibroblasts. Incubation of CFLC with ascorbic acid (50 μ g/ml) for three days caused an expression of actin and a down regulation of the expression of vimentin.

Table 2.1. Staining of cardiac fibroblast-like cells (CFLC), isolated from adult rats, to several antibodies after control incubation or after stimulation with ascorbic acid (50 µg/ml) for three days.

antibody	control	ascorbic acid
vimentin	++	+
desmin	-	-
actin	-	+

- : no staining, + : moderate staining, ++ : strong staining.

2.5 SUMMARY

A reliable technique to isolate and subcultivate cardiomyocytes, endothelial cells and fibroblasts from adult rat heart is described. Cardiomyocytes were for more than 95% rod shaped and attached to dishes, no other cell types adhered to the dishes. The cells retained their morphology for some days when kept under the described culture conditions. The endothelial cells were pure and positively characterized by uptake of DiI-Ac-LDL and staining with VWF and RECA-1. They could be passaged till passage six. Fibroblast-like cells were identified as fibroblasts in their morphology, expression of intermediate filaments, presence of collagen, and could be kept in culture for more than 25 passages.

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CHAPTER 3

CONTINUOUS ENDOTHELIAL CELL LINES OBTAINED FROM RAT HEART

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3.1 INTRODUCTION

As a barrier between blood and interstitial space, endothelial cells (EC) play a prominent role in the blood-tissue exchange of molecules (17). Besides, EC can synthesize physiologically important compounds, such as vasoactive and anti-thrombogenic agents, and secrete them to the blood and/or to the subendothelial matrix (9).

To investigate endothelial cell physiology in more detail, cultured cells are increasingly used in a variety of experimental models. In spite of numerous attempts only few investigators have succeeded in isolating and culturing endothelial cells from rat heart (2, 7, 18). The main problem to be solved is the presence of contaminating non-endothelial cells which easily overgrow the endothelial cells in culture and, hence, influence the outcome of the experiments. In our laboratory a technique has been implemented to isolate and cultivate cardiac EC (CEC), cardiomyocytes (CMC) and fibroblast-like cells (CFLC) from adult rat heart (13, 14, and chapter 2). Because of the relative low yield and the limited life-span of the cultured CEC, extensive studies on the (patho)physiological properties of CEC were found to be seriously hampered. For that reason attempts were made to obtain continuous cell lines originating from the rat CEC cultures.

The presence of a spontaneously transformed and immortalized line of human umbilical vein endothelial cells (HUVEC) has been described (19), but to our best knowledge a rat heart EC line has not yet been reported so far. In this chapter the establishment of two continuous cell lines of CEC cultures isolated from adult rat is described, designated rat heart endothelial cell line-50 (RHEC-50) and RHEC-116. These CEC lines show characteristics similar to both freshly isolated CEC and CEC *in vivo*. The RHEC-50 and RHEC-116 will be useful to study cardiovascular physiology.

3.2 METHODS

Isolation of cells

CEC were isolated from adult rat heart as described in chapter 2. Briefly, hearts were removed from 12 weeks old male Wistar or Lewis rats under ether anaesthesia, and perfused for 30 min with a modified Krebs Ringer buffer solution (MKR) containing: 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 10 mM NaHCO_3 , 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 11 mM glucose, 4 mM taurine, 25 μM CaCl_2 and 0.06% collagenase; pH was set at 7.4 and the buffer solution was gased with 95% O_2 plus 5% CO_2 . Thereafter the EC of one heart were collected and plated on two, fibronectin-precoated Falcon tissue culture dishes of 3.5 cm diameter in EC culture medium. The latter medium consisted of 40% M199, 40% RPMI1640,

20% fetal calf serum (FCS), and supplemented with 2 mM L-glutamine, 13 mM NaHCO_3 , 10 mM HEPES, 50 $\mu\text{g/ml}$ gentamicin, 20 U/ml heparin sodium, approximately 40 $\mu\text{g/ml}$ bovine brain extract and 2.5 $\mu\text{g/ml}$ fungizone. The primary cultures were provided with fresh culture medium every day. When almost confluent, the CEC clones were washed once with phosphate buffered saline (PBS), trypsinized with PBS containing 0.67 mM ethylenediaminetetraacetate (EDTA) and 1.25 mg/ml trypsin to detach the cells, and subsequently plated on culture dishes in a split ratio of 1 to 3 in EC culture medium without fungizone (passage one). Thereafter, the cells were further cultured, usually up to passage 6. However, occasionally (2 out of 60 successful isolation procedures) some batches grew also beyond passage 6. These cells could be plated in a split ratio of at least 1 to 6.

Cells were screened for mycoplasma contamination by the Department of Medical Microbiology and by the Centralised Experimental Animal Facilities, University of Limburg, Maastricht, The Netherlands, using generally accepted standard techniques (1).

Characterization of endothelial cells

The CEC were tested for uptake of 1,1'-dioctadecyl-1-3,3',3'-tetramethyl-indocarbocyanine-perchlorate-acetylated low density lipoprotein (DiI-Ac-LDL). To this end, cells cultivated on 96 well plates and 50-75% confluent, were incubated with 10 μg DiI-Ac-LDL/ml culture medium at 37°C for four hours. The medium was washed away and the cells were studied with the use of a Zeiss fluorescence microscope equipped with appropriate barrier filters. For immunofluorescence microscopy cells on 96 well plates were fixed in 3% paraformaldehyde in PBS and treated with 0.05% Nonidet P40 in PBS to permeabilize the membranes. After four times washing with PBS, cells were incubated with rabbit anti-human-Von Willebrand Factor (VWF), diluted 1:100 in PBS containing 1% bovine serum albumin (BSA V) and 0.1% NaN_3 in a humidified atmosphere at 37°C for 30 min, or incubated with RECA-1 (a rat endothelial cell antibody; ref. 3), diluted 1:500 in PBS containing 1% BSA plus 0.1% NaN_3 at 37°C for 30 min. Alternatively the cells were incubated with anti-vimentin, anti-cytokeratins or anti-desmin under the same conditions. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit-IgG or FITC-conjugated rabbit anti-mouse-IgG. The cells were washed and mounted in glycine buffered glycerol (pH 8.6) for microscopic evaluation.

For quantitative analysis cells were grown to confluency on 75 cm^2 tissue culture flasks. Photographs from the cells were taken with a Zeiss inverted phase contrast microscope, whereupon the cells were harvested by trypsinization. Cell protein content was determined according to Lowry and colleagues (15) and DNA was measured with the Hoechst 33258 dye as described by Labarca and Paigen (11).

Figure 3.1. Phase-contrast photographs of rat cardiac endothelial cells (CEC). A: passage 3 of a representative primary culture of freshly isolated CEC. B: rat heart endothelial cell line-50 (RHEC-50) passage 28. C: RHEC-50 passage 101. D: RHEC-116 passage 121. Bar represents 0.1 mm.

Karyotyping of cells was performed by routine procedures at the Department of Clinical Genetics, University of Limburg, Maastricht, The Netherlands. To this end, subconfluent cells were harvested by trypsinization, incubated with colcemid, fixed with acetic acid/methanol (1:3), and spotted on microscope slides.

Expression of membrane determinants was monitored after stimulation with interferon- γ (IFN- γ). Fresh culture medium containing 100 U/ml IFN- γ was applied to confluent EC-monolayers on 96 well plates. Incubation occurred for 3 days. Subsequently cells were washed with washing medium (RPMI1640 containing 10% FCS and 20 mM HEPES) and incubated with monoclonal antibodies OX-6 (anti-MHC class II), OX-18 (anti-MHC class I), and 1A29 (anti-ICAM-1). After 1 hour of incubation at room temperature cells were washed 3 times with washing medium and incubated with FITC conjugated goat anti-mouse F(ab)₂ IgG for 30 min. After washing, the cells were fixed in 1 % paraformaldehyde in PBS and examined by fluorescence microscopy.

Materials

Unless otherwise stated, all chemicals were obtained from Merck (Darmstadt, FRG) and were of the highest purity available. Collagenase CLS, batch 67047M, was purchased from Cooper Biomedical Worthington (Malvern, PA USA), fibronectin and anti-VWF from the Central Laboratory of the Netherlands Red Cross Transfusion Service (CLB, Amsterdam, The Netherlands), M199, RPMI1640, trypsin and fungizone from Gibco (Life Technologies Ltd., Paisley, Scotland UK) FCS; batch 701064 from Sera-Lab Ltd., Crawley Down, UK, or batch 0135827 from Flow Laboratories Ltd., Irvine, Scotland UK, or batch 40G3700P from Gibco). L-glutamine was obtained from Sigma Chemical Co. (St. Louis, MO USA), heparin sodium from Serva GmbH (Heidelberg, FRG), DiI-Ac-LDL from Biomedical Technologies Inc. (Stoughton, MA USA) Falcon tissue culture dishes were purchased from Becton Dickinson Ltd. (Playmouth, UK) and microtiter plates and culture flasks from Costar Corp. (Cambridge, MA USA), FITC-conjugated swine anti-rabbit-IgG and FITC-conjugated rabbit anti-mouse IgG from Dakopatts (Copenhagen, Denmark), FITC-conjugated goat anti-mouse F(ab)₂ IgG from Cappel Lab. Inc. (Cochranville, PA USA). IFN- γ was kindly donated by Dr. P. van der Meide, OX-6 and OX-18 were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK), 1A29 was kindly donated by Dr. M. Miyasaka (ref. 20). Anti-vimentin (RV202), anti-desmin (RD301) and anti-cytokeratins (RGE53, RCK102, M20, RCK105, LP2K) were generously provided by Prof. Dr. F. Ramaekers. BSA V was obtained from Boehringer Mannheim GmbH (Mannheim, FRG).

3.3 RESULTS AND DISCUSSION

Establishment of the endothelial cell lines

In our routine procedure for isolating CEC from adult rat (13, 14), the CEC obtained from one heart are essentially pure but few in number (10^2 - 10^3). Therefore, the cells usually are plated on about 20 cm² culture dishes. It takes on the average 10 days for these cultures to reach the stage of confluency. In primary culture, the CEC have a typical clonal form of growing, but after the first passage, they form a more homogeneous monolayer with elongated cells. Till passage three or four there are no changes in cell morphology. Usually, between passage four and six CEC have reached their proliferative life-span, the cells don't divide anymore and start to senesce. This process is accompanied by large increases in cell area, cell volume and protein content (16).

In attempts to obtain continuously dividing cells, the CEC in senescent condition were kept in culture. Two out of 60 successful CEC isolations showed an atypical growth pattern after passage six. These cell lines, RHEC-50 isolated from a 14 week-old Wistar rat and RHEC-116 isolated from a 13 week-old Lewis rat, showed initially the following growth pattern. Plated cells hardly grew after passage six for about two weeks. Thereafter some cells started to divide and formed a confluent monolayer again. The cells could be passaged, and in the subsequent 12 months more than 100 passages have been achieved. Figure 3.1 shows light micrographs of RHEC-50 and RHEC-116 and, for comparison, passage three of a representative primary CEC culture. There is a morphological difference in cell size; RHEC-50 and RHEC-116 are markedly smaller than the CEC of passage three.

The growth characteristics of the cell lines have been determined. The protein contents of the RHEC-50 and RHEC-116 were about 50 pg per cell based on counting cell numbers on photographs of the tissue culture flasks made just before trypsinization. The DNA contents of the cell lines were on the average 4.5 pg per cell when based on cell counting of the photographs. This value is in the same range as reported earlier (21). RHEC-50 and RHEC-116 each showed a constant protein/DNA ratio of about 10. For young passages of a representative primary CEC culture, this ratio was found to be higher and increased with passage number, being 25, 33 and 43 at passages one, two and three, respectively. These findings are in agreement with the morphological observation of difference in cell size between the continuous cell lines and the passages one to six of primary CEC.

The RHEC-50 and RHEC-116 were routinely subcultivated in a split ratio of 1 to 6 and grew to confluency within two to three days. In this time one 10 cm diameter dish gave a yield of about 7.10^6 cells (ca. 350 μ g protein). The omission of growth factor/ heparin or fibronectin precoating of the culture dishes reduced the proliferative rate by 50-60%. Reduction of the percentage FCS from 20% to 10% also diminished the growth rate markedly.

Figure 3.2. Karyotypes after Giemsa staining of rat heart endothelial cell lines
A: RHEC-116 passage 113.
B: RHEC-50 passage 20.
C: RHEC-50 passage 93.

The karyotype of the RHEC-116 had a modal number of 42 chromosomes which is the regular number for rat cells (12). Fig. 3.2a shows the chromosomes of RHEC-116 at passage 113. Each cell has 20 pairs of autosomes and each one of a X and one of a Y chromosome. In contrast, the population of RHEC-50 has a trisomy 6 and trisomy 19. This was already present at passage 20 and 59 (fig. 3.2b). At passage 93 an additional trisomy 8 was detected in this cell line (fig. 3.2c). In both RHEC-50 and RHEC-116 the occurrence of tri- or tetraploidy was found to be less than 10%.

When screened for mycoplasma contamination neither RHEC-50 nor RHEC-116 showed a positive reaction.

Characteristics of RHEC-50 and RHEC-116

The cell lines have been checked for several features characteristic for EC, including the presence of VWF, staining with RECA-1, a specific antibody directed against endothelial cell surface antigen (3), and internalization of DiI-Ac-LDL. In addition, RHEC lines were compared to young passages of primary cultures of CEC for the examined features. Also staining to the intermediate filament proteins vimentin, desmin and cytokeratins has been performed. For negative controls CFLC, also isolated from adult rat heart (13, 14), were used.

The VWF staining was not homogeneous; some cells showed a higher VWF content than others in the same well. This was found for CEC of passages one to four of primary cultures as well as for RHEC-50 and RHEC-116 (table 3.1, fig. 3.3). CFLC did not show VWF staining.

Table 3.1. Characteristics of cell types isolated from adult rat heart.

cell type	passage number	RECA-1	VWF	DiI-Ac-LDL
RHEC-50	12	+	+	++
RHEC-50	87	+	+	++
RHEC-116	8	+	+	++
RHEC-116	103	+	+	++
freshly isolated CEC	3	+	+	++
fibroblast-like cells	20	-	-	-

- : no staining, + : moderate staining, ++ : strong staining, RECA-1 : staining of mouse monoclonal antibody against rat endothelial antigen, VWF : staining of polyclonal rabbit antibody against human Von Willebrand Factor, DiI-Ac-LDL : uptake of 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indocarbocyanine-acetylated low density lipoprotein, CEC : cardiac endothelial cells, RHEC : rat heart endothelial cell line.

Figure 3.3. Fluorescence photographs of cardiac endothelial cells (CEC). A: staining with VWF of rat heart cell line-50 (RHEC-50) passage 87. B: staining with RECA-1 of RHEC-50 passage 87. C: staining with vimentin of RHEC-50 passage 43. D: uptake of DiI-Ac-LDL of RHEC-116 passage 121. Bar represents 0.1 mm.

RECA-1 reacted weakly but clearly with RHEC-50, RHEC-116 and with passages one to four of primary CEC cultures (table 3.1), whereas CFLC did not react.

Vimentin is the intermediate filament protein of cells derived from the mesenchym, and is generally thought to be the only intermediate filament protein that is expressed by endothelial cells (7). However, co-expression of vimentin and cytokeratins (10), or vimentin and desmin (6) in EC have been reported. The RHEC-50 and the RHEC-116, as well as the CFLC stained positive for vimentin (fig. 3.3c) and negative for desmin and most cytokeratins tested. Only RHEC-50 reacted weakly positive to one cytokeratin antibody (LP2K).

The DiI-Ac-LDL uptake by the EC was very distinct from that of the CFLC. At various passages RHEC-50 and RHEC-116 were clearly positive and showed a staining intensity similar to that of passages one to four or primary culture of representative CEC (table 3.1, fig. 3.3).

Because the expression of membrane determinants is well studied in HUVEC (4) and neonatal rat heart EC (5), this feature was also investigated in the present cell lines. Both RHEC-50 and RHEC-116 demonstrated no expression of class II major histocompatibility (MHC) antigen, a distinct expression of class I MHC antigen, and a weak expression of the adhesion molecule ICAM-1 (table 3.2).

Table 3.2. Expression of membrane determinants by the rat heart endothelial cell lines (RHEC-50 and RHEC-116), before and after stimulation with interferon- γ .

membrane determinants	RHEC-50 no IFN- γ	RHEC-50 with IFN- γ	RHEC-116 no IFN- γ	RHEC-116 with IFN- γ
class II MHC	-	++	-	+
class I MHC	+	++	+	++
ICAM-1	$\pm$	++	$\pm$	+

MHC : major histocompatibility complex, ICAM : intercellular adhesion molecule. Confluent cultures were stimulated with 100 U/ml interferon- γ (IFN- γ) for 3 days. Expression was measured by immunofluorescence, - : negative, $\pm$: weakly positive, + : positive, ++ : strongly positive.

Stimulation of the cell lines with 100 U/ml IFN- γ for 3 days induced expression of MHC class II molecules and upregulation of MHC class I and ICAM-1 antigens. Induction and upregulation of MHC class II and ICAM-1 antigens, respectively, was stronger in RHEC-50 than in RHEC-116. In general, the expression and induction patterns of the tested antigens in the cell lines were comparable with passages one to four of primary cultures of isolated CEC. These observations are

in agreement with earlier findings in human and rat EC (4, 5) and underline the endothelial properties of the RHEC-50 and RHEC-116.

Application of RHEC-50 and RHEC-116

At present, the continuous cell lines originating from EC of adult rat heart are successfully used in a variety of studies in our laboratory. The transport characteristics of glucose across endothelial preparations grown on inserts with porous membranes are currently investigated. Because the continuous cell lines offer the possibility to generate appreciable amounts of EC mRNA and protein, these cell preparations are also employed in molecular biological studies on the intracellular transport of fatty acids and the role of fatty acid-binding protein (FABP) therein. Finally, the continuous cell lines represent powerful tools for transfection studies aiming at overexpression of cellular proteins in order to explore the specific effects of these macromolecules in EC function.

3.4 SUMMARY

The described continuous CEC lines have shown to possess the same characteristics of CEC (presence of VWF, uptake of DiI-Ac-LDL, staining of RECA-1, vimentin and upregulation of membrane determinants after stimulation with IFN- γ) as primary cultures of freshly isolated CEC and EC in situ. These characteristics were preserved for at least more than 100 passages. Moreover, the cell lines retained a constant behaviour in growth and could yield a large number of cells (from $1 \cdot 10^6$ to $7 \cdot 10^6$ cells in two days). Therefore, both RHEC-50 and RHEC-116 are suitable for experimental studies.

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CHAPTER 4

FATTY ACID OXIDATION CAPACITY AND FATTY ACID-BINDING PROTEIN CONTENT OF DIFFERENT CELL TYPES ISOLATED FROM RAT HEART

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4.1 INTRODUCTION

Although the heart can utilize a variety of substrates to fulfill its energy demands, fatty acids are preferred under normal conditions (11). Since *de novo* synthesis of fatty acids in cardiomyocytes is limited, these substances are mainly derived from exogenous sources, including fatty acids bound to albumin in the vascular compartment. In the heart cardiomyocytes are considered to be the principal group of fatty acid consuming cells, although coronary endothelial cells are also capable of fatty acid oxidation (12).

The exact mechanism underlying the transport of fatty acids from the vascular compartment to the mitochondrial site of consumption in the cardiomyocytes is incompletely understood (2). It has been hypothesized that fatty acids cross the endothelial cells, lining the microvasculature, through the luminal cell membrane, the endothelial cytoplasm and the abluminal cell membrane, then diffuse through the interstitial space towards the myocytal membrane (sarcolemma), and cross the sarcolemma and the cytoplasm of the myocyte to finally reach the mitochondrial outer membrane, where conversion of the fatty acyl moiety into acylCoA occurs (13, 15).

Fatty acid-binding proteins (FABPs) are thought to be involved in both the transendothelial and the intramyocytal transport of fatty acids (2, 14, 15). The FABPs belong to a family of relatively low molecular weight proteins, are abundantly present in most cell types and show a high affinity for long-chain fatty acids (1). Recently it has been shown that the fatty acid-oxidizing capacities of several tissues run in parallel with their FABP contents (7).

The FABP type present in the cytosolic fraction of homogenized rat hearts (H-FABP_c) has been extensively characterized and quantified (4, 6). Information on the cellular distribution of H-FABP_c in the heart is scanty. Immunocytochemical studies have shown that bovine and rat cardiomyocytes contain H-FABP_c (3, 5), whereas considerable staining was also observed in rat cardiac endothelial cells *in situ* (5). However, reliable quantitative data are lacking.

The present study was conducted to quantify H-FABP_c in various cell types isolated from adult rat heart, including cardiomyocytes, endothelial cells and fibroblast-like cells. The content of H-FABP_c was related to the capacity to oxidize fatty acids by these particular cardiac cell types.

4.2 METHODS

Isolation and cultivation of cells

Cardiomyocytes (CMC), cardiac endothelial cells (CEC) and fibroblast-like cells (CFLC) were isolated, as described in chapter 2.

Experimental conditions and assay techniques

Cultured CEC and CFLC between passage two and six, and primary CMC were washed three times with prewarmed Hanks' Balanced Salt Solution (HBSS) containing: 137 mM NaCl, 5.4 mM KCl, 1.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.9 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mM Na_2HPO_4 , 5 mM glucose, 10 mM HEPES and 4.1 mM NaHCO_3 ; pH 7.4. Cells (approximately 1 mg protein in 300 μl HBSS per dish) were scraped from the dishes with a rubber policeman and subsequently homogenized. The homogenization fluid contained 250 mM sucrose and 1 mM EDTA. This suspension was homogenized in a Potter-Elvehjem homogenizer, using successive pestle clearances of 0.1 and 0.05 mm.

The oxidation of 120 μM [$1\text{-}^{14}\text{C}$]palmitate bound to albumin (molar ratio 5:1) was measured at 37°C for 30 minutes with 0.2 to 0.3 mg cellular protein per incubation. Oxidation rates were calculated from the radioactivity of the formed $^{14}\text{CO}_2$ and ^{14}C -labeled acid-soluble intermediates as described by Glatz et al. (8). Protein content was determined by the method of Lowry et al. (10). Cytochrome C oxidase activity of the homogenates was measured at 25°C as described before (9).

The H-FABP_c content in the different cell homogenates was measured by enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit antibodies raised against rat H-FABP_c. After centrifugation (10,000 g, 5 min) of the homogenates, the supernatants were directly coated to PVC microtiterplates and quantified by comparing them with a series of H-FABP_c standards in a concentration range of 2 to 20 $\mu\text{g/l}$. After incubation with anti-H-FABP_c antibodies and goat anti rabbit-IgG/HRPO, the oxidation rate of o-phenylenediamine was monitored in a Flow Multiscan plus MKII.

Materials

Unless otherwise stated, all chemicals were obtained from Merck (Darmstadt, FRG). The culture media RPMI1640, M199 and the trypsin solution were purchased from Flow (Irvine, Scotland UK), FCS from Boehringer Mannheim GmbH (Mannheim, FRG) and Seralab Ltd. (Crawley Down, UK) BSA V from Boehringer Mannheim GmbH. The Falcon tissue culture dishes and microtiter plates were obtained from Becton Dickinson Ltd (Plymouth, UK). Goat anti rabbit/HRPO was obtained from Nordic (Tilburg, the Netherlands), [$1\text{-}^{14}\text{C}$]palmitate (50-60 mCi/mmol) from Amersham International (Buckinghamshire, UK).

4.3 RESULTS

The cardiomyocytes used in this study were kept in culture during four hours after isolation. They were mechanically at rest. Microscopic evaluation showed that over 95% of the cells were rod-shaped. The CEC were grown to confluency.

The CFLC preparation, at passage two or higher lacked endothelial cells. CFLC formed a monolayer if confluent; in the post-confluent state they overgrew each other and had a more spindle-like appearance.

Palmitate oxidation was measured radiochemically in homogenates of the cells at concentrations of substrate, coenzymes and cofactors that gave maximal oxidation rates (18). The oxidation capacity of CMC was 2.31 ± 0.31 nmol/min per mg tissue protein. CEC and CFLC had a considerably lower oxidation capacity (table 4.1).

The distribution of the activity of the mitochondrial marker enzyme cytochrome C oxidase, measured in the homogenates of CMC, CEC and CFLC (table 4.1), indicated that the mitochondrial density was highest in CMC.

The content of H-FABP_c was found to be 4.5 ± 1.4 μ g /mg tissue protein in CMC, and 0.008 ± 0.004 μ g/mg tissue protein in CEC and 0.003 ± 0.004 μ g/mg tissue protein in CFLC (table 4.1). The detection level of the FABP-assay was 0.002 μ g/mg tissue protein.

Table 4.1. Palmitate oxidation rate, cytochrome C oxidase activity and H-FABP_c content in various cell types isolated from adult rat heart.

Cell type	CMC (n=4-6)	CEC (n=6)	CFLC (n=5)
Palmitate oxidation rate (nmol/min per mg tissue protein)	2.31 ± 0.31	0.09 ± 0.05	0.31 ± 0.19
Cytochrome C oxidase activity (U/mg tissue protein)	7.36 ± 2.42	0.28 ± 0.10	0.52 ± 0.12
H-FABP _c content * (μ g/mg tissue protein)	4.5 ± 1.4	0.008 ± 0.004	0.003 ± 0.004
Ratio H-FABP _c content to palmitate oxidation rate (μ g.min/nmol)	2.1 ± 1.6	0.096 ± 0.055	0.009 ± 0.006

Data are expressed as means $\pm$ SD of the indicated number of cell preparations. CMC; cardiac myocytes, CEC; cardiac endothelial cells, CFLC; cardiac fibroblast-like cells.

**Assayed on 10,000 g supernatants of homogenates of the cell preparations.*

4.4 DISCUSSION

The present findings indicate that the main locus of cardiac fatty acid oxidation is the cardiomyocyte. Assuming that in the order of 90% of the cardiac protein is of cardiomyocytal origin, cardiomyocytes contribute by about 98% to the total cardiac fatty acid oxidation capacity. Other cardiac cells, such as endothelial cells take a very limited portion of the overall fatty acid oxidation capacity of cardiac tissue. This notion supports the observations made in isolated coronary microvascular cells as reported earlier (12).

On the basis of the specific activity of the mitochondrial marker enzyme cytochrome C oxidase it can be concluded that the contents of mitochondria in CEC and CFLC are considerably less than in CMC. The capacity to oxidize fatty acids is closely related to the activity of cytochrome C oxidase and, hence, to the mitochondrial density of cells under investigation. Moreover the palmitate oxidation rate/cytochrome C oxidase activity ratio in the three cell types studied is mutually not significantly different (0.34 ± 0.02 , 0.36 ± 0.23 and 0.60 ± 0.32 for CMC, CEC and CFLC, respectively) using the Mann-Whitney U test.

The bulk of fatty acids used for oxidative degradation is supplied to the heart from the vascular compartment. Fatty acids are assumed to cross the endothelium, separating the vascular and interstitial compartment, directly through the cytoplasm of the endothelial cells. Since fatty acids have a low aqueous solubility, intracellular carriers are most likely required for their transport from the luminal to the abluminal site of the endothelial cell. Analogously, carrier moieties are also needed in cardiomyocytes to transport fatty acids from the site of entry, i.e. the myocyte plasma membrane to the mitochondria, which are the main locus of fatty acid oxidation in heart tissue. FABP has been proposed to fulfill this fatty acid-carrier role (5, 14). In this respect, it should be mentioned that for a number of organs a quantitative relation exists between the fatty acid oxidation capacity and the cytosolic capacity to bind fatty acids (7), suggesting a close functional relationship between fatty acid binding, most likely by a specific cytoplasmic fatty acid-binding protein (FABP_c), and mitochondrial fatty acid oxidation.

In concert with this notion, in isolated CMC a relatively high fatty acid oxidation capacity was observed to be associated with a relatively high content of FABP. However, a divergent picture emerges from data obtained in CFLC and CEC. Although, the palmitate oxidation rate to cytochrome C oxidase activity is not significantly different from that of CMC, the H-FABP_c content to palmitate oxidation rates are significantly lower, being approximately 0.5-5% of that measured in CMC (table 4.1).

The present findings of very low H-FABP_c levels in CEC disagree with earlier observations by Fournier and colleagues (5), who calculated from electron microscopic immunocytochemical studies that the level of FABP in the cardiac endothelial cell is about half of that in the cardiomyocyte. A possible explanation for this striking discrepancy is that CEC contain FABP(s) to which the polyclonal

antibody (raised against rat H-FABP_c), as used in the present study, does not react to any significant extent. When this is true, at least two different FABPs have to be present in the heart, one localized in the cardiomyocytes and a second one in endothelial and/or fibroblast-like cells. Experiments have to be done to measure the capacity of a cytosolic protein preparation from isolated CEC to bind fatty acids, so as to identify the possible presence of an endothelial-specific FABP. Alternatively, with CEC and CFLC the recovery of H-FABP_c in the supernatant after centrifugation of the homogenate could be highly incomplete, e.g. due to protein binding to membrane structures. It should be stressed that this is a rather theoretical explanation, since no other type of FABP shows this particular feature that might underestimate the actual content of this binding protein in the cytosolic fraction. Finally, during culture, CEC and CFLC could possibly lose their ability to produce FABP. However no differences in H-FABP_c content was measured in passages one, two or three of CEC (12.9, 5.7 and 8.3 ng/mg tissue protein respectively).

Assuming that extrapolation of the present in vitro findings of very limited amounts of H-FABP_c in CEC to cardiac endothelial cells in situ is allowed, the virtual lack of fatty acid-binding proteins in this particular cell type might have consequences for the mechanism of transendothelial transport of long-chain fatty acids and their transfer from the cell membrane to the mitochondrial site of oxidation inside the endothelial cells. However, low cytoplasmic FABP levels do not automatically imply that endothelial cells form an impermeable barrier for fatty acyl moieties. Because the relatively short distance between the luminal and abluminal membrane of the endothelial cell, non-protein bound diffusion of fatty acids may be sufficiently high to fulfill the requirement of the endothelial and parenchymal cells for this important substrate.

4.5 SUMMARY

Heart tissue contains an appreciable amount of H-FABP_c. FABP is thought to play a crucial role in the transport of fatty acids from the cellular membrane to the intracellular site of oxidation and also, in case of endothelial cells, in the transfer of fatty acids from the vascular to the interstitial compartment through the endothelial cytoplasm. The present study was designed to assess the content of FABP in various cell types, including CMC, CEC and CFLC isolated from adult rat heart. Moreover, the possible quantitative relationship between the capacity of different cell types in the heart to oxidize fatty acids and the presence of FABP was delineated. Palmitate oxidation capacities, measured in homogenates of the isolated cells, were 2.31 nmol/min per mg tissue protein in freshly isolated CMC, but only 0.09 and 0.31 nmol/min per mg tissue protein in cultivated CEC and CFLC, respectively. Palmitate oxidation rates were closely related to the cytochrome C oxidase activity and, hence, to the mitochondrial density in the cells under investigation. In CMC the content of H-FABP_c was about 4.5 μg /mg tissue protein. However, in CEC and CFLC the H-FABP_c content was 0.008 and 0.003

$\mu\text{g}/\text{mg}$ tissue protein, respectively, corresponding to at maximum 0.2% of the H-FABP_c content of CMC. These findings indicate a marked difference between CMC and non-myocytal cells in the heart regarding their capacity to oxidize fatty acids, and a marked disproportion between the fatty acid oxidation capacity and immunochemically determined H-FABP_c content in both CEC and CFLC. The functional implication of these observations remains to be elucidated.

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CHAPTER 5

PRODUCTION OF ARACHIDONIC ACID METABOLITES IN ADULT RAT CARDIAC MYOCYTES, ENDOTHELIAL CELLS, AND FIBROBLAST-LIKE CELLS

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5.1 INTRODUCTION

Arachidonic acid (AA) can be enzymatically oxidized to eicosanoids. Eicosanoids are substances acting as modulators in a multitude of (patho)physiological processes, such as thrombosis, inflammation, immune-response, tissue rheology and atherosclerotic disease (27, 36). Nowadays three metabolic pathways are known by which eicosanoids are generated (see refs. 32, 36 for review). The formation of prostaglandins (PG's) and thromboxanes (Tx's) is catalyzed in the cyclo-oxygenase pathway. The production of hydroxyeicosatetraenoic acids (HETE's) and leukotrienes (LT's) occurs in the lipoxygenase pathway. More recently the synthesis of epoxides, HETE's and diHETE's in an epoxigenase pathway has been described (31).

Under basal conditions the concentration of non-esterified AA in the heart is low (13, 16, 26). The production and release of eicosanoids is enhanced when the heart is made ischemic and reperfused. Stimulation also occurs by agents such as the calcium ionophore A23187 or bradykinin (6), and by increasing the levels of exogenous AA (17, 19, 26). Recent studies have shown that degradation of membrane phospholipids is a factor contributing to the irreversible loss of cardiac function during ischemia and reperfusion (12, 41, 42). Hydrolysis of phospholipids is associated with an increase in the tissue level of non-esterified AA (8, 42), but it is unclear whether there is a direct relationship between the increased availability of AA and enhanced eicosanoid production in these conditions (6, 16, 34, 39).

Despite a number of attempts to identify the cellular site of eicosanoid production in cardiac tissue, no agreement exists regarding the cell type responsible for the production of eicosanoids in the heart (19, 20, 24, 34, 43). Some investigators have suggested that vascular cells are the primary source of PG-synthesis, while contradictory results have been published regarding the capacity of cardiomyocytes to produce these substances (1, 2, 18, 23). No clear information is available about the capacity of fibroblast-like cells to synthesize eicosanoids.

The aims of this study were, firstly, to compare the capacity of various cell types of the heart to produce eicosanoids. To this end cardiomyocytes (CMC), endothelial cells (CEC) and fibroblast-like cells (CFLC) from adult rat hearts were used as experimental model. CMC were freshly used, whereas CEC and CFLC were investigated after subcultivating the cells for 2 to 6 passages. The second aim of the study was to determine the type of eicosanoids formed and the amount released by the three above mentioned cell types under various conditions. Agents such as A23187, histamine and bradykinin were used to stimulate eicosanoid production. Eicosanoids were monitored with the use of a sensitive HPLC assay technique (44). In a subset of experiments 6-keto-PGF_{1 α} , the stable product of prostacyclin, and PGE₂ were measured with a more sensitive radioimmunoassay. Thirdly, since the endothelial cells were found to display the highest production rate of eicosanoids *in vitro*, the time course of eicosanoid production by cardiac endothelial cells as a function of the concentration of

exogenous AA, in the absence or presence of the Ca^{2+} ionophore A23187, was investigated in more detail. Besides, the effect of the addition of albumin on endothelial eicosanoid production was monitored since exogenous AA is supplied to endothelial cells in vivo mainly complexed to albumin. These findings will be discussed in relation to previous studies (15, 16, 26, 40, 42) carried out to examine the relationship between AA accumulation and eicosanoid formation in cardiac tissue.

5.2 METHODS

Animals and isolation of cells

Male adult Wistar or Lewis rats of 12 weeks were used. CMC, CEC and CFLC were isolated from the hearts in sterile conditions by a modification of the methods of Piper et al. (33) and Diglio et al. (14) and described in chapter 2.

Incubation of cells for eicosanoid production

CEC and CFLC after passage two and primary CMC on dishes with a diameter of 10 cm were washed three times with Hanks' Balanced Salt Solution (HBSS) with 10 mM HEPES and covered with 4 ml incubation medium HBSS. Calcium ionophore (A23187) and/or AA were present when indicated.

The cells were incubated in a humidified 95% air plus 5% CO_2 stove at 37°C for 30 min. Thereafter the supernatant was collected for eicosanoid determination and the remaining cells were lysed in 1 M NaOH for 30 min at 37°C for protein determination according to Bradford et al. (4). In a subset of experiments cells were scraped from the plate and their eicosanoid content was analyzed. On the average, a 10 cm dish with a confluent layer of non-cardiomyocytes (approximately $2 \cdot 10^6$ cells), contained 0.1-0.4 mg cellular protein. A dish with on the average 10^5 CMC contained about 0.5 mg cellular protein.

A23187 was dissolved in dimethyl sulfoxide (10 mM), and bradykinin and histamine were dissolved in water (1 mM and 0.1 mM, respectively) and prior to administration diluted one to thousand in HBSS. Before use, AA was purified by silicagel column chromatography; elution was performed with 90% hexane plus 10% diethylether and yielded a purified AA fraction. After drying under a stream of nitrogen, purified AA was dissolved in ethanol. The concentration of AA in ethanol amounted to 80 mM.

In a subset of experiments cells were treated with indomethacin (10 μM). The drug was dissolved at a concentration of 20 mM in ethanol. Cells were preincubated with indomethacin for 15 min. This drug was also present in the final incubation medium.

In some experiments an AA-BSA complex was used. To this end 8 mM KOH was added to 8 mM AA in ethanol. After evaporation of ethanol by a stream of nitrogen at 37°C fatty acid free BSA, dissolved in water, was added. The AA-BSA stock solution concentration was 1.6 mM AA/2 mM BSA. The final AA concentration was varied by diluting the stock solution with HBSS containing 200 μ M BSA.

Since the production of AA metabolites by cultured human umbilical vene endothelial cells (HUVEC) has been extensively studied (3, 21, 22, 25, 37), these cells were used to obtain reference values. HUVEC were isolated and cultured according to Bruggeman et al. (5).

Electron microscopy

For transmission electron microscopy cells were grown on thermanox tissue culture coverslips incubated with AA and/or A23187, fixed and further treated as described in chapter 2.

Determination of prostaglandins, thromboxane, hydroxy fatty acids and leukotrienes

Prostaglandins, thromboxane, hydroxy fatty acids [hydroxyeicosatetra-enoic acid (HETE), 12-hydroxyheptadecatrienoic acid (HHT) and leukotrienes (LT)] were determined as previously described (15, 17). Hereto the supernatants of cell incubations were purified on a C₁₈ reversed-phase column after addition of internal standards and acidification to pH 4.0-4.5. After pre-elution of these columns with methanol/water (15/85, by vol) followed by acidified water (pH=4.0), the eicosanoid fraction was eluted with methanol and dried with a stream of nitrogen at 40°C. The dried residues obtained from the cell supernatants were used directly to measure leukotrienes (17) or further purified with silicagel column chromatography (15). Stepwise elution of these columns yielded purified fractions containing HETE/HHT and prostaglandins/thromboxane, respectively. The HETE/HHT fraction was directly applied to reversed-phase HPLC with ultraviolet detection at 235 nm (15). The eluted prostaglandin/thromboxane fraction was methoximated, esterified with panacyl bromide and analyzed by reversed-phase HPLC with fluorimetric detection (15). Figure 5.1 shows a representative HPLC profile. The practical lower limits of detection of prostaglandins/thromboxane B₂, HETE/HHT and LT were 0.15 pmol injected on column. Routinely one fifth (20 μ l) of purified sample was injected on the column. This implies that at least 0.75 pmol should be present in the incubation fluid or cell extract to be detectable by the HPLC assay technique. The overall recoveries of prostaglandins/thromboxane B₂ and HETE/HHT in the assay were 61-72% and 74-86%, respectively.

Figure 5.1. Representative high-performance liquid chromatography profile. Data reflect analysis of a typical experiment of cardiac endothelial cells incubated in the presence of arachidonic acid (AA; 80 μ M) for 30 min. 6-Keto-PGF₁ α , 6-keto-prostaglandin F₁ α ; PGF₁ α and PGE₂, prostaglandins F₁ α and E₂, respectively; TxB₂, thromboxane B₂.

Radioimmunoassay of 6-keto-PGF₁ α and PGE₂

Since we failed to detect 6-keto-PGF₁ α (the stable metabolite of prostacyclin) in the supernatants of CMC with the use of the earlier described HPLC method, a more sensitive radioimmunoassay was applied for the detection of this eicosanoid. Hereto, the cell supernatants (4 ml) were acidified to pH 5.5-6.0 and applied to C₁₈ reversed-phase columns as described by Zijlstra et al. (44).

Figure 5.2. Phase contrast light micrographs of cultured endothelial cells (CEC) and myocytes (CMC) from adult rat heart. A: confluent CEC after 60 min control incubation. B: confluent CEC after 30 min incubation with 80 μ M of arachidonic acid (AA). C: confluent CEC after 60 min incubation with 80 μ M of AA. D: confluent CEC after 60 min of incubation with 10 μ M of A23187.

(Figure 5.2 continued). E: confluent CEC after 10 min incubation with 10 μM of A23187 and 80 μM of AA. F: confluent CEC after 30 min of incubation with 10 μM of A23187 and 80 μM of AA. G: confluent CEC after 60 min of incubation with 10 μM of A23187 and 80 μM of AA. H: CMC after 30 min of incubation with 10 μM of A23187 and 80 μM of AA. Bar represents 0.1 mm.

In a subset of experiments cells were collected in water with the use of a cellscraper and extracted in chloroform/water. Thereafter the cellular extracts were also applied to C₁₈ reversed-phase columns. The eluted fractions were dried with a stream of nitrogen at 40°C and resuspended in radioimmunoassay buffer. The radioimmunoassay of 6-keto-PGF_{1α} and PGE₂ was performed according to the manufacturer's instructions (Advanced Magnetics Inc., Cambridge, MA, USA). The practical lower detection limit of 6-keto-PGF_{1α} and PGE₂ was estimated to be 27 fmol per assay. Routinely one fifth (200 μl) of purified cell extracts or incubation fluids were assayed. This implies that at least 135 fmol should be present in the incubation medium or cell extract to be detectable. Validation of the radioimmunoassays was performed by comparing the values of 6-keto-PGF_{1α} produced by CFLC and determined by radioimmunoassay with those assessed by the HPLC method. An acceptable correlation ($r_s=0.91$) between both assays could be established (data not shown).

Materials

Unless otherwise stated all chemicals were obtained from Merck (Darmstadt, FRG). A23187, bradykinin, histamine, arachidonic acid, indomethacin, BSA V (essentially fatty acid free) and L-lysine were obtained from Sigma Chemical Co. (St Louis, MO USA). The Falcon tissue culture dishes were obtained from Becton Dickinson Ltd. (Plymouth, UK). Thermanox tissue culture coverslips were obtained from Miles Laboratories Inc. (Naperville, IL USA).

Statistics

The data are presented as mean values $\pm$ SD or SEM. The number of experiments per group varied from 4 to 9. Differences between groups were tested with the nonparametric Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

5.3 RESULTS

Morphological changes of CEC during incubation with AA and/or A23187

In a subset of experiments the changes in morphology of CEC during incubations with exogenous AA and/or the calcium ionophore A23187 were monitored. No changes in morphology were light and electron microscopically observed when the cells were incubated in the plain incubation buffer, i.e. in the absence of AA and A23187, for at least 60 minutes (figs. 5.2a, 5.3a). When CEC were incubated in the presence of 80 μM AA, morphological changes at the light microscopic level were absent during the first 30 min of incubation (fig. 5.2b). Less than 20% of the cells incubated in the presence of 80 μM AA for 30 min showed ultrastructural changes (fig. 5.3b). Thereafter the cells became more elongated and frayed (fig. 5.2c). When CEC were exposed to A23187, the cells started to round up above the nucleus after 10 min.

Figure 5.3. Electron micrographs of cultured endothelial cells (CEC) from adult rat heart. A: CEC after 30 min control incubation. B: CEC after 30 min incubation with 80 μ M of arachidonic acid (AA). C: CEC after 30 min incubation with 10 μ M of A23187. D: CEC after 30 min incubation with 10 μ M of A23187 and 80 μ M of AA. Bar represents 0.01 mm.

With longer exposure time the cells formed cytoplasmic extrusions (fig. 5.2d). Electron microscopic evaluation revealed that about 50% of CEC exposed to 10 μ M A23187 were necrotic after 30 min of incubation (fig. 5.3c). Addition of AA to an A23187 containing incubation buffer considerably attenuated the effect of the calcium ionophore. When both A23187 (10 μ M) and AA (80 μ M) were added to the incubation medium, the cells started to round up after 10 to 20 min of incubation (fig. 5.2e). After 20 to 30 min extrusions became visible (fig. 5.2f) and after 60 min of incubation more or less elongated cells with grubby appearance were seen (fig. 5.2g). Electron microscopic observation showed that

ultrastructurally 85% of the cells were intact after 30 min of incubation with 10 μ M of A23187 and 80 μ M of AA (fig. 5.3d).

The CMC generally did not reveal significant morphological changes at the light microscopical level during 30 min of incubation with 10 μ M A23187, 80 μ M AA, or both (fig. 5.2h). Occasionally the percentage rod shaped cells was slightly reduced after incubation with 10 μ M A23187 and 80 μ M AA.

Production of eicosanoids

Comparison between different cell types. Isolated CMC, CEC and CFLC were routinely incubated in the presence of 10 μ M A23187 and 80 μ M AA for 30 min, unless otherwise stated. As shown in table 5.1 the amounts of individual eicosanoids produced and released into the incubation medium, and the summated amounts greatly differed among the various cell types studied. The highest production rate was displayed by CEC. On the average 23 nmol eicosanoids per mg cellular protein were synthesized and released by CEC per 30 min incubation (table 5.1).

Table 5.1. Release of eicosanoids in CEC, CFLC, and CMC isolated from adult rat heart and in HUVEC.

	CEC	CFLC	CMC	HUVEC
n	7	6	5	9
sum	23.2 $\pm$ 4.5	2.01 $\pm$ 0.38		3.82 $\pm$ 0.73
11-HETE	1.57 $\pm$ 0.20	0.04 $\pm$ 0.04	<0.008	0.07 $\pm$ 0.02
15-HETE	2.02 $\pm$ 0.52	0.11 $\pm$ 0.05	<0.008	0.40 $\pm$ 0.11
HHT	7.21 $\pm$ 1.55	0.26 $\pm$ 0.04	<0.008	0.77 $\pm$ 0.13
6-keto-PGF _{1α}	0.12 $\pm$ 0.09	0.62 $\pm$ 0.20	<0.008	0.25 $\pm$ 0.06
PGE ₂	7.09 $\pm$ 2.22	0.97 $\pm$ 0.30	<0.008	1.69 $\pm$ 0.32
PGF _{2α}	1.23 $\pm$ 0.50	0.02 $\pm$ 0.01	<0.008	0.64 $\pm$ 0.47
TxB ₂	3.97 $\pm$ 2.22	<0.008	<0.008	<0.008

Values are means $\pm$ SD expressed in nmol.mg protein⁻¹.30 min⁻¹; n, no. of experiments. Cells were incubated with 10 μ M of A23187 and 80 μ M of arachidonic acid at 37°C for 30 min. CEC, cardiac endothelial cells; CFLC, cardiac fibroblast-like cells; CMC, cardiomyocytes; HUVEC, human umbilical vein endothelial cells; 11-HETE, 11-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; HHT, 12-hydroxyheptadecatrienoic acid; 6-keto-PGF_{1 α} , 6-keto-prostaglandin F_{1 α} ; PGE₂, prostaglandin E₂; PGF_{2 α} , prostaglandin F_{2 α} ; TxB₂, thromboxane B₂. Eicosanoids present in incubation medium were determined with the use of a high-performance liquid chromatography technique.

The production by CFLC was considerably less and amounted to 2 nmol/mg per 30 min. The amount of eicosanoids produced by isolated CMC was below the detection limit of the HPLC assay used (<0.00015 nmol/assay corresponding with on the average 0.008 nmol/mg protein per 30 min). The production of eicosanoids in HUVEC was on the order of 4 nmol/mg protein per 30 min, a value comparable with that found by other investigators (22, 25).

When a more sensitive radioimmunoassay was applied to monitor the release of 6-keto-PGF_{1 α} , the stable degradation product of prostacyclin, by CMC a value of about 2 pmol/mg protein per 30 min was obtained (data not shown). The production of 6-keto-PGF_{1 α} by CEC was found to be 120 pmol/mg protein per 30 min, whereas HUVEC and CFLC released 250 and 620 pmol/mg protein per 30 min, respectively (table 5.1). For all the other metabolites measured, CEC produced the largest amounts. Only CEC were capable of producing thromboxane B₂ (TxB₂), the stable degradation product of TxA₂. Formation of leukotrienes was undetectable in either cell preparation.

Comparison between the physiological agonists bradykinin and histamine, and the calcium ionophore A23187. In order to determine the effect of bradykinin and histamine, CMC and CEC were incubated in the presence of 1 μ M bradykinin or 0.1 μ M histamine for 30 minutes. No exogenous AA was added. The effect of the physiologically relevant stimuli on cellular eicosanoid production were compared with that of the Ca²⁺ ionophore A23187. The amount of 6-keto-PGF_{1 α} and PGE₂ was measured in both the incubation medium above the cells, and the cells themselves after being scraped from the plate. This experiment enables us to discriminate between the amount of eicosanoids produced and released into the incubation medium, and the amount sequestered in the cells at the bottom of the dish.

Table 5.2 indicates that under influence of bradykinin and histamine small amounts of 6-keto-PGF_{1 α} and PGE₂ were released into the incubation medium from both CEC and CMC. Only the histamine stimulated release of 6-keto-PGF_{1 α} by CEC was significantly different from that of control, non-stimulated cells. The stimulating effect of A23187 on cellular release however, was found to be highly significant when compared with control incubations in both CEC and CMC.

Analysis of the cellular fraction revealed that the increase in the content of 6-keto-PGF_{1 α} and PGE₂ in CMC reached the level of significance ($p < 0.05$) during 30 min of incubation in the absence of stimulating agents. When bradykinin, histamine or A23187 were included in the incubation medium the CMC content of 6-keto-PGF_{1 α} and PGE₂ did not further increase. In contrast, no significant increase in the content of 6-keto-PGF_{1 α} and PGE₂ was observed when CEC were incubated under control conditions for 30 min. Addition of bradykinin, histamine and A23187 resulted in a significant increase of the cellular content of the two eicosanoids.

Table 5.2. Amount of 6-keto-PGF_{1α} and PGE₂ in the incubation medium and cells after incubation with different stimuli.

cell type	medium		cellular content	
	6-keto-PGF _{1α}	PGE ₂	6-keto-PGF _{1α}	PGE ₂
<i>t=0 min</i>				
CEC			0.7±0.6	34.7±22.7
CMC			0.2±0.1	11.1±3.8
<i>t=30 min</i>				
control				
CEC	<0.3	1.7±2.4	3.0±3.6	62.3±94.2
CMC	0.3±0.5	1.8±2.9	12.7±12.1*	98.5±96.7*
bradykinin, 1 μM				
CEC	0.5±0.6	5.7±4.9	4.3±3.3*	117.8±93.4
CMC	2.0±2.9	6.5±10.3	14.2±14.4*	87.8±57.0*
histamine, 0.1 μM				
CEC	0.8±0.5 [#]	4.3±2.6	5.3±4.4*	145.3±150.8*
CMC	2.5±2.5	1.8±2.2	14.7±6.9*	94.2±46.5*
A23187, 10 μM				
CEC	20.5±17.6 [#]	2268±2709 [#]	4.2±4.2*	189.4±203.5*
CMC	7.9±5.7 [#]	12.6±10.4 [#]	8.1±8.1*	37.2±26.9*

Values are means ± SD expressed as pmol/mg protein. Eicosanoids were determined with the use of a radioimmunoassay technique. Detection level of the radioimmunoassay is 135 fmol per sample. Each incubation contained 0.1–0.5 mg protein. Number of experiments varied from 4 to 8. * Significant different from cellular content at *t*=0. # Significant different from control *t*=30 in the Mann-Whitney U test (*p*<0.05).

Time course of eicosanoid production. The time course of the production of AA metabolites was studied in more detail in CEC preparations. Data presented in figure 5.4 indicate that the rate of eicosanoid release was highest during the first 5 to 10 min of incubation with either 10 μ M A23187 or 80 μ M AA present in the incubation medium. The release leveled off thereafter. When both A23187 and AA were added the releasing rate reached its highest value within 5 min after the start of the addition of the stimulating agents. In general, comparable time patterns were seen for the individual eicosanoids released during the first 30 min (data not shown) as for the sum of AA metabolites (Fig. 5.4).

Figure 5.4. Time course of the sum of eicosanoids released from cultured cardiac endothelial cells. Data are expressed as nmol metabolites per mg cellular protein $\pm$ SE. Cells were stimulated with either the Ca^{2+} ionophore A23187 (Ca-i; 10 μ M) or arachidonic acid (AA; 80 μ M) or with a combination of both.

Influence of A23187 and of varying concentrations of exogenous AA on the production of eicosanoids. The production of eicosanoids in cultured cardiac endothelial cells was below the practical lower limit of detection of the currently applied HPLC technique (i.e. 0.75 pmol/sample) when the cells (0.1-0.5 mg protein) were incubated in plain incubation medium. Addition of AA to the incubation medium stimulated the release of eicosanoids when the extracellular concentration of AA exceeded 10 μ M (Fig. 5.5a). For most eicosanoids maximal stimulation was reached at 80 μ M AA. The release of TxB_2 , PGE_2 and $\text{PGF}_{2\alpha}$ declined at higher AA levels. In contrast, the release of HHT leveled off, whereas the amount of 11- and 15-HETE increased linearly with increasing concentrations of extracellular AA above 80 μ M.

Addition of 10 μM Ca^{2+} ionophore A23187 had a substantial effect on the total amount of eicosanoids released when the exogenous concentration of AA was below 40 μM (fig. 5.5b compared with 5.5a). PGE_2 , TxB_2 and HHT were released in significant amounts. Interestingly, A23187 stimulated also the basal release (i.e. in the absence of exogenous AA) of TxB_2 , HHT and PGE_2 in cardiac endothelial cells. At an extracellular AA concentration above 40 μM the Ca^{2+} ionophore A23187 greatly enhanced the release of PGE_2 (fig. 5.5b compared with 5.5a).

When AA was added to the incubation medium as fatty acid-albumin complex (at a fixed final concentration of 200 μM BSA) in the presence of 10 μM A23187, the stimulating effect of exogenous AA on the release of eicosanoids by cardiac endothelial cells was appreciably attenuated (fig. 5.5c). At AA concentrations below 40 μM the release was almost absent; higher concentrations of AA, up to 160 μM , stimulated linearly the release of prostaglandins, TxB_2 and HETE's.

Effect of indomethacin on the production of eicosanoids in CEC. When in a subset of experiments CEC were incubated in the presence of indomethacin (10 μM) and AA (80 μM), the release of products of the cyclooxygenase pathway (HHT, 6-keto- $\text{PGF}_{1\alpha}$, PGE_2 , $\text{PGF}_{2\alpha}$ and TxB_2) was completely abolished. In contrast, AA induced release of the lipoxygenase pathway metabolites (11-HETE, 15-HETE and 5-HETE) remained unaffected by indomethacin (data not shown).

5.4 DISCUSSION

The findings in the present study on isolated CMC, CEC and CFLC of adult rat hearts strongly suggest that the main site of cardiac eicosanoid release is the endothelial cell, although fibroblast-like cells are also capable of producing these biologically active substances in appreciable amounts. Exogenous AA is required to fully stimulate eicosanoid release in endothelial and fibroblast-like cells. Ca^{2+} ionophore, A23187, administration in combination with AA has only an additional stimulatory effect on eicosanoid release when the AA concentration is below 40 μM . Exogenous AA complexed to albumin reduces the release of eicosanoids.

Figure 5.5 (facing page). Release of individual eicosanoids from rat cardiac endothelial cells. A: varying concentrations of arachidonic acid (AA) in the absence of the Ca^{2+} ionophore A23187 ($n=5$). B: varying concentrations of AA in the presence of 10 μM A23187 ($n=8$). C: Varying concentrations of AA bound to 200 μM (end concentration) bovine serum albumin in the presence of 10 μM A23187 ($n=6$). Data are the mean values $\pm$ SE. For the sake of clarity variation bars are only shown for the sum of the eicosanoids (sum eicos; variation bars of individual eicosanoids are in the same range). Release is expressed in nmol AA metabolite per mg tissue protein per 30 min. 5-, 11-, and 15-HETE, 5-, 11-, and 15-hydroxyeicosatetraenoic acid respectively; HHT, 12-hydroxy-heptadecatrienoic acid; 6-keto- $\text{PGF}_{1\alpha}$, 6-keto-prostaglandin $\text{F}_{1\alpha}$; PGE_2 , prostaglandin E_2 ; $\text{PGF}_{2\alpha}$, prostaglandin $\text{F}_{2\alpha}$; TxB_2 , thromboxane B_2 .

Release of eicosanoids by cultured endothelial cells
(nmol eicosanoids per mg protein per 30 min)

Arachidonic acid metabolites

None of the cell types studied released leukotrienes. As the production rate in isolated AA and A23187-stimulated cells surpasses the rate of formation in the intact heart many folds and the types of *in vitro* produced eicosanoids greatly differ from the pattern released in intact hearts, a possible role of cardiomyocytes in *in vivo* cardiac eicosanoid, and in particular prostacyclin production, cannot be excluded.

Assuming that the maximal rates of eicosanoid release, as observed *in vitro*, reflect the amounts that can be produced in each specific cell type *in vivo*, the present data indicate that the contribution of cardiomyocytes to the overall formation of eicosanoids in the intact heart is limited. Taking into account that cardiac endothelial cells contribute on the order of 5% to the total cardiac protein content, whereas about 80% of the protein is of cardiomyocyte origin, the contribution of the latter cell type to the total amount of eicosanoids produced will not exceed 1% when all cell types are maximally stimulated. In a recent paper from our laboratory (17) it was reported that in isolated rat hearts, reperfused after a period of 60 min no-flow ischemia the production of 6-keto-PGF_{1 α} was on the order of 400 pmol/g wet weight per 30 min. This value corresponds with about 3 pmol 6-keto-PGF_{1 α} produced per mg protein per 30 min. Comparable amounts were found to be released from isolated rat hearts stimulated with the Ca²⁺ ionophore A23187 (17). It is worthy to note that the amounts of 6-keto-PGF_{1 α} released in intact hearts, stimulated by different factors, are on the same order of magnitude as the amounts released in isolated rod shaped CMC when stimulated with A23187 (table 5.2). Therefore, it cannot be excluded that the total production of 6-keto-PGF_{1 α} , as observed in the intact heart, is released by the cardiomyocytes, despite their limited overall capacity to synthesize eicosanoids. However, it is unlikely that cardiomyocytes are the sole site of production *in vivo* since earlier studies of Hsue and colleagues (24) have convincingly shown that endothelial cells *in situ* play an active role in cardiac prostaglandin formation. Hence, the conclusion has to be drawn that the contribution of cardiomyocytes to the release of eicosanoids in fully stimulating conditions is limited, although *in situ* cardiomyocytes may contribute for an unknown proportion to the overall synthesis of eicosanoids in the heart since the production in intact hearts is very low compared to the release from isolated and cultured CEC and CFLC.

The present findings show that the pattern of eicosanoids released by cells isolated from adult rat hearts appreciably differs from the spectrum of AA metabolites produced by the intact heart. Studies on isolated, intact rat hearts subjected to either ischemia and reperfusion or Ca²⁺ ionophore treatment produced mainly 6-keto-PGF_{1 α} (16, 17, 26). In the reperfused hearts minor amounts of TxB₂ were produced as well (16). Administration of A23187 to the perfusion medium results in the formation of leukotrienes D₄ and E₄ in addition to 6-keto-PGF_{1 α} (17). The release of other eicosanoids obviously remains below the practical detection limit of the HPLC assay procedure applied. It cannot be excluded that isolation and subcultivation of cardiac cells alter the activity of the various enzymes involved in

the production of AA metabolites. In this respect it is interesting to note that we found an 8-fold increase in prostacyclin release in isolated CEC when endothelial cell growth factor and heparin were excluded from the culture medium (data not shown). Comparable findings have been reported for HUVEC (3, 22) and bovine pulmonary microvessel endothelial cells (9). In addition, other investigators have reported that variations in the rate of population doubling (25) and differences in cell density (21, 31) affect the amount of prostacyclin produced in vitro. The nature of the stimulus may also influence the most favored route in cellular eicosanoid synthesis (11, 35). When the concentration of exogenous AA is increased in the medium covering mouse cerebromicrovascular endothelial cells, the production of PGE₂ is stimulated rather than that of prostacyclin (7, 31). Similar effects were observed in rat cardiac endothelial cells when the Ca²⁺ ionophore A23187 was applied (fig. 5.5b).

The failure of maximally stimulated isolated CMC, and CEC and CFLC to produce leukotrienes strongly suggests that other cell types are responsible for the production of these substances in vivo. Mast cells commonly present in cardiac tissue (28) may be likely candidates for the site of synthesis of leukotrienes in the intact rat heart.

The findings in the present study clearly indicate that CEC synthesize thromboxane A₂ (fig. 5.5a, b, c). Similar findings have been reported in HUVEC (21, 37, 38) and rat aortic endothelial cells (10, 30). This notion implicates that CEC are capable of releasing both prostacyclin and TxA₂, eicosanoids with in general biologically adverse effects on microcirculatory function and platelets (35).

Earlier studies on intact ischemic and reperfused rat hearts have shown that enhanced eicosanoid production is a transient event (16, 17, 26). Since under these circumstances AA steadily accumulated in the affected cardiac structures, lack of substrate is not a likely cause of the decline in eicosanoid formation. This conclusion is supported by the present findings, i.e. return of the rate of eicosanoid release to basal levels despite excess of exogenous AA. Autodestruction or self-deactivation of the enzymes involved may explain this characteristic pattern in the production rate (35). Mechanistically, oxygen free radicals produced in the course of eicosanoid synthesis may negatively affect the metabolic activity of the eicosanoid producing enzymes (35). However it cannot be excluded that toxic properties of A23187 and/or the detergent action of AA contribute to the loss of metabolic action. In line with this assumption is the observation that endothelial cells change ultrastructurally when incubated in the presence of either A23187 or a combination of exogenous AA and the Ca²⁺ ionophore. Similar signs of damage of stimulated endothelial cells were observed by Revtyak et al. (35) in cultured bovine endothelial cells. However, the measured decrease in eicosanoid release occurs already after 10 min of incubation with AA and/or A23187 (fig. 5.4). Whereas the morphological changes occur after 30 min of incubation with AA (fig. 5.2c), after 20 min of incubation with AA and A23187 (fig. 5.2f), and after 10 min of incubation with A23187.

It should be noted that the effect of physiological agents, such as bradykinin and histamine on the production of eicosanoids by isolated CEC and CMC was found to be limited when compared with the Ca^{2+} ionophore A23187. Although mean values of 6-keto-PGF_{1 α} and PGE₂ concentration in the medium above the cells after 30 minutes of stimulation with bradykinin or histamine was higher than control incubations in the absence of stimulating agents, the level of significance ($p < 0.05$) was not reached with the exception of histamine stimulated release of 6-keto-PGF_{1 α} in CEC. Interestingly, the cellular content of 6-keto-PGF_{1 α} and PGE₂ increased during incubation under control conditions. The addition of stimulating agents to the incubation medium did not significantly influence the increase of the amount of eicosanoids sequestered in the cells (table 5.2). These findings strongly suggest that both CEC and CMC have a basal cellular eicosanoid level. In case of a strong stimulus (for instance A23187), the amount of eicosanoids produced after the addition of the stimulating agent is quantitatively released into the surrounding medium.

CEC *in situ* are surrounded by albumin, present in both the vascular and interstitial space. In order to investigate the influence of this fatty acid-carrying protein on the production of eicosanoids in isolated CEC, in a subset of experiments AA complexed to 200 μM albumin was administered to the incubation medium. The presence of albumin significantly altered the stimulating effect of exogenous AA in endothelial eicosanoid formation (fig. 5.5c). This effect was most noticeable at AA concentrations up to 40 μM , because no release of eicosanoids could be observed below this level. In the absence of albumin AA probably binds to cellular structures and substantially increases the availability of AA for enzymatic conversion. When albumin is present, the majority of AA is most likely kept in solution in the surrounding medium. In view of the fact that the AA concentration in arterial plasma is less than 20 μM (40, 42), the conclusion can be drawn that under physiological conditions extracellular AA is no strong stimulus for endothelial eicosanoid production. However, in hearts with compromised perfusion, the content of AA in the ischemic myocardium significantly increases (8, 12, 42). In this respect, it is interesting to note that under these circumstances the significantly elevated tissue AA content induces only limited amounts of eicosanoids, probably due to compartmentalization of AA in irreversibly damaged heart cells (39).

5.5 SUMMARY

Despite a number of attempts to identify the cellular site of production of eicosanoids in cardiac tissue, no agreement exists about the cell type responsible for the production of eicosanoids in the heart. The capacity of various cell types, isolated from adult rat heart, to synthesize eicosanoids was studied. Cells were routinely incubated in the presence of the Ca^{2+} ionophore A23187 (10 μM) and exogenous arachidonic acid (AA, 80 μM). The release of total eicosanoids in the incubation medium amounted to 23.3 ± 4.5 and 2.0 ± 0.4 nmol/mg cellular

protein per 30 min in subcultivated CEC and CFLC, respectively. The release in isolated CMC remained below the detection limit of the HPLC assay technique applied (<0.00075 nmol/incubation). The production rate of eicosanoids in subcultivated CEC was highest in the first 5 to 10 min of incubation and declined thereafter, suggesting deactivation of the enzymes involved.

PGE_2 , $\text{PGF}_{2\alpha}$, 12-HHT, 11- and 15-HETE and TxB_2 were the main eicosanoids released by endothelial cells. 6-Keto- $\text{PGF}_{1\alpha}$, the stable degradation product of prostacyclin, contributed relatively little to the total amount of eicosanoids formed by CEC. CFLC released predominantly PGE_2 and 6-keto- $\text{PGF}_{1\alpha}$ and to a lesser extent 12-HHT and 15-HETE. Neither CEC nor CFLC released leukotrienes. With the use of a radioimmunoassay technique cardiomyocytes were found to release 0.002 ± 0.0001 nmol of the stable prostacyclin-derivate 6-keto- $\text{PGF}_{1\alpha}$ /mg cellular protein per 30 min. In comparison CEC and CFLC released 0.12 ± 0.09 and 0.62 ± 0.20 nmol 6-keto- $\text{PGF}_{1\alpha}$ /mg cellular protein per 30 min, respectively.

Since CEC were the main eicosanoid producing cells in vitro, the effect of the extracellular stimulants AA and A23187 was studied in more detail in this cell type. Exogenous AA ($80 \mu\text{M}$) was required to fully stimulate endothelial eicosanoid formation. Addition of A23187 stimulated the release of eicosanoids at AA concentrations below $80 \mu\text{M}$. No additional effect was observed at higher extracellular levels of AA. Because exogenous AA is supplied to endothelial cells in vivo complexed to albumin, the effect of this protein on endothelial eicosanoid release was studied. Extracellular albumin ($200 \mu\text{M}$) was found to reduce the stimulatory effect of AA and A23187 especially at low levels of AA.

The effect of the more physiological stimuli histamine and bradykinin was studied in CEC and in CMC in comparison with A23187. Histamine and bradykinin did not significantly increase the release of 6-keto- $\text{PGF}_{1\alpha}$ or PGE_2 in CMC. Only histamine gave rise to a slight but significant higher release of 6-keto- $\text{PGF}_{1\alpha}$ in CEC. The cellular content of 6-keto- $\text{PGF}_{1\alpha}$ and PGE_2 was not influenced by addition of bradykinin, histamine or A23187 to the incubation medium.

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CHAPTER 6

ARACHIDONIC ACID INCORPORATION IN CARDIO- MYOCYTES, ENDOTHELIAL CELLS AND FIBROBLAST- LIKE CELLS ISOLATED FROM ADULT RAT HEART

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6.1 INTRODUCTION

Phospholipids are the fundamental constituents of cardiac membranes and play an important role in the preservation of cellular integrity. Under pathophysiological conditions cellular integrity can become compromised with concomitant physico-chemical alterations in membrane phospholipids (17).

In cardiac tissue phospholipids are present in a variety of subclasses, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, each with a different fatty acid composition. The fatty acid arachidonic acid (AA) comprises about 15% of the total fatty acid content in rat heart and the majority of AA is incorporated in the phospholipid (PL) fraction (96.4%), whereas 3.5% is found in the triacylglycerol (TG) pool and less than 0.1% is present as unesterified fatty acid (FA, refs. 14, 15).

Alterations in myocardial membrane structure and function play most likely a crucial role in the development of myocardial cell injury during ischemia and reperfusion (15). Several hypotheses have been proposed to explain the mechanism of cell death. Lack of energy and intracellular accumulation of calcium have been proposed to be important factors in the development of irreversible tissue damage upon ischemia followed by reperfusion (7). The loss of control of the cytoplasmic calcium concentration could be attributed to the observed decline in energy status of the cell. This could lead to a reduction in the calcium-pumping activity of the sarcolemma, mitochondria and sarcoplasmic reticulum and possibly to partial opening of the slow calcium channels and other non-specific pores. Then the rise in calcium could cause a higher enzymatic activity of calcium-dependent enzymes such as phospholipases. As shown in previous studies, the AA content rises after 30 min ischemia suggesting disturbed phospholipid homeostasis during the ischemic insult (2, 14, 17, 18, 19). Changes in the content and composition of membrane phospholipids may play an important role in the transition from reversible to irreversible cell injury. As a result of stress on the membrane by osmotic pressure or contracture of the myofibrils, proper attachment of the membrane to the cytoskeleton can be further impaired causing disruption of the membrane (6). Free radicals can cause additional damage of the membrane by attacking the unsaturated bonds in the fatty acyl chains of membrane phospholipids.

Information on the incorporation and release of AA is important to delineate the changes in phospholipids at the point of reversible to irreversible cell damage. Since the heart is composed of different cell types, e.g. endothelial cells, cardiomyocytes, and fibroblasts, inter-cellular differences in phospholipid and AA homeostasis may be anticipated. Knowledge about the AA incorporation under control conditions in the different cell types is still incomplete and needs further investigation. This knowledge is required to explain in a proper fashion changes in AA metabolism during early events of cell damage. For that reason the aim of this study was to get more insight into the incorporation of AA in the cellular phospholipid pool under normoxic conditions. To this end [^3H]-AA was

incorporated in cardiomyocytes (CMC), endothelial cells (CEC) and fibroblast-like cells (CFLC) isolated from adult rat heart. Its cellular distribution was analyzed by autoradiography at the light and electron microscopic level. The effect of the duration of the incubation and the concentration of AA on incorporation of labeled AA in the phospholipid and triacylglycerol pools was monitored by biochemical measurements.

6.2 METHODS

Isolation and culturing of cells

CEC, CFLC and CMC were isolated as described in chapter 2. CMC were kept only 20 hours in culture after isolation; then the attached CMC were still rod shaped and excluded trypan blue. The non-attached cells were washed away. CEC and CFLC were grown under tissue culture conditions. In the experiments described, CEC and CFLC between passage 2 and 6 were used.

Labeling for autoradiography

Before labeling the cells were washed with Hanks' Balanced Salt Solution with 10 mM HEPES (HBSS). [5,6,8,9,11,12,14,15- ^3H]-AA was dissolved in ethanol in a concentration of 5 μM and was diluted in HBSS to 10 nM before the experiment.

For autoradiography 0.5 ml of HBSS and 1 μCi , 10 nM [^3H]-AA was added to the cells grown on coverslips and incubations were performed for 30 min at 5% CO_2 , 95% air, in a 37°C stove. For controls, cells were incubated with HBSS alone under further identical conditions as the incubations with [^3H]-AA. Thereafter cells were fixed in 2% glutaraldehyde with 2% tannine and post fixed in 1% osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated and embedded in epon. For light microscopy ultrathin sections on glass slides were covered with Ilford K2 emulsion according to the dipping technique and exposed for 3-6 days. Then the emulsion was developed with D-76, 1:1 diluted, for 4 min and fixed in X55 profix, 1:8 diluted, for 5 min. After washing with water for 20 min and drying, the coupes were stained with toluidine blue and examined under the light microscope.

For electron microscopic autoradiography ultrathin sections were mounted on collodion (2% in amyl-acetate) or formvar coated gilded grids of 200 mesh. The specimens were stained with lead citrate and coated with a thin layer of carbon by evaporation. Then mounted on corks (about 1 cm in diameter) by the use of double-sided tape. The autoradiographic emulsion Ilford L4 was mounted on the corke-borne specimens with the wire loop technique. After 30 to 150 days of exposure the emulsion was developed with Microdol X for 3 min, rinsed in distilled water for 20 sec, fixed in 30% thiosulfate for 4 min, extensively rinsed in water, and finally dried before examination under the electron transmission microscope (CM 10, Philips).

Labeling of endogenous lipid pool

For biochemical analysis of AA incorporation in the cellular lipid pool 0.5 ml HBSS containing 1 μCi [^3H]-AA, with or without additional unlabeled AA (1 μM or 10 μM), was administered to the cells on 3.5 cm diameter dishes. Incubations were performed for 30 or 90 min under 5% CO_2 , 95% air, at 37°C. Aspecific binding of [^3H]-AA to the dishes was measured by incubating control dishes without cells under similar conditions. After washing with PBS, cells and controls were collected by scraping in 0.25 ml, 4°C PBS and additional washing of the dish was performed with 0.25 ml PBS.

The lipids were extracted from the 500 μl (cell) homogenates in 9.5 ml chloroform/methanol (2:1, by vol) containing 0.01% butylated hydroxy toluene according to Folch (4). The lipid classes were separated by thin layer chromatography (TLC) using plates coated with silicagel (Kieselgel 60). A mixture of fatty acids (FA), triacylglycerols (TG) and phospholipids (PL) was used as external standard. The plates were predeveloped with chloroform/methanol/acetic acid/water (10:10:1:1, by vol) until the solvent front had moved 1 cm from its origin. After drying, the plates were further developed with petroleumether/diethylether/acetic acid/water (120:25:1.5, by vol). The lanes with the lipids standards were visualized with UV light after spraying with rhodamine G. Than comigrated spots on the parallel lanes were scraped from the plate and counted in a liquid scintillation counter (Beckman LS 3801). For these experiments protein was determined from parallel dishes according to Bradford (1).

Determination of the endogenous fatty acid, triacylglycerol and phospholipid pool

For analysis of the quantity of cellular FA, TG, PL and the amount of AA present in these pools in the different cell types, in a subset of experiments lipids were extracted from homogenates of cells with hexane/isopropanol according to Rodriguez-Vico et al. (13). The several lipid pools were separated by TLC as described above, scraped from the plates, methylated and quantified by gas chromatography as described previously (16). The protein content in the pelleted residual fraction was dissolved and determined by the standard Lowry method (9).

Materials

Unless otherwise stated all chemicals were obtained from Merck (Darmstadt, FRG). [5,6,8,9,11,12,14,15- ^3H]-AA (209.6 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK), Ilford K2 and L4 from Ilford Ltd. (Mobberly Cheshire, UK), developers D-76 and Microdol X from Kodak (Chalon s./Saone, France), the fixative X55 profix from Amaloco (Ommen, The Netherlands). The gilded grids were obtained from Agar Scientific (Stansted, UK).

Figure 6.1. Light microscopic photographs of isolated rat heart cells after incorporation of $0.01\ \mu\text{M}$ $[^3\text{H}]$ -arachidonic acid for 30 min and exposure to autoradiographic emulsions.

A: endothelial cells.

B: fibroblast-like cells.

C: cardiomyocytes.

Bar represents $10\ \mu\text{m}$.

Statistics

The data are presented as mean values $\pm$ SD. Differences between groups were tested with the nonparametric Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

6.3 RESULTS

Light microscopic autoradiographic analysis of [^3H]-AA incorporation in isolated cells

When isolated cells were incubated with 1 μCi [^3H]-AA for 30 min, in general a significant number of grains could be detected on the autoradiographic emulsions after 3 to 6 days of exposure (figs. 6.1a, b and c). In addition, the morphology of the cells was well preserved as seen under the light microscope. In CEC and CFLC the nuclear compartment was very well visible (figs. 6.1a and b), and the lined up myofibrils, characteristic for striated muscle cells including cardiomyocytes, were clearly present (fig. 6.1c).

The localization in the cytoplasmic extranuclear compartment (including intracellular membranes and plasmalemma) was very prominent. When the number of grains were counted in the cells under investigation, the density of grains amounted to 0.30 ± 0.12 , 0.15 ± 0.06 and 0.20 ± 0.10 , grains per square μm cytoplasmic surface area of CEC, CFLC and CMC, respectively.

Table 6.1. Light microscopic autoradiographic analysis of different cell types, isolated from adult rat heart, after 30 min incorporation of 1 μCi , 0.01 μM [^3H]-AA. Controls were incubated in the absence of labeled AA.

cell type	condition	# cells	# grains/ μm^2
CEC	[^3H]-AA	45	0.30 ± 0.12
	control	22	0.005 ± 0.007
CFLC	[^3H]-AA	15	0.15 ± 0.06
	control	10	0.002 ± 0.003
CMC	[^3H]-AA	24	0.20 ± 0.10
	control	20	0.006 ± 0.001

CEC, CFLC and CMC refer to endothelial cells, fibroblast-like cells and cardiomyocytes, respectively. [^3H]-AA to [^3H]-arachidonic acid. Data are presented as mean values $\pm$ SD and are expressed as number of grains per μm^2 cytoplasm, corrected for the number of grains counted per μm^2 in the extracellular space.

The differences between the cell types did not reach the level of significance. The number of grains above the nuclear compartment (table 6.1, figs. 6.1a and b) was in the range of the grain density of the extracellular space. On the average a grain density of 0.01 grains/ μm^2 could be counted in the extracellular space (see also figs. 6.1a, b and c).

The values of the above mentioned cellular grain density were obtained by analyzing 15 to 45 individual cells. Considerable differences (on the order of 50%) were observed between cell preparations incubated on different days. Figs. 6.1a, b and c indicate that within one preparation also inter-individual differences in grain density are present. In some experiments sequential coupes were made of one cell. After covering these coupes with autoradiographic emulsion, using appropriate exposure times, also coupe-to-coupe variations were detectable. The latter variation was on the order of 30%.

Electron microscopic autoradiographic analysis of [^3H]-AA incorporation in isolated CEC and CMC

For a further ultrastructural observation of the distribution of [^3H]-AA, electronmicroscopic autoradiographs were developed and qualitatively analyzed. The CMC showed a clear cytoplasmic labeling (fig. 6.2a, b). Mitochondria as well as the lipid droplets seemed to contain labeled material. Only after overexposure of 5 months, sequestration of [^3H]-AA in the sarcolemma and in its close vicinity (fig. 6.2b) became visible. The overexposed specimens of CMC showed that there was a clear labeling of the lipid droplets, mitochondria and probably the nuclear membrane.

The CEC also demonstrated a perinuclear, cytoplasmic labeling (fig. 6.2c, d). Lipid droplets were less frequent or hardly detectable. [^3H]-AA was found to be associated with both the plasmalemma and the nuclear membrane (fig. 6.2c, d).

Incorporation of labeled AA in the cellular lipid pool (biochemical analysis)

Incubation of CMC, CFCL and CEC with exogenous [^3H]-AA resulted in a significant incorporation of label in the cellular lipid pool. In general 600-8000, 200-2000, 1500-15000 dpm [^3H]-AA/mg cellular protein were found to be sequestered by CMC, CFLC and CEC, respectively. The incubation conditions were 0.01, 10, or 10 μM AA for 30 or 90 min. In these experiments the amount of [^3H]-AA incorporated in the total cellular lipid pool was only slightly influenced by the extracellular AA concentration and incubation time. The incorporated quantities correspond with 10-25% of the amount of [^3H]-AA present in the incubation medium.

The labeling of the FA fraction was low, i.e. in the range of the aspecific binding of [^3H]-AA to the dishes. The labeled AA was mainly incorporated in the cellular phospholipid and triacylglycerol pool. At low AA concentrations (0.01 μM) CEC and CFLC sequestered predominantly [^3H]-AA in the phospholipid pool.

Figure 6.2. Electron micrographs isolated rat heart cells, after incorporation of $0.01 \mu\text{M}$ [^3H]-AA for 30 min and exposure to autoradiographic emulsion. A: cardiomyocyte (CMC) after two months of exposure. B: CMC after 5 months of exposure. C and D: cardiac endothelial cell after 5 months of exposure. Bar represents $1 \mu\text{m}$.

The proportion of labeled AA incorporated in triacylglycerols was small, resulting in a high phospholipid/triacylglycerol ratio (table 6.2). When the incubation time was extended from 30 to 90 min a relative change of deposition of label could be observed in the direction of the triacylglycerols (decline in phospholipid/triacylglycerol ratio). When the extracellular AA concentration was increased from $0.01 \mu\text{M}$ to $1.0 \mu\text{M}$, in CEC and CFLC the preference of the phospholipid pool as site of AA incorporation was less marked and incorporation in the triacylglycerol fraction became more prominent. In general, at 1 and 10

μM AA, longer incubation times did not decrease the ratio phospholipid/triacylglycerol (^3H)-AA) in CEC and CFLC, but tended to increase this ratio (even significant, from 4.9 ± 1.0 to 7.2 ± 0.9 , in CEC at $1 \mu\text{M}$ AA). In contrast to CEC and CFLC, in CMC at 0.01 and $1 \mu\text{M}$ AA, no significant differences could be observed between the phospholipid and the triacylglycerol pool with respect to the amount of ^3H -AA incorporated, as similar quantities of label were incorporated in the phospholipid and triacylglycerol pool during 30 and 90 min of incubation (table 6.2). Raising the extracellular AA concentration to $10 \mu\text{M}$ AA promoted the incorporation of ^3H -AA in cellular triacylglycerols. Under this condition the triacylglycerol pool was found to be the preferred site of deposition of label as indicated by a ^3H -AA phospholipid/triacylglycerol ratio significantly lower than 1 (table 6.2).

Table 6.2. The ^3H -AA phospholipid/triacylglycerol ratio in CEC, CFLC and CMC after 30 and 90 min of incubation. The concentration of added AA varied from 0.01 to $10 \mu\text{M}$.

cell type	AA (μM)	n	time (min)	
			30	90
CEC	0.01	4	31.1 ± 28.1	15.1 ± 7.9
	1	6	4.9 ± 1.0^d	$7.2 \pm 0.9^{a,d}$
	10	8	$1.0 \pm 0.4^{d,e}$	$1.4 \pm 0.5^{d,e}$
CFLC	0.01	4	158.7 ± 32.7^b	49.4 ± 30.8^a
	1	4	$17.0 \pm 4.8^{b,d}$	$18.0 \pm 1.6^{b,d}$
	10	4	$2.3 \pm 0.4^{b,d,e}$	$2.7 \pm 0.5^{b,d,e}$
CMC	0.01	6	$0.7 \pm 0.4^{b,c}$	$0.9 \pm 0.3^{b,c}$
	1	6	$0.8 \pm 0.3^{b,c}$	$1.3 \pm 0.7^{b,c}$
	10	6	$0.3 \pm 0.1^{b,c,d,e}$	$0.4 \pm 0.3^{b,c,e}$

CEC, CFLC and CMC refer to cardiac endothelial cells, fibroblast-like cells and cardiomyocytes, respectively; AA to arachidonic acid; n to the number of experiments. Data are depicted as mean values $\pm$ SD. Differences between groups were tested with the nonparametric Mann-Whitney U test, p values < 0.05 were considered to be statistically significant; a) significant different from the corresponding value at 30 min; b) significant different from the corresponding value in CEC; c) significant different from the corresponding value in CFLC; d) significant different from the corresponding value obtained at $0.01 \mu\text{M}$ AA in the same cell type; e) significant different from the corresponding value obtained at $1 \mu\text{M}$ AA in the same cell type.

The content of fatty acids, triacylglycerols and phospholipids in isolated cells

Since differences in incorporation of labeled AA in the endogenous lipid pools between the various cell types can be caused by differences in pool size of fatty acids, triacylglycerol and phospholipids, the content of these lipid fractions was analytically assessed (table 6.3a). In addition, the proportion of AA in the fatty acid, triacylglycerol and phospholipid pool was monitored (table 6.3b).

The phospholipid content in the three cell types was on the order of 250-350 nmol fatty acyl chains per mg cellular protein, corresponding with about 125-175 nmol phospholipid per mg protein. No significant differences could be observed between the various cell types. In contrast, the size of the triacylglycerol pool was significantly higher in CMC than in CEC and CFLC. The content in the latter two cell types was mutually not significantly different. Also the FA content differed between the cell types studied, in CEC it was twice as high than in CFLC and CMC. The size of the unlabeled AA fraction in the cellular FA pool was not different in the three cell types. Interestingly, the content of unlabeled AA in the cellular phospholipid pool was found to be twice as high in CMC than in CEC and CFLC (table 6.3b), despite similar total phospholipid levels in these cells. The content of AA in the cellular triacylglycerol pool was higher in CMC than in the other two cell types, as could be expected on the basis of the total triacylglycerol content. As a consequence, the ratio of AA present in the phospholipid over the triacylglycerol pool was significantly lower in CMC (i.e. 28.2) than in CEC and CFLC (i.e. 83.9 and 98.4, respectively).

6.4 DISCUSSION

The present study shows that [^3H]-AA added to cells isolated from adult rat hearts is readily incorporated into the cellular lipid pool.

Autoradiography at the light and electron microscopic level revealed that label was mainly present in the extranuclear space of the cell. When CMC, CEC and CFLC were incubated under comparable conditions, the number of grains per square μm cytoplasmic surface were on the same order of magnitude in the three cell types. Since [^3H]-AA is mainly incorporated in the cellular phospholipid and triacylglycerol pool (table 6.2), the grains observed do most likely reflect deposition of labeled AA in the cellular membranes and lipid droplets being the storage sites of phospholipids and triacylglycerols, respectively. Although the electron microscopic technique does not allow identification of the exact site of deposition of label unless extended statistic analysis will be performed, the present findings strongly suggest that in addition to the sarcolemmal phospholipids also phospholipids in intracellular membranes (i.e. membranes surrounding the matrix of subsarcolemmal and intrafibrillar mitochondria, and intracellular lipid droplets) have sequestered a substantial quantity of [^3H]-AA. Discrimination between intracellular lipid droplets and neighbouring mitochondria also requires

extended cross-fire analysis techniques. In this regard, Miyazaki and coworkers (10, 11) observed in cultured neonatal cardiomyocytes with electron microscopic autoradiography that deposition of [^3H]-AA mainly occurred in mitochondrial and perinuclear membranes. In their study, the incorporation of label in the sarcolemma of normoxic cells appeared to be minimal (10, 11).

Chemical analysis corroborated the notion that the extracted [^3H]-AA is incorporated in the cellular phospholipid and triacylglycerol pool. The relative distribution of the labeled fatty acid over the two esterified lipid pools depended on a variety of factors such as type of cells studied, concentration of AA added to the incubation medium and to a lesser extent the incubation interval.

Table 6.3. The cellular fatty acid content of FA, TG and PL in isolated CEC, CFLC and CMC (panel A) and the content of AA in the FA, TG and PL fractions of these cell types (panel B). The ratio's of the total content of PL and TG and of the amounts of AA in these lipid pools are given as well.

<i>A) content of fatty acyl moieties in FA, TG and PL</i>							
cell type	FA	n	TG	n	PL	n	PL/TG
CEC	7.3 $\pm$ 3.0	7	12.7 $\pm$ 6.9	12	313 $\pm$ 65	11	30.7
CFLC	4.0 $\pm$ 0.6 ^b	5	10.2 $\pm$ 5.5	9	274 $\pm$ 87	10	31.7
CMC	2.6 $\pm$ 1.2 ^b	5	55.3 $\pm$ 25.8 ^a	9	350 $\pm$ 102	7	8.2 ^a
<i>B) total amount of AA in FA, TG and PL</i>							
cell type	FA	n	TG	n	PL	n	PL/TG
CEC	0.10 $\pm$ 0.01	2	0.55 $\pm$ 0.37	4	28.7 $\pm$ 19.4	11	83.9
CFLC	0.15 $\pm$ 0.04	2	0.31 $\pm$ 0.12	5	29.5 $\pm$ 15.8	8	98.4
CMC	0.18 $\pm$ 0.09	5	2.27 $\pm$ 0.53 ^a	8	63.5 $\pm$ 23.4 ^a	7	28.2 ^a

CEC, CFLC and CMC refer to cardiac endothelial cells, fibroblast-like cells and cardiomyocytes; AA to arachidonic acid; FA to fatty acids; TG to triacylglycerols and PL to phospholipids. Data are depicted as means $\pm$ SD and are expressed as nmol fatty acyl moieties per mg cellular protein; n refers to number of experiments. Differences between groups were tested with the nonparametric Mann-Whitney U test, p values <0.05 were considered to be statistically significant; a) significant different from the corresponding values in CEC and CFLC; b) significant different from the corresponding value in CEC.

In general, phospholipids are the preferred pool of deposition of labeled AA in CEC and CFLC when the extracellular concentration of AA is low. Apparently at higher concentrations of AA the incorporation into the phospholipid pool of the cells gets saturated and the esterification of this fatty acid into the well-known storage form of cellular fatty acids, the triacylglycerol pool is enhanced. It should be noted that the high phospholipid/triacylglycerol ratio of [^3H]-AA incorporated corresponds with the relative abundance of unlabeled AA in the two lipid classes (table 6.3). A marked difference was observed between CMC on the one hand and CEC and CFLC on the other. At AA concentration of $1.0\ \mu\text{M}$ and lower, in CMC the quantities of labeled AA sequestered in the phospholipid and triacylglycerol pools were comparable. At high AA concentration, the triacylglycerol pool was found to be the preferred site of incorporation. This is in agreement with the findings of Hohl and Rösen (5), who also demonstrated that in adult cardiomyocytes with increasing concentrations of exogenous AA, a shift occurs in cellular storage of extracted AA from phospholipids to triacylglycerols. The relatively high rate of sequestration of [^3H]-AA in the cardiomyocytic triacylglycerol pool can partly be explained by the fact that the total amount of triacylglycerol is about 5 times higher in CMC than in CEC or CFLC [the percentage of (unlabeled) AA in cardiomyocytic, CEC and CFLC triacylglycerols was approximately 3-4%]. However, it is noteworthy that the incorporation of labeled AA in the CMC triacylglycerol pool is appreciably higher than could be expected on the basis of relative abundance of unlabeled AA in phospholipids and triacylglycerols, as the phospholipid/triacylglycerol ratio of [^3H]-AA is on the order of 1 and the ratio for unlabeled AA is about 28. This difference most likely indicates that the cardiomyocytic triacylglycerol turnover is operating at a higher rate than the deacylation-reacylation cycle of membrane phospholipids. In contrast to the findings in adult cardiomyocytes, in cultured neonatal cardiomyocytes the deposition of labeled AA is most marked in the cellular phospholipid pool (10).

The relatively high rate of incorporation of labeled AA in the cellular triacylglycerol pool in adult cardiomyocytes should be taken into account when release of labeled AA from endogenous lipid pools are interpreted in terms of membrane damage evoked by insults like ischemia and reperfusion or energy depletion.

The measured FA content of the isolated CMC, the cell type that in situ comprehends more than 75% of the cardiac volume, amounted to $2.6\ \text{nmol/mg}$ cellular protein. This value is in agreement with findings of Myrmel and coworkers (12) and is on the average ten times higher than the FA content as determined in isolated working intact hearts ($150\ \text{nmol FA/g}$ dry weight, ref. 3, equivalent to $0.2\ \text{nmol FA/mg}$ tissue protein). A possible explanation for this striking difference could be the mechanical inactive state in which the isolated CMC in culture are, compared to the working condition of isolated hearts. The rise of the FA content in isolated CMC is most likely due to the culture medium that contains fetal calf serum in which FA are present. As a consequence of the

stationary condition, isolated CMC have lower metabolic needs and have to store the surplus of FA. This may also explain the two times higher content of the storage pool of FA, the TG, in isolated CMC compared to the size of the TG fraction of isolated working hearts (42 nmol versus 12-28 nmol TG/mg tissue protein, ref. 3).

6.5 SUMMARY

The incorporation of radiolabeled arachidonic acid ($[^3\text{H}]\text{-AA}$) in normoxic CMC, CEC and CFLC isolated from adult rat heart was studied. Deposition of $[^3\text{H}]\text{-AA}$ in the cellular lipid pool was assessed with biochemical and autoradiographic techniques.

Extraction and subsequent analysis of lipids from the three different cell types revealed that CMC contained significantly more triacylglycerols than CEC and CFLC. The proportion of (unlabeled) AA was also higher in triacylglycerols in CMC than in CEC and CFLC. The quantity of phospholipids did not differ among the three cell types studied. However, the content of (unlabeled) AA in the CMC phospholipid pool was twice as high as in CEC and CFLC.

The amount of $[^3\text{H}]\text{-AA}$ incorporated in the cellular lipid pool of CMC, CEC and CFLC depended on the concentration of AA in the incubation medium and the incubation time. In CEC and CFLC incorporation of $[^3\text{H}]\text{-AA}$ was highest in the cellular phospholipid pool (0.01 μM AA, 30 min incubation). With increased concentration of AA and longer incubation times, the cellular triacylglycerol pool became more important as site of $[^3\text{H}]\text{-AA}$ incorporation. In CMC, comparable amounts of $[^3\text{H}]\text{-AA}$ were incorporated in the cellular triacylglycerol and phospholipid pools (0.01 and 1 μM AA). At higher AA concentrations (10 μM) the triacylglycerol pool was the preferred site of $[^3\text{H}]\text{-AA}$ deposition.

Autoradiographic analysis at light and electron microscopic level revealed that the extra-nuclear space was readily stained when the three cell types were incubated with $[^3\text{H}]\text{-AA}$. These findings indicate that all cellular lipid pools and membranes are most likely site of deposition of radiolabeled arachidonic acid.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION AND CONCLUSIONS

The present study was performed to investigate aspects of fatty acid metabolism in cells isolated from cardiac tissue that are thought to play an important role in overall cardiac fatty acid homeostasis.

The use of isolated and/or cultivated cells, offers a possibility to identify the metabolic properties of a certain cell type in a complex organ like the heart. It cannot be neglected, however, that the application of an *in vitro* cell model has the disadvantage that a rather non-physiological situation is created, by disconnecting the cells from their environment and thus their endocrine, neural and other physiological regulation mechanisms. In order to perform experiments with isolated cardiac cells we developed non-contaminated cultures of cardiomyocytes (CMC), cardiac endothelial cells (CEC) and fibroblast-like cells (CFLC) from adult rat hearts. In culture more than 95% of CMC were rod shaped and attached to dishes; no other cell types adhered to the plates. CEC and CFLC were subcultivated to increase the number of cells, since appreciable amounts of material are needed for biochemical/physiological studies. The CEC were pure and positively characterized by endothelial markers, CFLC were identified as fibroblasts in their morphology and expression of intermediate filaments. Two stable cell lines were obtained from immortalized CEC cultures; the RHEC-50 and RHEC-116. The advantages of using cell lines is their constant behavior in growth and the possibility to generate appreciable amounts of cellular material.

Fatty acid homeostasis is characterized by a multitude of transport routes and metabolic conversions (12). One feature is myocardial fatty acid oxidation. The main locus of myocardial fatty acid oxidation is the cardiomyocyte, as indicated by the present findings. CMC contribute by about 98% to total cardiac fatty acid oxidation capacity. The contribution of other cardiac cells to this oxidation capacity is very limited which supports earlier observations (11). On the basis of the specific activity of the mitochondrial marker enzyme cytochrome C oxidase it can be concluded that the content of mitochondria is considerably less in CEC and CFLC than in CMC. The capacity to oxidize fatty acids is closely related to the activity of cytochrome C oxidase and, hence, the mitochondrial density of the cells under investigation. This conclusion is based on the findings that the palmitate oxidation rate/cytochrome C oxidase activity ratio in the three cell types studied is mutually not significantly different.

The transport of fatty acids from the vascular compartment is thought to be mediated by albumin in the vascular and interstitial space and by cytoplasmic fatty acid binding proteins (FABP_c) in the CMC. There is a quantitative relation between fatty acid oxidation capacity and cytoplasmic capacity to bind fatty acids (2), suggesting a close functional relationship between protein-mediated fatty acid intracellular transport, most likely by a specific FABP_c, and mitochondrial fatty acid oxidation. This is in agreement with the finding that in isolated CMC a relatively high palmitate oxidation capacity is observed, accompanied by a relatively high content of the heart type, H-FABP_c. However, in CEC and CFLC,

the ratio H-FABP_C over palmitate oxidation was found to be significantly lower than in CMC. The very low H-FABP_C levels in CEC suggest that in these cells other mechanisms are involved in the transport of fatty acids. For instance, the relative short distances between luminal and abluminal membrane probably allows transendothelial transport to occur by passive diffusion. However the presence of another, non-cardiomyocyte specific FABP, undetectable with the present techniques, has to be considered.

A physiologically important fatty acid is arachidonic acid (AA). It can be enzymatically converted to eicosanoids, compounds with specific biological activity. Cardiac tissue has the ability to produce a variety of eicosanoids from endogenous as well as exogenous AA. The capacity to synthesize eicosanoids by the various cardiac cell types was investigated on isolated cells. The findings strongly suggest that endothelial cells are the main site of cardiac eicosanoid production, although fibroblast-like cells are also capable of producing these biologically active substances in appreciable amounts. The production of eicosanoids (expressed on mg cellular protein) was very low in isolated CMC. The Ca²⁺ ionophore A23187 had a strong stimulating effect on eicosanoid production. The effect of more physiological agents, such as bradykinin and histamine, was less profound and depended on the cell type studied. It should be noted that the pattern of individual eicosanoids differed from cell type to cell type but also from the pattern observed in intact rat heart, perfused under *ex vivo* conditions. Moreover, the amount of eicosanoids produced by isolated cells under stimulating circumstances surpassed substantially the total amount of AA metabolites produced in the intact rat heart. The stable product of prostacyclin, 6-keto-PGF_{1α}, was found to be the main product in isolated hearts, whereas HHT, PGE₂, TxB₂, 15-HETE, 11-HETE, PGF_{2α} and to a lesser extent 6-keto-PGF_{1α}, were the main products in isolated (and subcultivated) cells. Interestingly, the amount of 6-keto-PGF_{1α} produced by stimulated CMC was found to be on the same order of magnitude as the amount produced by reperfused isolated rat heart. This finding might suggest that also cardiomyocytes play a role in overall eicosanoid production in the intact heart. However, earlier studies of Hsue and colleagues (5) have convincingly shown that endothelial cells *in situ* play an important role in cardiac prostaglandin formation. Hence, it may be concluded that the contribution of cardiomyocytes to the release of eicosanoids in fully stimulating conditions is limited, although *in situ* cardiomyocytes may contribute to the overall synthesis of eicosanoids in the heart since the production in intact hearts is very low compared to the release of these compounds from isolated cells from cardiac origin.

Because of the different patterns of eicosanoids released by isolated cells, compared to intact hearts, it cannot be excluded that isolation and subcultivation of cardiac cells alter the activity of the various enzymes involved in the production of AA metabolites. In this respect the present finding, that addition of cell growth factors and heparin alters the prostacyclin production, is worth to mention. Moreover, other investigators have reported that variations in the rate

of population doubling (6) and differences in cell density (3, 8) affect the amount of prostacyclin produced *in vitro*. Also the nature of the stimulus may also influence the most favored route in cellular eicosanoid synthesis (1, 10). The present results suggest that both CEC and CMC have a basal cellular eicosanoid level and that the amount of eicosanoids produced after the addition of the stimulating agent e.g. A23187 is quantitatively released into the surrounding medium. The inability of maximally stimulated isolated CMC, CEC and CFLC to produce leukotrienes strongly suggests that other cell types are responsible for the production of these substances *in vivo*. Mast cells commonly present in cardiac tissue (7) are likely candidates for the site of synthesis of leukotrienes in the intact rat heart.

Normally, in cardiac tissue AA is mostly present in membrane phospholipids. The features of incorporation of exogenous arachidonic acid in the lipid pools of various cardiac cell types was biochemically followed by [^3H]-AA incorporation in the cellular lipid pools of isolated CEC, CFLC and CMC. The label was mainly incorporated in the cellular phospholipid and triacylglycerol pool. There was a difference between CEC and CFLC on the one hand, and CMC on the other, in the relative distribution of the labeled fatty acid. In CEC and CFLC at low AA concentrations, the phospholipids were the preferred site for AA incorporation; at higher AA concentration an equal distribution of labeled AA in both pools was observed. In general the CMC phospholipid pool was earlier saturated, resulting in a more pronounced labeling of the triacylglycerol fraction. Especially at higher concentrations of AA the triacylglycerol pool was the preferred site of incorporation in CMC. This is in agreement with findings of Hohl and Rösen (4), but different from reports on neonatal CMC (9). The relatively high rate of incorporation of labeled AA in the cellular triacylglycerol pool of CMC may have consequences for the interpretation of the release of AA from endogenous lipid pools as indicator of membrane damage during pathophysiological processes. Autoradiographical studies at the electron microscopic level showed that exogenous radiolabeled AA is predominantly incorporated in cellular membranes, including mitochondrial and nuclear membranes. These results confirm the biochemical findings that AA is incorporated in both phospholipids and triacylglycerols.

In conclusion, the results presented indicate that the use of an *in vitro* cell system offers the possibility to gain more insight into cardiac fatty acid homeostasis. The cell types studied behave differently for the several investigated aspects; the CMC play a more prominent role in the myocardial palmitate oxidation, whereas the CEC and the CFLC are the more capital producers of AA metabolites. Also for uptake and incorporation in the cellular lipid pool the CMC clearly diverge from CEC and CFLC. This isolated cell model may be a helpful tool for other studies; especially for those that require a large quantity of cellular material, the use of the cell lines can be useful. However for proper interpretation of the results, the findings in the models always have to be related to the more physiological *in vivo* situation.

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SUMMARY

In the present study a variety of aspects of fatty acid metabolism are investigated in cells isolated from cardiac tissue. First a reliable technique to isolate, subcultivate and characterize cardiomyocytes (CMC), cardiac endothelial cells (CEC) and cardiac fibroblasts (CFLC) from adult rat hearts is described. In chapter 3 continuous CEC lines obtained from CEC cultures are reported. These cell lines have shown to possess the same characteristics (presence of VWF, uptake of DiI-Ac-LDL, staining of RECA-1 and vimentin, and upregulation of membrane determinants after stimulation with IFN- γ) as primary cultures of freshly isolated CEC and endothelial cells *in situ*. These characteristics were preserved for at least more than 100 passages. Moreover, the cell lines retain a constant behavior in growth and yield a large number of cells.

Heart tissue contains an appreciable amount of cytosolic fatty acid binding protein (H-FABP_c), that is thought to play a crucial role in the transport of fatty acids from the cellular membrane to the intracellular site of oxidation. Besides, in case of endothelial cells fatty acid binding proteins are supposed to be involved in the transfer of fatty acids from the vascular to the interstitial compartment through the endothelial cytoplasm. In chapter 4 the content of H-FABP_c in various cell types, isolated from adult rat heart, and the quantitative relationship between the capacity of different cell types in the heart to oxidize fatty acids and the presence of H-FABP_c is investigated. In CEC the palmitate oxidation was low compared to CMC (0.09 and 2.31 nmol/mg tissue protein, respectively). Palmitate oxidation rates were closely related to the cytochrome C oxidase activity and, hence, to the mitochondrial density in the cells under investigation. In CMC the content of H-FABP_c was about 4.5 μ g/mg tissue protein. In contrast, in CEC and CFLC the H-FABP_c content was 0.008 and 0.003 μ g/mg tissue protein, respectively, corresponding to at maximum 0.2% of the H-FABP_c content of CMC. The ratio H-FABP_c/palmitate oxidation in CEC was found to be appreciably lower than in CMC. These findings indicate a marked difference between CMC and non-myocytal cells in the heart regarding their capacity to oxidize fatty acids, and a disproportion between the fatty acid oxidation capacity and the cellular content of immunochemically determined H-FABP, suggesting that other mechanisms than involvement of H-FABP_c are responsible for the transport of fatty acids in endothelial cells.

The capacity of various cell types, isolated from adult rat heart, to synthesize eicosanoids is described in chapter 5. Cells were routinely incubated in the presence of the Ca²⁺ ionophore A23187 (10 μ M) and exogenous arachidonic acid (AA, 80 μ M). The release of total eicosanoids in the incubation medium amounted to 23.3 $\pm$ 4.5 and 2.0 $\pm$ 0.4 nmol/mg cellular protein per 30 min in subcultivated CEC and CFLC, respectively. The release in isolated CMC remained below the detection limit of the HPLC assay technique applied. With the more sensitive RIA technique CMC were found to release 2 pmol/mg protein per 30 min 6-keto-PGF_{1 α} , the stable degradation product of prostacyclin. PGE₂,

PGF_{2α}, 12-HHT, 11- and 15-HETE and TxB₂ were the main eicosanoids released by endothelial cells. 6-Keto-PGF_{1α} contributed relatively little to the total amount of eicosanoids formed by CEC (120 pmol/mg protein per 30 min). CFLC released predominantly PGE₂ and 6-keto-PGF_{1α} and to a lesser extent 12-HHT and 15-HETE. Neither CEC nor CFLC released leukotrienes. Because CEC were the main eicosanoid producing cells in vitro, the effect of the extracellular stimulants AA and A23187 was studied in more detail in this cell type. So the influence of the AA concentration and the duration of the incubation was followed in CEC. The effect of the physiologically relevant stimuli histamine and bradykinin was studied in both CEC and CMC in comparison with A23187. Histamine and bradykinin did not significantly increase the release of 6-keto-PGF_{1α} or PGE₂ in CMC. Only histamine gave rise to a slight, but significant higher release of 6-keto-PGF_{1α} in CEC. The cellular content of 6-keto-PGF_{1α} and PGE₂ was not influenced by addition of bradykinin, histamine or A23187 to the incubation medium. In cultured cells the amount of eicosanoids released after stimulation was many times more compared to the amount released by stimulated intact hearts.

In chapter 6 the incorporation of radiolabeled arachidonic acid ([³H]-AA) in normoxic CMC, CEC and CFLC isolated from adult rat heart is summarized. Deposition of [³H]-AA in the cellular lipid pool was assessed with biochemical and autoradiographic techniques. Extraction and subsequent analysis of lipids from the three different cell types revealed that CMC contained significantly more triacylglycerols than CEC and CFLC. The proportion of (unlabeled) AA was also higher in triacylglycerols in CMC than in CEC and CFLC. The quantity of phospholipids did not differ among the three cell types studied. However, the content of (unlabeled) AA in the CMC phospholipid pool was twice as high as in CEC and CFLC. The amount of [³H]-AA incorporated in the cellular lipid pool of CMC, CEC and CFLC depended on the concentration of AA in the incubation medium and the incubation time. In CEC and CFLC incorporation of [³H]-AA was highest in the cellular phospholipid pool. With increasing concentrations of AA and longer incubation times, the cellular triacylglycerol pool became more important as site of [³H]-AA incorporation. In CMC, comparable amounts of [³H]-AA were incorporated in the cellular triacylglycerol and phospholipid pools. At higher AA concentrations (10 μM) the triacylglycerol pool was the preferred site of [³H]-AA deposition. Autoradiographic analysis at light and electron microscopic level revealed that the extra-nuclear space was readily stained when the three cell types were incubated with [³H]-AA. These findings indicate that all cellular lipid pools and membranes are the most likely site of deposition of radiolabeled arachidonic acid.

SAMENVATTING

In deze studie zijn bepaalde aspecten van het vetzuurmetabolisme onderzocht in cellen geïsoleerd uit volwassen rattehartweefsel. Allereerst is een techniek beschreven om cardiomyocyten (CMC), cardiale endotheelcellen (CEC) en fibroblasten (CFLC) uit volwassen ratteharten te isoleren, in kweek te brengen en te karakteriseren. Continue cellijnen, ontstaan uit CEC culturen, zijn in hoofdstuk 3 beschreven. De cellijnen blijken dezelfde endotheliale karakteristieken te bezitten als primaire culturen van CEC en endotheelcellen in situ. Te weten: aanwezigheid van Von Willebrand factor en vimentine, en kleuring tegen een specifiek rat endotheelcel antigeen. Voorts hebben de cellen uit deze lijnen het vermogen om Dil-acetylated low density lipoproteïne op te nemen, en vertonen zij een toename in expressie van membraan determinanten na stimulatie met interferon- γ . Deze endotheliale kenmerken werden voor meer dan honderd passages behouden. Een voordeel van deze cellijnen is dat ze een constant groei gedrag hebben en dat ze grote hoeveelheden cellen kunnen opbrengen.

In hartweefsel is een aanzienlijke hoeveelheid cytosolisch vetzuurbindend eiwit (H-FABP_c) aangetoond. Aan dit eiwit wordt een belangrijke rol toegeschreven bij het transport van vetzuren in cardiomyocyten, van de celmembraan naar de intracellulaire plaats van oxidatie, de mitochondrieën, en in endotheelcellen, bij de overdracht van vetzuren van de vasculaire naar de interstitiële ruimte via het cytoplasma van de endotheelcel. In hoofdstuk 4 is het gehalte aan H-FABP_c in de verschillende celtypen, geïsoleerd uit ratteharten, en de quantitative relatie tussen capaciteit van de verschillende cellen om vetzuren te oxideren en de aanwezigheid van H-FABP_c beschreven. In CEC was de palmitaat oxidatie laag vergeleken met die in CMC (respectievelijk 0.09 en 2.31 nmol/mg weefsel eiwit). De palmitaat oxidatie waarden blijken goed te correleren met de activiteit van het cytochroom C oxydase, en dus met de mitochondrieële dichtheid van de onderzochte cellen. In CMC was het gehalte aan H-FABP_c ongeveer 4.5 μ g/mg cellulair eiwit. In CEC and CFLC echter was het H-FABP_c gehalte respectievelijk maar 0.008 en 0.003 μ g/mg cellulair eiwit, hetgeen overeenkomt met maximaal 0.2% van de hoeveelheid H-FABP_c aanwezig in CMC. Dit wijst op een aanzienlijk verschil tussen CMC en niet-myocyten in capaciteit om vetzuren te oxideren en een disproportie tussen de vetzuuroxidatie capaciteit en immunochemisch bepaalde gehalte aan H-FABP_c. Bij het vetzuur transport in endotheelcellen lijken dan ook andere mechanismen betrokken te zijn dan transport via H-FABP_c.

Voor het hart is arachidonzuur (AA) een belangrijk vetzuur. AA is een bouwsteen van membranen en kan omgezet worden tot eicosanoiden, stoffen met verschillende fysiologische effecten. De capaciteit van de verschillende celtypen om eicosanoiden te synthetiseren is bestudeerd in hoofdstuk 5. De cellen werden hiertoe routinematig geïncubeerd in de aanwezigheid van Ca^{2+} -ionofoor A23187 (10 μ M) en exogeen AA (80 μ M). De totale afgifte van eicosanoiden aan het medium bedroeg 23.3 ± 4.5 en 2.0 ± 0.4 nmol/mg cellulair eiwit per 30 min in gekweekte CEC en CFLC respectievelijk. In geïsoleerde CMC kon de hoeveelheid

afgegeven eicosanoiden niet gedetecteerd worden met de HPLC techniek. Werd echter met de gevoelige RIA techniek het gehalte aan 6-keto-PGF_{1α}, het stabiele degradatie produkt van prostacycline, gemeten, dan bleek dit 2 pmol/mg eiwit per 30 min te zijn. PGE₂, PGF_{2α}, 12-HHT, 11- en 15-HETE en TxB₂ waren de belangrijkste eicosanoiden gevormd door de endotheelcellen. 6-Keto-PGF_{1α} droeg relatief weinig bij aan aan de totale hoeveelheid eicosanoiden door CEC geproduceerd. CFLC produceerden voornamelijk PGE₂ en 6-keto-PGF_{1α} en in mindere mate 12-HHT en 15-HETE. Noch bij CEC, noch bij CFLC kon een meetbare productie van leukotrienes aangetoond worden. Omdat CEC de voornaamste eicosanoïd producerende cellen in vitro zijn, is het effect van de extracellulaire stimuli AA en A23187 uitgebreid bestudeerd in dit cell type. Zo werden variaties in de incubatieduur en in AA concentratie bekeken. Het effect van de meer fysiologische stimuli histamine en bradykinine is onderzocht in zowel CEC als CMC en vergeleken met A23187 stimulatie. Histamine en bradykinine veroorzaakten geen significante toename in 6-keto-PGF_{1α} en PGE₂ afgifte in CMC. Alleen in CEC gaf histamine een kleine, maar significante, verhoging in de productie van 6-keto-PGF_{1α} te zien. Het cellulaire gehalte van beide celtypen aan 6-keto-PGF_{1α} en PGE₂ werd niet beïnvloed door toevoeging van bradykinine, histamine of A23187 aan het incubatiemedium. De hoeveelheid eicosanoiden in vitro geproduceerd na maximale stimulatie overtreft die van gestimuleerde intacte harten vele malen.

De inbouw van radioactief gemerkt arachidonzuur ([³H]-AA) en de hoeveelheid AA aanwezig in de verschillende lipide pools in normoxische CMC, CEC en CFLC geïsoleerd uit volwassen ratteharten is beschreven in hoofdstuk 6. De meting van de hoeveelheid ingebouwde [³H]-AA in de cellulaire lipide pools is uitgevoerd met biochemische en autoradiografische technieken. Extractie en analyse van de lipiden van de drie verschillende celtypen liet zien dat CMC significant meer triacylglycerolen bevatten dan CEC en CFLC. De grootte van de fosfolipide pools (uitgedrukt per mg cellulair eiwit) verschilde niet in de bestudeerde celtypen. Echter, het gehalte aan niet-radioactief gemerkt AA in de CMC fosfolipide pool was twee keer zo hoog als in de CEC en CFLC. De hoeveelheid ingebouwde [³H]-AA in de cellulaire lipide pool hing af van de concentratie van AA in het incubatiemedium en van de incubatieduur. In CEC en CFLC was de inbouw van [³H]-AA het hoogste in de cellulaire fosfolipide pool. Met toenemende concentratie van AA en langere incubatietijden werd de triacylglycerol pool belangrijker voor inbouw van [³H]-AA. In CMC werden vergelijkbare hoeveelheden [³H]-AA in de cellulaire triacylglycerol en fosfolipide pool ingebouwd. Bij hogere concentraties AA (10 μM) was de triacylglycerol pool de voorkeursplaats voor de inbouw van radioactief gemerkt AA. Autoradiografische analyse op licht- en elektronenmicroscopisch niveau liet zien dat de extra-nucleaire ruimte van de drie bestudeerde celtypen de plaats is waar na incubatie met [³H]-AA en blootstelling aan fotografische emulsie zwarte korrels worden gevormd. Deze bevindingen wijzen in de richting van een inbouwen van het radioactief gemerkte AA in alle cellulaire lipide pools en membranen.

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