Mechanisms for and consequences of cellular lipid accumulation – The role of the Very Low Density Lipoprotein (VLDL) receptor

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Till Anders och Junior ♥

POPULÄRVETENSKAPLIG SAMMANFATTNING

I denna avhandling har mekanismer och konsekvenser av fettansamling i olika vävnader studerats.

Fett används, tillsammans med bl.a. socker, av kroppen som bränsle för att framställa energi. De enda cellerna i kroppen som är specialiserade på att förvara fett är fettcellerna men av olika anledningar kan andra celler börja lagra fett. Anledningar kan vara höga nivåer av fett i blodet som t.ex. vid övervikt eller om cellen slutar att använda fett som bränsle. De flesta celler har en förmåga att förvara en liten mängd fett, men om för mycket fett lagras i andra celler än fettceller kan detta göra att de slutar fungera och dör.

Delarbete I: I detta arbete har fettinlagring i hjärtat efter en hjärtinfarkt undersökts. En hjärtinfarkt uppkommer när tillförseln av blod till en del av hjärtat blir försämrad eller helt stoppad vilket försämrar leveransen av syre och näring till hjärtat. Försämrad blodtillförsel till hjärtat kan orsakas av en förträngning av eller en propp i de kärl som förser hjärtat med blod. Försämrad näringstillförsel medför att hjärtat inte kan framställa energi som det skall vilket gör att hjärtat inte klarar av att pumpa blod till resten av kroppen på ett effektivt sätt. Syrebrist i hjärtat, exempelvis vid en hjärtinfarkt, ökar produktionen av ett protein på hjärtmuskelcellens yta. Proteinet (VLDLr) har bl.a. funktionen att ta upp fett ur blodet in i cellen. Konsekvensen av ökningen av VLDLr blir att hjärtat börjar lagra upp fett. Det fett som lagras upp är skadligt för hjärtat och kan orsaka att hjärtcellerna dör. Om VLDLr tas bort (kan göras i möss) eller blockeras så minskar mängden fett i hjärtat efter infarkten. Vidare minskar infarktstorleken och antalet döda celler, slutligen ökar överlevnaden. Resultaten visar också att människor med dåligt syresatt hjärta har mer VLDLr och mer fett i sina hjärtan jämfört med friska människor.

Delarbete II: I första delarbetet visades att VLDLr ökar vid lågt syretryck och vilka konsekvenser detta har för hjärtat. I detta arbete visas hur cellen styr det ökade uttrycket av VLDLr. Resultaten visar att mängden av ett styrprotein (Hif-1α) ökar under syrebrist. Hif-1α binder specifikt till en viss del av VLDLr genen vilket styr hur mycket VLDLr som cellen producerar. Samma specifika del av VLDLr genen finns i både mus- och människohjärta och båda binder Hif-1α.

Delarbete III: Syftet med detta arbete var att titta på hur ökad mängd VLDLr påverkar andra vävnader än hjärtat. I detta arbete studeras en vanlig typ av njurcancer, som kännetecknas *(1)* av mycket fett i cancercellerna och *(2)* av att cellerna har ett fel, en mutation, som gör att de alltid har höga nivåer av styrproteinet Hif-1α. Resultaten visar att den stora mängden Hif-1α ger ökad mängd VLDLr och att om VLDLr tas bort så minskas mängden fett i cancercellerna. Dessa resultat visar att Hif-1α styr VLDLr i fler vävnader än hjärtat och att VLDLr orsakar fettinlagring i fler vävnader än i hjärtat.

Det viktigaste resultatet i denna avhandling är att VLDLr orsakar skadlig fettinlagring i hjärtat vid en hjärtinfarkt. I framtiden skulle ett läkemedel som blockerar VLDLr kunna användas för att öka överlevnaden efter en hjärtinfarkt.

ABSTRACT

Lipid accumulation in non adipose tissue is associated with various cases of tissue dysfunction and tissue failure. Reduced availability of oxygen is known to cause intracellular lipid accumulation in cardiomyocytes as well as in hearts. Cardiac lipid accumulation has been shown to cause impaired cardiac function but it is not fully clear how the lipids accumulate in the hypoxic myocardium.

We have studied a model of hypoxic/ischemic myocardium using HL-1 cardiomyocytes incubated in hypoxic condition as well as an *in vivo* model where mice were subjected to a myocardial infarction causing cardiac ischemia.

We found that the very low density lipoprotein receptor (VLDLr), a member of the low density lipoprotein receptor (LDLr) family suggested to be able to mediate uptake of lipids, was significantly upregulated in response to hypoxia and that this upregulation was mediated through hypoxic activation of transcription factor Hif-1α. The VLDLr induced an increase in intracellular triglycerides which were mediated not primarily through increased uptake of fatty acids but from an increased uptake of extracellular triglyceride-rich lipoproteins. The uptake of lipoproteins was rapid in response to hypoxia. The increase in intracellular lipids caused an accumulation of cardiotoxic ceramides in the cardiomyocytes which induced myocardial endoplasmatic reticulum (ER) -stress. ER-stress initially induces a cardioprotective response but prolonged ER-stress cause apoptosis which was increased when the VLDLr was expressed. Ablation of the VLDLr reduced the ER-stress. The mice lacking VLDLr expression showed a reduced infarct size which could be dependent on a reduced amount of toxic ceramides and apoptosis.

We could also show that it was possible to block the harmful actions of the VLDLr by using VLDLr specific antibodies. Treatment with these antibodies reduced the lipid accumulation, ER-stress and apoptosis otherwise following a myocardial infarction.

The hypoxic VLDLr expression is not restricted to species or tissue. We could see that the VLDLr was increased in human ischemic myocardium compared to non-ischemic biopsies. We could also see that the VLDLr expression was increased in human clear-cell renal carcinoma where in this case the increased VLDLr expression was not due to hypoxia but on constitutive Hif-1α activation. Like in the myocardium the VLDLr caused an accumulation of intracellular triglyceride in the cancer, which already contained great amounts of cholesterol esters.

These results indicate that the VLDLr is an important mediator of post-ischemic intramyocardial lipid accumulation and that the blocking of this lipid uptake improves survival.

LIST OF ABBREVIATIONS

AMPK	AMP-activated Protein Kinase
ApoB100	Apolipoprotein B100
ApoE	Apolipoprotein E
ATF6	Activating Transcription Factor 6
ChIP	Chromatin Immunoprecipitation
CHOP	CCAAT/enhancer-binding protein homologous protein
DG	Diacylglycerol / Diglyceride
DMOG	Dimethyloxalylglycine
ER	Endoplasmatic Reticulum
ERSE	ER-stress Responsive Element
FA	Fatty Acid
FAT/CD36	Fatty Acid Translocase (CD36)
FATP	Fatty Acid Transport Protein
FCS	Foetal Calf Serum
Hif	Hypoxia Inducible Factor (1, 2, α, β)
HRE	Hypoxia Responsive Element
HSPG	Heparane Sulfate Proteoglycane
IRE 1	Inositol-Requiring Enzyme 1
LAD	Left Anterior Descending Coronary Artery
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LXR	Liver X Receptor
MI	Myocardial Infarction
NF-Y	Nuclear Factor Y
PERK	pancreatic ER-kinase (PKR)-like ER kinase
PPAR	Peroxisome Proliferator-Activated Receptor (α, β, γ, δ)
pVHL	von Hippel–Lindau tumour suppressor protein
RAP	Receptor Associated Protein
RCC	Renal Cell Carcinoma
ROS	Reactive Oxygen Species
siRNA	Small Interference Ribonucleic Acid
Sp1	Specificity Protein 1
SREBP	Sterol Regulatory Element Binding-Protein
TG	Triacylglycerol / Triglyceride
TGL	Triglyceride-rich Lipoproteins
UPR	Unfolded Protein Response
VLDL	Very Low Density Lipoprotein
VLDLr	Very Low Density Lipoprotein Receptor
XBP1	X box-binding protein 1

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their roman numerals:

I

The VLDL receptor promotes lipotoxicity and increases mortality in mice following an acute myocardial infarction

Perman JC, Boström P, Lindbom M, Lidberg U, Ståhlman M, Hägg D, Lindskog H, Scharin Täng M, Omerovic E, Mattsson Hultén L, Jeppsson A, Petursson P, Herlitz J, Olivecrona G, Strickland DK, Ekroos K, Olofsson SO, Borén J.

J Clin Invest. 2011 Jul 1;121(7):2625-40.

II

Hypoxia-induced regulation of the very low density lipoprotein receptor

Jeanna C. Perman, Ulf Lidberg, Ali Moussavi Nik, Peter Carlsson, Sven-Olof Olofsson and Jan Borén

Submitted

III

Increased expression of the very low-density lipoprotein receptor mediates lipid accumulation in clear-cell renal cell carcinoma

Jeanna C. Perman, Marcus Ståhlman, Max Levin, Sven-Olof Olofsson, Martin E. Johansson and Jan Borén

Submitted

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...every day’s a school day...

INTRODUCTION

Lipid Metabolism

Lipids are together with glucose the most important substrates for generating energy for the different tissues in the body. Besides energy generation lipids are important for energy storage, signal transduction and membrane structure.

Lipids are a broad group of molecules which among others include fatty acids, glycerolipids and sphingolipids. Fatty acids (FA) are important building blocks for other lipid classes as well as involved in signal transduction, inflammation and as energy substrate. Glycerolipid are lipids that include a glycerol backbone, most important glycerolipids are the mono- di- and triglycerols (MG, DG and TG) witch all have FAs of different lengths esterified to the glycerol backbone. The most important of the glycerolipids is the TGs functioning as the main storage form of lipids. The glycerolipid group also contain the phospholipids which are the major component of cell membranes forming lipid bilayers. Most phospholipids contain a DG and a phosphate head group, which is hydrophilic making the phospholipid hydrophilic, in contrast to lipids that are hydrophobic. It is the hydrophilic nature of the phospholipid that makes them form membranes. Spingolipids are a complex family of compounds that share a common structural feature, the sphingosine backbone and a FA. A biologically important sphingolipid is ceramide which has been implicated in a variety of cellular functions including apoptosis, cell growth arrest, and differentiation[1, 2].

At some points hydrophobic lipids needs to be transported in the bloodstream, therefore they do so in an organized manner. Such an organized manner is the lipoprotein. Lipoproteins are spherical lipid particles consisting of a hydrophobic core of TG and cholesterol esters surrounded by a monolayer of phospholipids interspersed by unesterified (free) cholesterol (Figure 1). On the surface of the particles a number of structural proteins called apolipoproteins (apo) are embedded. The apolipoproteins are of varying size and are divided into different groups such as apoAs, apoB, apoCs and apoEs with different functions. There are five different main classes of lipoproteins divided by their density, the chylomicrons, the very low density lipoproteins (VLDL), the intermediate density lipoproteins (IDL), the low density lipoproteins (LDL) and the high density lipoproteins (HDL) all with different or slightly different biological functions.

The chylomicrons are the largest lipoproteins, they are TG rich and synthesized by the intestine to carry dietary lipids to peripheral tissues and the liver. VLDL is large triglyceride rich lipoproteins (TGL) secreted by the liver. As VLDL is metabolised in the circulation, the particles are gradually deprived of their TG and accumulation cholesterol resulting in the generation of IDL, and subsequently LDL (Figure 1).

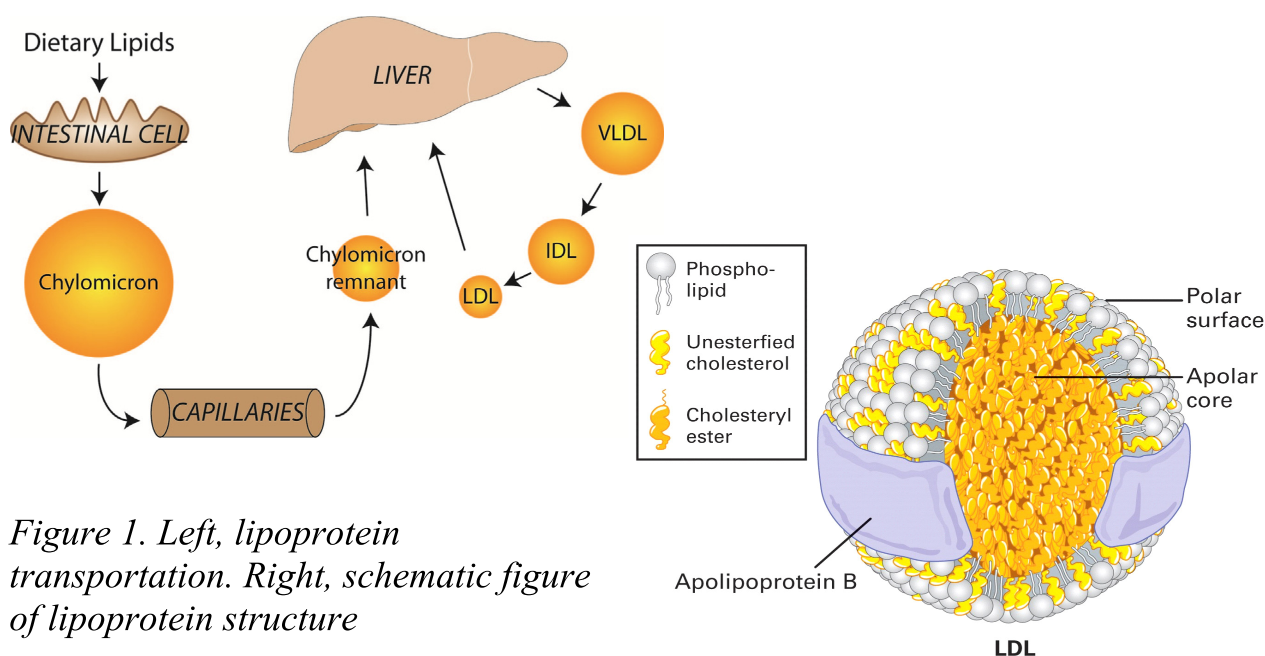

Figure 1. Left, lipoprotein transportation. Right, schematic figure of lipoprotein structure

Cellular Lipid Uptake

Fatty acids (FAs) are together with glucose the most important energy substrates for all tissues. FAs are transported to peripheral tissues either bound to albumin or esterified into glycerol, forming the triacylglycerol core of circulating chylomicrons and VLDL. In the peripheral tissues lipids can be taken up either as full lipoproteins or as fatty acids (FA). The lipids in the core of the lipoproteins are hydrolysed and liberated from the lipoprotein particles by lipoprotein lipase (LPL) and then taken up into the cell by diffusion or by receptor-mediated facilitated diffusion a process involving proteins such as FA translocase (FAT/CD36), FA transport protein (FATP) and plasma membrane bound FA binding protein ($FABP_{pm}$)[3-5]. The uptake of lipoproteins is facilitated by lipoprotein receptors[6].

Receptor-mediated Lipid Endocytosis

Lipoprotein receptors are membrane proteins able to internalize lipoproteins and manage the exchange of lipids on the cell surface[6]. The recognition between the receptors and the lipoproteins occurs via the apos on the lipoprotein surface. Apart from uptake of lipoproteins these receptors have been described to be involved in several aspects of brain function[7, 8] and to work as transcytotic molecules[9].

Receptor-mediated endocytosis of plasma lipoproteins is a critical step in the metabolism of lipids, there are several lipoprotein receptors but the most well

characterized is the LDL receptor which is known to play a key role in cholesterol homeostasis[6]. The LDL receptor mediates the uptake of plasma lipoproteins that contain apoB100 and/or apoE, and thereby supplies the cell with cholesterol. Examples of other lipoprotein receptors belonging to the LDL receptor family apart from LDLr itself are, the apoE receptor 2 (apoER2), the VLDL receptor (VLDLr) and the LDLr related protein (LRP)[10]. All receptors of the LDL receptor family exhibit similar structural features[10-12].

The endocytosis process of lipoproteins is well studied for the LDLr and was first described by Brown and Goldstein[13]. The LDLr is located in clathrin coated pits on the plasma membrane. When the lipoprotein binds to the receptor the pit is closed and pinched off from the membrane forming clathrin-coated vesicles inside the cell. This process occurs in all nucleated cells. Once the coated vesicle is internalized it will shed its clathrin coat and fuse with late endosomes, the receptors are recycled to the plasma membrane or degraded and the lipid can be stored or used for energy production[14] (Figure 2).

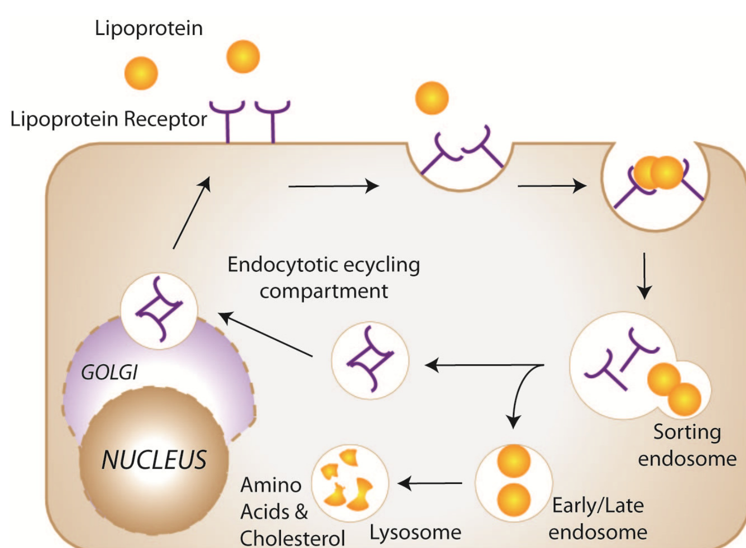

Figure 2. Schematic figure of lipoprotein endocytosis. The receptor is localized in clathrin coated pits, upon lipoprotein binding the complex is internalized into sorting endosomes and the receptors are recycled to the plasma membrane or degraded in lysosomes as the lipoproteins. Adapted from[14].

Uptake of Fatty Acids

The uptake of FA into peripheral tissues can occur either through diffusion or facilitated by plasma membrane bound transporters. For a schematic picture see figure 3. The major players involved in this process will be overview below.

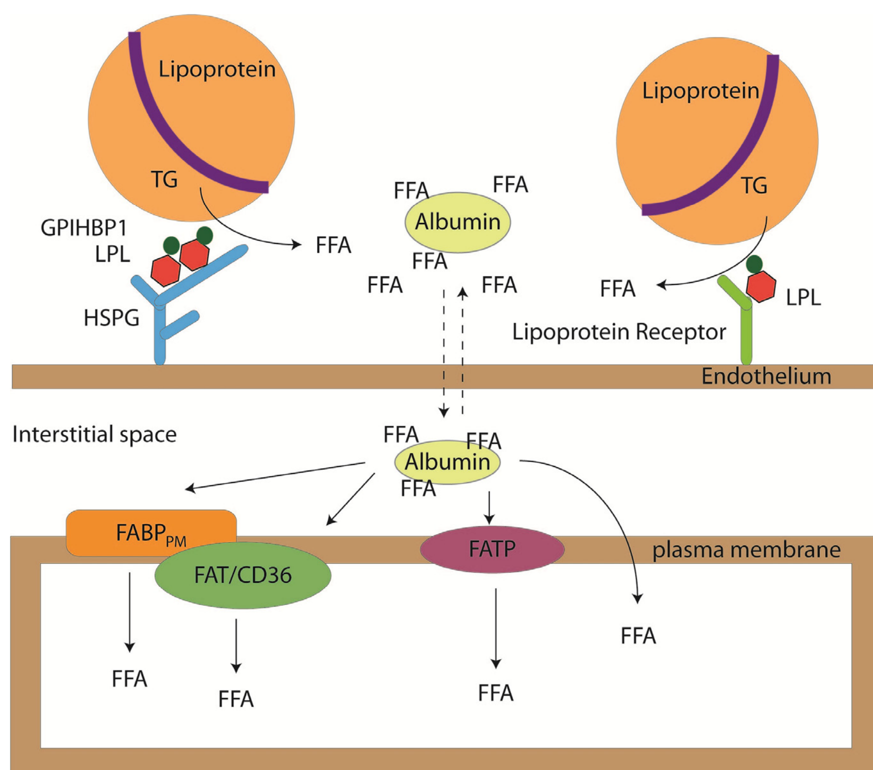

Figure 3. Schematic picture of tissue lipid uptake. Fatty acids are liberated by lipoprotein lipase from circulating lipoproteins. The FAs are then taken up by the cell. FABP$_{pm}$ plasma membrane bound fatty acid binding protein, FAT/CD36 fatty acid translocase, FATP fatty acid transport protein, FFA free fatty acid, GPIHBP1 glycosylphosphatidylinositol (GPI)-anchored high-density lipoprotein-binding protein 1, HSPG heparane sulphate proteoglycane, LPL lipoprotein lipase, TG triglyceride.

The triglycerides residing in the core of the circulating lipoproteins are hydrolysed by LPL which thereby makes the FA available for the tissues. LPL is a member of a gene family also containing hepatic lipase and pancreatic lipase[15]. LPL is present in a circulating and a plasma membrane anchored form, where LPL is anchored to the plasma membrane by interactions with heparane sulphate proteoglycanes (HSPG)[16, 17]. For LPL to be able to lipolytically process the triglyceride-rich lipoproteins (TGL) the endothelial cell protein, glycosylphosphatidylinositol (GPI)-anchored high-density lipoprotein-binding protein 1 (GPIHBP1), is required.

GPIHBP1 is 28kDa glycoprotein located within the lumen of capillaries of heart, skeletal muscle, and adipose tissue with an expression pattern almost identical to LPL[18]. Most of the liberated FAs are then taken up into the cell by transporters such as $FABP_{pm}$, FATP and FAT/CD36.

$FABP_{pm}$ is a 40kDa membrane bound protein that is ubiquitously expressed but is most prominent in heart, skeletal muscle, brain, liver, and kidney[19]. Proof of its FA binding properties came when antibodies raised against $FABP_{pm}$ inhibited FA uptake in hepatocytes, myocytes and adipocytes. Inhibition was never complete and suggested that $FABP_{pm}$ may account for up to 50% of the measured FA uptake rates[19-21].

FATP is a family of six 63 kDa fatty acid transport proteins (FATP1–6) with multiple transmembrane domains. FATP is expressed in most mammalian tissues tested with highest expression levels observed in brain, and tissues active in FA utilization like skeletal muscle, heart, and fat cells. Its high levels in the brain would suggest a role in brain FA metabolism and possibly signalling. Overexpression of FATP in cells is associated with an increase in the uptake of fluorescent FA. FATP is conserved over species, and especially a highly conserved domain related to ATP-binding and hydrolysis which appears essential for FA transport activity, however the mechanism is unclear. FATP is regulated by peroxisome proliferator-activated receptors (PPARs) and by nutrients in adipose tissue[19-21] as well as by vascular endothelial growth factor B in endothelial cells[22].

FAT/CD36 is the more extensively studied fatty acid transporter and is an 88 kDa membrane protein. It was designated FAT during its cloning from rat, the protein was later found to share amino acid homology with human platelet glycoprotein IV, also referred to as CD36, suggesting that these proteins are species homologs. FAT/CD36 is expressed in tissues with a high metabolic capacity for long-chain FA, like adipose tissue, oxidative muscles like soleus, heart and intestines, but is absent in liver, brain, and kidney. Differentiation of preadipocytes into adipocytes showed an induction of FAT which was paralleled with an increase in FA uptake. CD36 is also strongly induced by peroxisome proliferators. In humans, polymorphisms in the CD36 gene may underlie defective myocardial FA uptake and some cases of heart disease[19-21]. CD36 can also function as a scavenger receptor responsible for internalization of oxidized low density lipoproteins (oxLDL), high density lipoproteins (HDL) and very low density lipoproteins (VLDL)[23, 24] as well as a signalling transducer[25].

The lipids that are taken up into the cells can either be used as a source of energy or stored as TG in intracellular lipid droplets.

Role of Lipids in Pathogenesis

The adipocytes are the only cell in the body specialized to store lipids. Lipid accumulation in non adipose tissues is called ectopic lipid accumulation. Ectopic lipid accumulation can occur for instance when the balance between lipid uptake, synthesis and utilization is mismatched. The lipid is accumulated in the cytoplasm as cytoplasmic lipid droplets containing a core of neutral lipid surrounded by a monolayer of polar lipids interspersed by cholesterol and proteins[26].

Excessive ectopic lipid accumulation can lead to cell dysfunction or cell death, a phenomenon known as lipotoxicity. For example ectopic lipid accumulation can cause insulin resistance in muscle and liver as well as functional losses in pancreas and the heart linking obesity with type 2 diabetes and cardiovascular disease[27]. Another form of ectopic lipid accumulation is atherosclerosis where lipids are progressively accumulated within the vascular wall forming thickenings of the innermost layer of the intima. As the atheroma continues to grow the narrowing if the artery will lead to ischemia of the supported organ. Rupture of the atheroma can lead to clot formation and potential occlusion of downstream vessels causing ischemia for example myocardial infarction or stroke[28, 29].

Myocardial Energy Metabolism

Normal Heart Physiology

The heart has a very high energy demand and must continually generate ATP at a high rate to sustain contractile function, basal metabolic processes, and ionic homeostasis. In the normal adult heart primarily rely on FAs for energy production, compared to the foetal heart which almost exclusively relies on glucose as energy source.

The high ATP demand of the heart is sustained by the heart being able to maintain on many different energy sources with the main part, 50-70% coming from FA β-oxidation[30]. The β-oxidation is tightly regulated and depends on the FA supply to the heart, the availability of other substrates (glucose, lactate, ketones, amino acids), the energy demand of the heart, the oxygen supply to the heart, the FA uptake and esterification, as well as mitochondrial transport and function. Since the heart has a very low capacity for lipid storage the uptake and utilization of FAs in the heart is very tightly coupled. Of the entering FAs about 80% is oxidized and the remainder is stored as TG in lipid droplets, however the turnover of the cardiac TG pool in a healthy heart is five hours. Alterations in β-oxidation can have significant energetic and functional consequences on the heart[31].

The uptake of FAs into the heart occurs roughly as described for general lipid uptake, governed primarily by FAT/CD36, FATP1 and 6, $FABP_{pm,}$, FAT/CD36, FATP and cytoplasmic heart-type FABP (Figure 3)[32]. The FAs are transferred from the capillary lumen through the luminal membrane of the endothelial cells either bound to FABP and transferred by FAT/CD36 or FATP or unbound through diffusion, through the endothelial cytoplasm and through the endothelial abluminal membrane. In the interstitial space the fatty acids are transported bound to interstitial albumin to the sarcolemma, the transport across the sarcolemma occurs most likely the same way as the endothelial membranes. In the sarcoplasm the FAs are transferred bound to FABP and then esterified to fatty acyl CoA by fatty acyl CoA synthase (FACS). The fatty acyl CoA can then be esterified to complex lipids such as TG, or the acyl group transferred to carnitine via carnitine palmitoyltransferase (CPT) 1 before it is shuttled into mitochondria for FA β-oxidation (Figure 4)[30, 31].

The knowledge that proteins are involved in the uptake and intracardiac transport of FA have led to research about the possibility that specific proteins are involved in inherited or acquired cardiac myopathies. Apart from supplying energy to the heart, FA may take part in signal transduction as well as post translational modifications of proteins, such as acylation, activation of protein kinases and serve as precursors for eicosanoids[30].

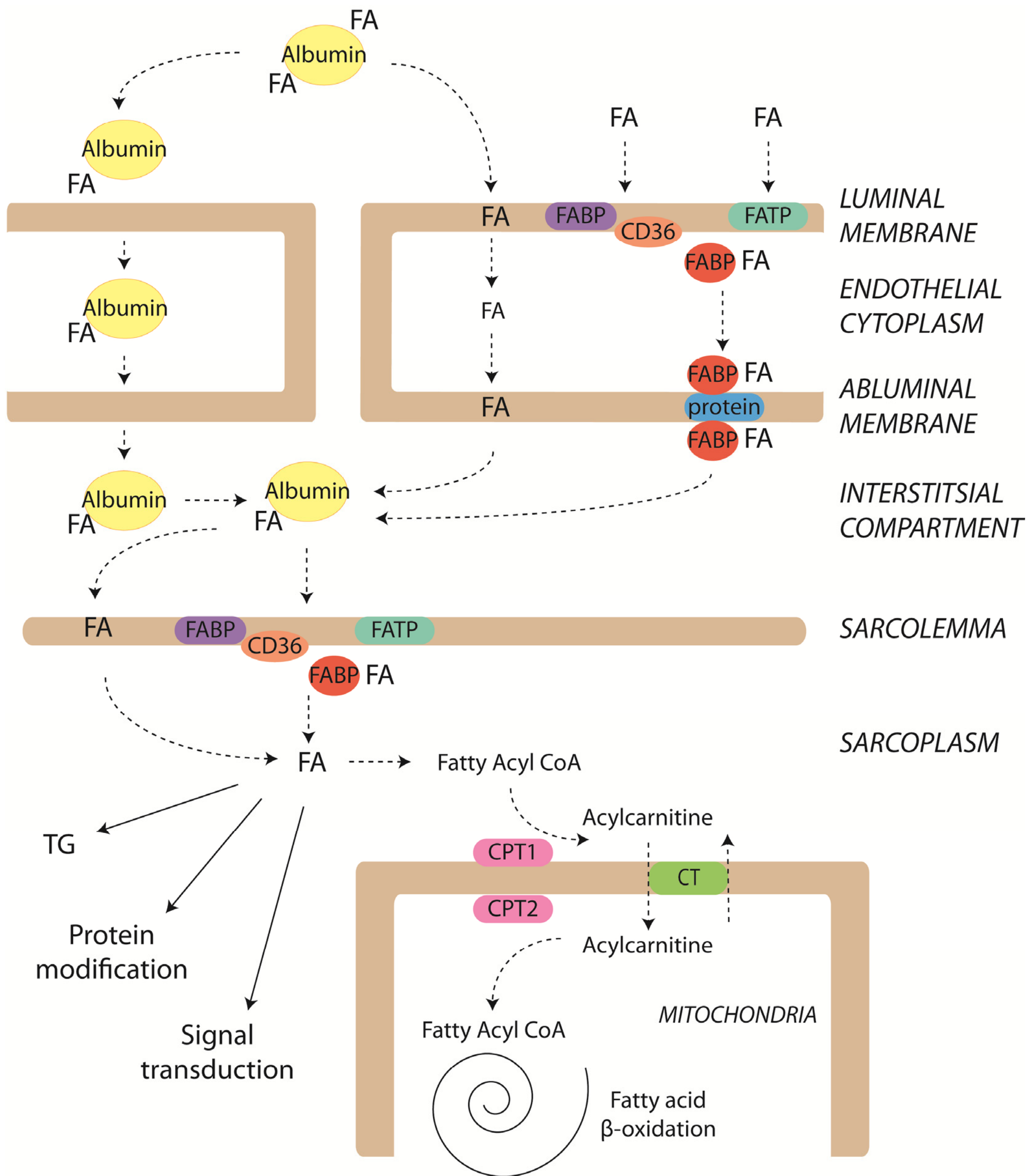

Figure 4. Myocardial uptake of fatty acids adapted from[30, 31, 33]. Various alternative pathways for the transport of FAs into the cell exist. FA can cross the endothelium either free or albumin-complexed or by protein-mediated facilitated diffusion. The protein-mediated transport (far right) is the most important. In the interstitial compartment the FAs are carried by interstitial albumin before they are transported into the cell.

The Failing/Ischemic Heart

Heart failure is not a disease but rather a complex clinical syndrome that is generally defined as an impaired ability of the ventricle to fill with and eject blood. Heart failure is roughly divided into two main categories: *1*) ischemic heart failure (patients with a history of coronary artery disease and/or myocardial infarction), and *2*) nonischemic idiopathic heart failure[31]. The most common cause of heart failure in the western world is coronary artery disease[34].

Myocardial ischemia occurs when coronary blood flow is inadequate, and therefore, the oxygen supply to the myocardium is not sufficient to meet oxygen demand. Ischemia increases the concentration of plasma norepinephrine which elevates the amount of circulating FA by promoting adipose tissue lipolysis and suppresses pancreatic insulin secretion and peripheral insulin sensitivity. An increase in circulating plasma FAs during and after ischemia thus increases the delivery of FA to the myocardium and can alter FA utilization during both the ischemic and postischemic period[31]. During an ischemic attack the metabolic preference of the heart switches to increase the glucose and lactate utilization, the heart goes through a "foetal switch"[35, 36] though the switch is debated[31]. The reason for this is that even though FA generates more ATP it also consumes more oxygen, which is problematic in an oxygen deprived environment. However, even though a switch occurs, β-oxidation still proceeds stimulated by the activation of AMP-activated Protein Kinase (AMPK). AMPK is an energy sensor in muscle and heart that is switched on by cellular stresses that interfere with ATP production (e.g., hypoxia, glucose deprivation, or ischemia)[37].

During low oxygen supply the expression of key enzymes in FA oxidation and glucose metabolism is shifted[38, 39] a schematic picture can be seen in figure 5. Reduced oxygen availability decrease the gene expression of acyl-coenzyme A synthetase (ASC) and carnitine palmitoyltransferase 1a (CPT1a), enzymes required for β-oxidation of FAs[40-42]. CPT1 has been suggested to be the major regulator of β-oxidation since it controls the influx of acyl groups into the mitochondria and to be regulated by PPARα[42]. ASC activates the FAs turning them into CoA esters making them available for CPT transport, ASC is inhibited by its product (FA-CoA)[40]. To increase the anaerobic glucose utilization, hypoxia increases the gene expression of glucose transport protein (GLUT) 1 together with key enzymes in the glucose metabolism and and increased translocation of GLUT1 and 4 to the plasma membrane[38]. For example, phosphofructokinase, which converts glucose to pyruvate and lactate dehydrogenase (LDH) promoting the conversion of pyruvate into lactate are increased. The entry of pyruvat into the TCA cycle is prevented by the increased expression of pyruvate dehydrogenase kinase which inhibits pyruvate dehydrogenase[43, 44]. These metabolic changes promote the ATP production from glucose and lactate while preventing the oxygen-consuming TCA-cycle and β-oxidation.

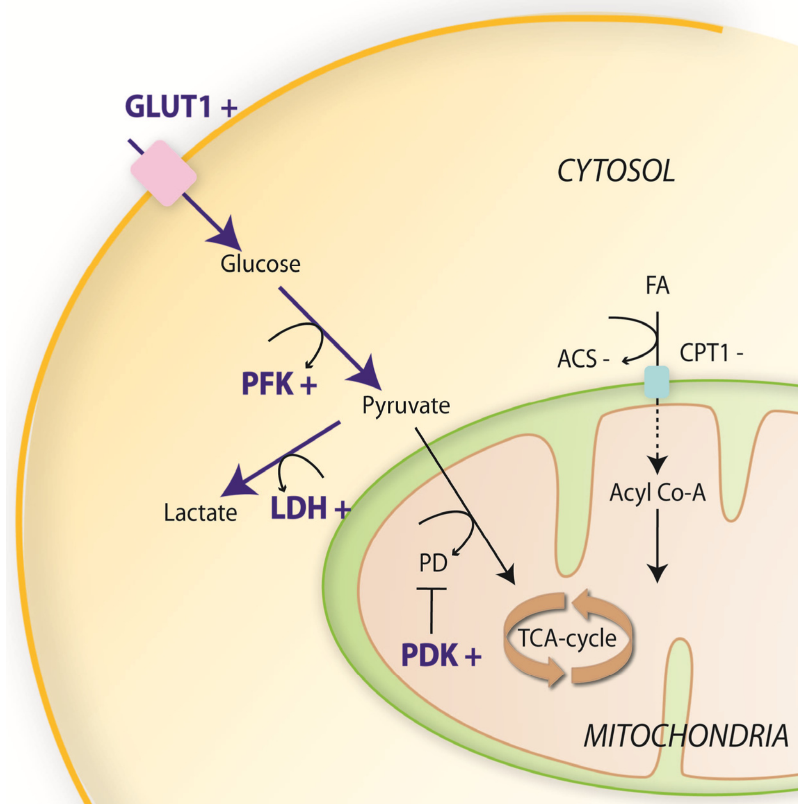

Figure 5. Schematic figure of the change in cardiac metabolism substrate preference during hypoxia ACS, acyl-coenzyme A synthtetase; CPT1, carnitine palmitoyltransferase 1; FA, fatty acid; GLUT1, glucose transporter 1; LDH, lactate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase.

A consequence of the decrease in ATP production is impaired contractile function. This impairment is due to the weakened function of the ion pump sarcoplasmic Ca^{2+}-ATPase. Sarcoplasmic Ca^{2+}-ATPase is responsible for the reuptake of Ca^{2+} following myocyte contraction and impaired function results in Ca^{2+} overload and contractile dysfunction. Many available drugs to ease the detrimental effects of cardiac ischemia aim at reducing β-oxidation in favour of increased glycolysis and reinstituting Ca^{2+} balance[31]. Another feature of ischemia is the accumulation of lipids in the failing myocardium leading to lipotoxicity[45]. Toxic intermediate products may further worsen cardiac function and metabolism with development of progressive myocardial atrophy, apoptosis and protein breakdown[46]. Cardiomyocyte apoptosis has been observed in animal models of heart failure and in human heart failure due to ischemic cardiomyopathy[47, 48].

Ischemia induced lipid accumulation in the myocardium has been known for a long time[49, 50]. Early the accumulation of lipid in the ischemic heart was showed to consist of intracellular lipid droplets rich in TG[49, 51]. The origin of the lipid was suggested to depend on an increased accumulation of extracellular FA[49, 51] a theory that was strengthen by Chabowski and colleagues who described an increased translocation of CD36 to the plasma membrane surface in cardiomyocytes in response to hypoxia[52] together with an concomitant increase in FA uptake. Increasing the uptake of FA and reducing the FA utilization will lead to an accumulation of intracellular lipid.

Lipotoxicity

Under physiologic conditions, most triglycerides are stored in adipocytes with only minimal accumulation of lipids in other tissues such as the liver or muscle. The heart has a very limited capacity to store intramyocardial lipid and therefore the uptake and oxidation is tightly coupled[53]. However, at times this balance can be disturbed and accumulation of intramyocardial lipid can occur through an increase in lipid uptake or an impairment of lipid oxidation.

Most studies on intramyocardial lipid accumulation and lipotoxicity has been made in models of obesity and diabetes[45, 46, 54], therefore most of our knowledge of the consequences of lipid accumulation in the myocardium are in the light of an obese or diabetic background. In these models triglyceride accumulation within cardiomyocytes is associated with impaired contractile function[46, 55]. However, increased amounts of intramyocardial lipid has also been detected after ischemia[49].

Current thinking suggests that cardiomyopathy is not a direct consequence of TG accumulation alone, but that cardiomyopathy develops secondary to an accumulation of by-products of lipid metabolism, such as ceramide or other fatty acid derivatives that are known to interfere with intracellular signalling pathways [46, 56, 57]. These are called “lipotoxic” effects. Collectively, the term cardiac lipotoxicity refers to this constellation of altered fatty acid metabolism, intramyocardial lipid overload, and contractile dysfunction. The lipid accumulation can lead to the production of toxic lipid intermediates, reactive oxygen intermediates, ceramide, and/or activate signalling pathways (e.g., PKCθ) which can induce cell death[45, 46, 58, 59]. Although the underlying molecular pathways are only partially understood, several groups have reported animal models of cardiac lipotoxicity. Reduction of the deposition of intramyocardial lipid, for instance by treatment with insulin-sensitizing drugs in a rat model of diabetes-induced lipid accumulation, reverses contractile dysfunction indicating that intramyocardial triglyceride accumulation is deleterious[46].

To study the effects of lipid accumulation in the myocardium various animal models have been used. The obese Zucker rat develops age-related intramyocardial triglyceride accumulation and contractile dysfunction[55]. However, the extreme obesity in the Zucker rat model makes it difficult to determine whether the cardiac maladaptations are related to excessive myocardial lipid accumulation or to increased expression of conventional risk factors for cardiovascular disease. To address this limitation, various lean genetic mouse models of cardiac-restricted steatosis have been developed. For example, cardiac-specific overexpression of acyl-CoA synthetase in mice results in a marked increase in fatty acid import resulting in intramyocardial lipid overload, cardiomyopathy and profound left ventricular systolic dysfunction[60] showing the deleterious effects of lipid accumulation. Problems with models like these are of course that these are

congenital disorders and not acquired as usually in the human situation (diet-induced obesity). Transcription factors that have been proposed to have direct impact on lipid accumulation and homeostasis are sterol regulatory element binding-protein (SREBP)-1c and PPARα and γ[61, 62]. SREBP-1c regulates lipogenesis and metabolism of glucose to FA and TG, PPARα and γ in the myocardium increase FA oxidation. Both PPARα and γ and SREBP-1c expression have been shown to correlate with left ventricular dysfunction in humans with metabolic syndrome[61].

Acutely following cardiac ischemia clinically high levels of circulation free FAs have been detected [63], levels that are also seen in for example obesity and diabetes. Cell culture studies have demonstrated that long-chain saturated FAs, such as palmitate (16:0) and stearate (18:0), induce programmed cell death in a variety of cell types[46, 58], including cultured cardiomyocytes. Cytochrome c release, caspase activation, and DNA laddering are detected in cultured neonatal cardiomyocytes following treatment with palmitate[64]. Excessive deposition of TG in nonadipose tissues (steatosis) enlarges the intracellular pool of fatty acyl-CoA, thereby providing substrate for nonoxidative metabolic pathways, such as ceramide synthesis and expression of nitric oxide synthase (iNOS) that lead to cell dysfunction and death through apoptosis[46]. However, these pathological changes have been difficult to specifically link to cardiac dysfunction, arrhythmias and cardiomyopathy.

Ceramides

Ceramides belongs to the sphingolipid class of lipids. They consist of a sphingoid backbone, which is a common structural feature of all the sphingolipids, and a FA. The fatty acid can vary in length mostly between 16 and 26 carbons and is attached via an amid linkage. Ceramides are mainly synthesized by *de novo* but they can also be generated by sphingomyelinase hydrolysis of membrane sphingomyelin glyceosphingolipids[65]. The *de novo* synthesis of ceramides occurs in the endoplasmatic reticulum (ER) and begins with the condensation of palmitoyl CoA with serine by serine palmitoyl transferase, which is the rate limiting step of the pathway. The ceramide is subsequently transported by ceramide transfer protein (CERT) or by vesicles to the golgi apparatus where it can be further processed into spingomyeline, glycosyl ceramides or lactosyl ceramides[1, 65, 66]. Ceramides are highly hydrophobic and are generated by membrane-associated enzymes and exert their effects either in close proximity to the generation site or require specific transport mechanisms to reach their targets in other intracellular compartments. Ceramides have also been described to be able to flip across the membrane with the help of transporters[67]. The ceramides are bioactive molecules and have been, as

mentioned above, been implicated in apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and insulin signalling as part of cellular lipotoxicity[1, 2].

Ceramides have been linked to disease states in different tissues, for instance in pancreatic β-cells excessive cytosolic accumulation of triglyceride and its by-product, ceramide, activated the inducible form of nitric oxide synthase, which accelerated cell death (apoptosis) and failure of the cell[68]. Ceramides have also been suggested to be involved in inflammation through IL-6[69], reactive oxygen species (ROS) production[70] as well as endoplasmatic reticulum (ER) stress[71]. Likewise in models of lipotoxicity, heart ceramide levels have been shown to be upregulated[46, 60] and inhibition of ceramide synthesis has been shown to correct the lipotoxicity leading to skeletal muscle insulin resistance in mice fed a high-fat diet[72]. For example, incubation of 3T3-L1 adipocytes with a membrane-permeable C2-ceramide inhibited insulin-stimulated glucose transport by 50% by reducing GLUT4 translocation through inhibiting the phosphorylation and activation of Akt[73]. Ceramide has been suggested to lead to inhibition of cell division and apoptosis in some cells[74]. Cardiac ceramide levels are elevated in models of cardiac lipotoxicity due to cardiac overexpression of longchain acyl CoA synthase[60], PPARα[75], PPARγ[62], and FATP[76] but also in hypoxic[77] and hypoxia-reoxygenation models[78, 79]. Reduction of the amount of intramyocardial ceramide, by inhibition of ceramide synthesis with myriocin, showed an improvement of cardiac function in mice together with an increased FA and reduced glucose oxidation[80].

More evidence has emerged that the FA chain length of ceramides is an important determinant of the biological effect mediated by the bioactive lipid[81, 82]. For example long chain ceramides (C24-ceramide) have been described to be involved in cell cycle arrest but not apoptosis in MCF-7 cells[83] and inhibition of C16-ceramide generation in B-cells rescues from cell death[84]. It has been shown that hypoxia acutely increases the amount of intramyocardial ceramide, foremost with shorter (16 carbon) FAs and that, apart from C16, hypoxia seems to favour the dominance of certain species such as C18:1-Cer and C24:1-Cer, over others, 24-Cer[77]. Furthermore, in a screening with mutated *c. elegans* it was shown that hypoxia-stimulated accumulation of long-chain ceramides (C20-22-ceramide) was associated with improved hypoxic tolerance and survival whereas accumulation of very long chain ceramides (C24-26-ceramide) was associated reduced hypoxic tolerance and death. It was also shown that these different chain-lengths ceramides were synthesized by different ceramide syntheases[85, 86]. These results indicate that the general concept of the harmfulness of ceramides might not be straight forward.

Endoplasmatic Reticulum - Stress

The endoplasmatic reticulum (ER) is a central coordinator of diverse cellular processes. The ER acts as gatekeeper to the secretory pathway by folding, modifying, and exporting nascent secretory and membrane-bound proteins as well as a storage for intracellular calcium for localized release by second messenger cascades. Lipogenic reactions (including those involved with synthesis of triacylglycerols, sterols, ceramides, and most cellular phospholipids) occur on the cytosolic side of the ER membrane. The ER forms the nuclear envelope and can contribute to biogenesis of peroxisomes, lipid droplets, and autophagic membranes. The ER makes close contacts with every other membranous structure in the cell, and these contacts likely facilitate the bidirectional transfer of lipids, calcium, and other molecules. Thus, disruption of ER function broadly impacts cellular function, and disruptions in other cellular processes typically redound to the ER[87].

Several factors are required for optimum protein folding, including ATP, Ca^{2+} and an oxidizing environment to allow disulphide-bond formation. As a consequence of this specialist environment, the ER is highly sensitive to stresses that perturb cellular energy levels, the redox state or Ca^{2+} concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins - a condition referred to as ER-stress. Protein aggregation is toxic to cells and, consequently, numerous pathophysiological conditions are associated with ER-stress, including glucose or nutrient deprivation, viral infections, lipids, increased synthesis of secretory proteins, expression of mutant or misfolded proteins as well as ischemia, neurodegenerative diseases and diabetes[87-89]. To combat the deleterious effects of ER-stress, cells have evolved various protective strategies, collectively termed the unfolded protein response (UPR, Figure 6). The UPR is a signalling pathway from the ER to the nucleus[87]. When stress occurs the UPR is mediated by signalling through ER-localized transmembrane receptors; pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). In resting cells, all three ER-stress receptors are in an inactive state through their association with the ER chaperone, GRP78. On accumulation of unfolded proteins, GRP78 dissociates from the receptors, which leads to their activation and triggering of the UPR[87-89]. These three receptors represent three different branches of the UPR, all three aims at signalling to transcription factors to increase expression of chaperones, genes involved in protein degradation, amino acid transport and metabolism proteins [87]. Persistent ER-stress or failure to initiate UPR may lead to apoptosis[88]. For simplicity I will throughout name ER-stress and UPR collectively as “ER-stress”.

After dissociation from GRP78, PERK phosphorylates eukaryotic initiation factor 2 (eIF2) which inhibits the translation of eIF2 dependent proteins, this inhibition can be bypassed by ATF4 which promotes cell survival by inducing genes involved in

amino-acid metabolism, redox reactions, stress response and protein secretion. After dissociation from GRP78, ATF6 translocates to the golgi apparatus where it is cleaved into its active form. Active ATF6 then moves to the nucleus and induces genes with an ER-stress response element (ERSE) in their promoter, such as GRP78, GRP94, protein disulphide isomerise (PDI), and the transcription factors CHOP and X box-binding protein 1 (XBP1). The signalling performed by ATF6 is purely pro-survival and aim to counteract ER-stress. On activation, the endonuclease activity of IRE1 removes a 26-nucleotide intron from the XBP1 mRNA (sXBP1). sXBP1 encodes a stable, active transcription factor targeting ER chaperones and the HSP40 family member P58^{IPK} which inhibits PERK[89].

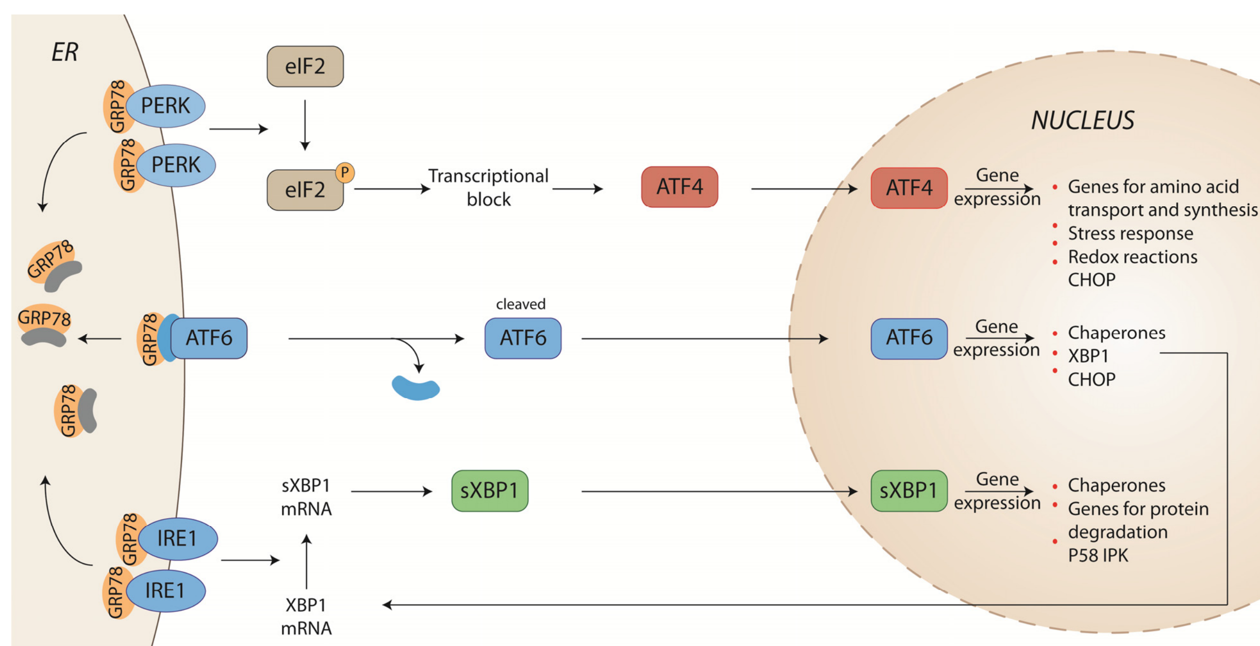

Figure 6. The three branches of ER-stress, adapted from[88-90].

During prolonged ER-stress signalling through PERK, ATF6 and IRE1 can trigger pro-apoptotic pathways. These pro-apoptotic pathways activate downstream effectors including CHOP and JNK, which further push the cell towards death. CHOP is also known as growth-arrest- and DNA-damage inducible gene 153 (GADD153) and is transcribed by all three branches of ER-stress, however the ATF4 branch is the most important. Target genes for CHOP includes BCL2, GADD34, ER oxidoreductin 1 (ERO1α) and Tribbles-related protein 3 (TRB3). The expression of BCL2 is downregulated by CHOP, this downregulation together with JNK phosphorylation of BCL2 induces apoptosis[87, 89]. Chemical chaperones, such as 4-phenyl butyric acid (PBA), trimethylamine N-oxide dihydrate (TMAO), and dimethyl sulfoxide, are a group of low molecular weight compounds known to stabilize protein conformation, improve the folding capacity of the ER, and facilitate protein trafficking. For example 4-PBA can normalize hyperglycemia,

restore systemic insulin sensitivity, and enhance insulin action in liver, muscle, and adipose tissues in obese diabetic mice[91].

Ischemia has in many models been shown to induce ER-stress. In the myocardium, ischemia induces ER-stress, by decrease of ATP, ER Ca^{2+} levels, and UDP-glucose, which interferes with protein folding. In a model of cardiac ischemia and cardiomyocyte hypoxia low oxygen pressure was shown to induce ER-stress primarily through XBP1, and blocking of the XBP1 branch significantly increased cardiac apoptosis indicating an initially protective role for ER-stress in cardiomyocytes[92]. In other models hypoxia but not reperfusion has been shown to induce the PERK and ATF6 branches[93, 94]. All three arms of ER-stress in cardiomyocytes have been suggested to be regulated by prolylhyroxylases, DMOG a stabilizer of prolylhyrdroxylase induced ER-stress in HL-1 cardiomyocytes and inhibition attenuated post-ischemic myocardial damage[95].

The Very Low Density Lipoprotein Receptor

The VLDLr is the member of the LDL receptor family that resembles the LDLr the most[10, 11, 96]. There are several other receptors belonging to the LDLr family such as the apolipoprotein E receptor 2 (apoER2), the LDLr related protein (LRP), LRP1B, megalin, LRP3, LRP4, LRP5, and LRP6[10]. All receptors of the LDL receptor family exhibit similar structural features. They consists of five domains (i) an aminoterminal ligand binding domain composed of multiple cysteine rich repeats; (ii) an epidermal growth factor (EGF) precursor homology domain, (iii) an O-linked sugar domain with clustered serine and threonine; (iv) a transmembrane domain; and (v) a cytoplasmic domain containing an NPVY sequence, which is required for receptor-mediated endocytosis via clathrin-coated pits[10-12] (Figure 7).

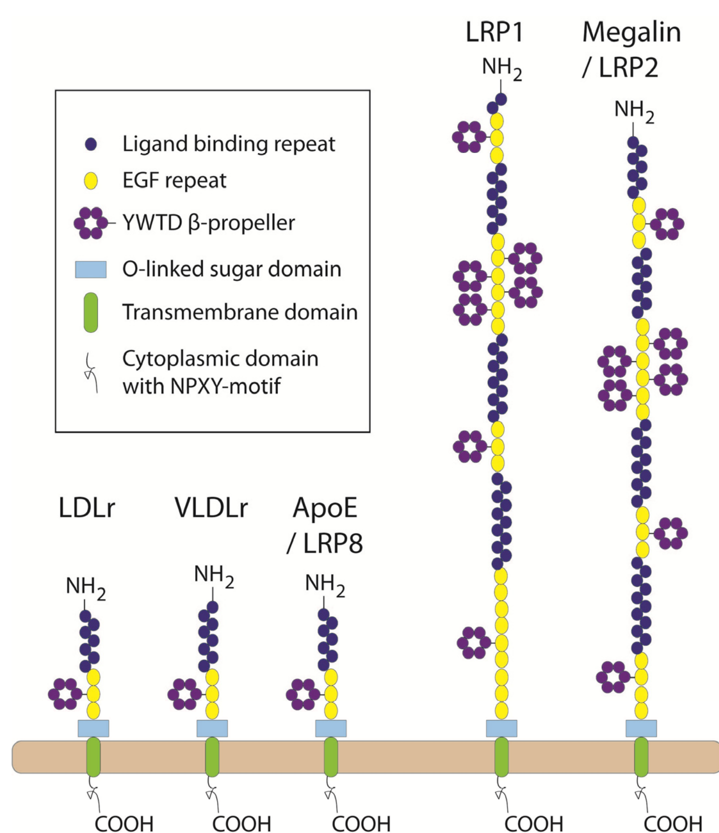

Figure 7. Architecture of the LDLr family. Adapted from[14, 97].

Structure

The VLDLr was originally cloned because of its homology to the LDLr by Oka et. al. in human[98] and Gåfvels and colleagues in mouse[99]. The human VLDLr gene contains 19 exons spanning approximately 40kb[99, 100]. The intron-exon organisation of the gene resembles that of the LDLr except that the VLDLr gene contains an extra exon that encodes the additional repeat of the ligand binding domain[11]. The eight ligand-binding domain of the VLDLr are each about 40 amino acids long, their conformation is maintained by a Ca^{2+} ion that is chelated by the carboxylates

of Glu and Asp residues in the acidic cluster and two backbone oxygens. Further stabilization is achieved via three disulfide bonds present in each of the modules[101, 102]. The VLDLr mRNA produce two splice variants, VLDLr type 1 and VLDLr type 2. The VLDLr type 2 lacks the O-linked sugar domain encoded by exon 16[11, 100, 103]. The gene can be differentially spliced in humans, rats, rabbits and bovines[11, 103-106], but not in mice[98]. The VLDLr have an about 95% amino-acid conservation between human[11, 99, 104, 107], mouse[98, 108], rabbit[96] and rat[105], and 84% amino-acid conservation between human and chicken[109]. The remarkably good interspecies conservation of the VLDLr indicates an important physiological role for this receptor.

Tissue Distribution

The VLDLr is highly abundant in the heart, skeletal muscle, adipose tissue, placenta and brain but are barely detectable in the liver[99, 104, 110] which is where the LDL receptor is expressed in large quantities. Expression of the VLDL receptor has also been detected in different cell types such as THP-1 macrophages, HL-60 cells, human monocyte derived macrophages, and rat cardiomyocytes[11, 111-113]. The VLDLr expression is also found in endothelial and smooth muscle cells of arteries and veins[114], as well as on macrophages and smooth muscle cells *in vivo* in human and rabbit in atherosclerotic lesions[114-116]. The type 1 and type 2 VLDLr differ not only in structure but also in tissue distribution, however overlap occurs in some tissues. Type 1 VLDLr has been shown to be expressed in the heart, brain, and skeletal muscle compared to type 2 VLDLr, that have been shown to be expressed in primarily non-muscular tissues including cerebrum, cerebellum, kidney, spleen, adrenal gland, testis, ovary, uterus and aortic endothelial cells[100, 103].

Ligand Binding - Lipids and Lipoproteins

The VLDLr binds to lipoproteins through interactions with ApoE, compared to the LDLr which binds to ApoB. ApoE is present on all lipoprotein subclasses, whereas the full length apoB100 is present on all lipoprotein subclasses except chylomicrons which instead contain the truncated apoB48. The difference in ligand preference is suggested to be dependent on the difference in the ligand binding repeats[96]. Results have shown that the VLDLr binds VLDL and IDL but not LDL[117, 118]. Furthermore enrichment of apoE on the VLDL surface increases the binding affinity to the VLDLr[119-121]. The binding affinity to VLDL is not decrease upon lowering to 4 degrees as is the case with the LDLr[118].

Other ligands that have been shown to bind to the VLDLr include receptor-associated protein (RAP)[122], thrombospondin-1 (TSP-1)[123], LPL[119, 120], urokinase plasminogen activator (uPA)/plasminogen activator inhibitor-1 complex[124] and

several other proteinase-serpin complexes[125]. RAP is a 39kDa protein that has been shown to bind to both LDLr and VLDLr with high affinity inhibiting lipoprotein ligand binding and uptake by the receptors. The biological role of RAP is not fully understood, however it has been suggested that RAP could be important for the early processing of the receptors in the ER perhaps in regulating receptor transport or trafficking to the cell surface[122, 126]. LPL has been shown to be able to bind[118] and be internalized by the VLDLr[124] exactly how though is not understood. The purpose of the LPL binding to the VLDLr has been suggested to be to hydrolyse the core TG to make the lipoproteins smaller and thereby facilitating endocytosis[119, 121]. For TSP-1 and uPA little is known but that the VLDLr and LDLr binds and internalize the protein where it is degraded[123, 124].

There are few differences in ligand binding between the type 1 and type 2 VLDLr, no differences in internalization, dissociation, and degradation of ligand have been described, however type 1 VLDLr has relatively stronger affinity to VLDL and is more stable[103, 106]. Since the difference between the type 1 and type 2 VLDLr does not reside in the ligand binding domain but rather in a part important for stability the few differences found is not surprising[103].

Functions

Lipid Uptake

As previously discussed the VLDLr is not expressed in the liver, but predominantly in heart, muscle and adipose tissue[99, 104, 110]. Because heart and skeletal muscle use FAs as fuel source and adipose tissue use FAs for energy storage, such observations led to the hypothesis that it could be involved in the delivery of FA to peripheral tissues by mediating peripheral uptake of triglyceride-rich, apoE-containing lipoproteins[96, 98, 99, 108, 117]. This theory is supported by observations from the chicken, in which a protein very similar to the type 2 VLDLr is essential for the accumulation of VLDL-derived lipid in developing eggs[109, 127, 128]. The importance of VLDLr in FA uptake was questioned when VLDLr knock-out mice showed a normal lipoprotein profile,[129] if the VLDLr was important for peripheral lipid uptake the mice would have been expected to have higher circulating plasma lipids. More results questioning the involvement of VLDLr in FA uptake was that the VLDLr and LPL was not coordianately regulated[105, 108] and that fasting and refeeding did not influence the VLDLr expression[105] which would have been expected. Also the involvement of VLDLr in neuronal migration in the developing brain[130] further increased the doubts of the VLDLr involvement in peripheral tissue FA delivery in normal physiology[105]. Some years ago it was shown by Goudriaan and colleagues that VLDLr knock-out mice are protected against diet-induced obesity and that leptin deficient *ob/ob* mice have a less pronounced weight gain in the absence of VLDLr[131]. Other studies showed that a double VLDLr and LDLr

knock-out mouse fed a high fat diet had significantly increased serum TG levels compared to LDLr knock-out mice and that a VLDLr over-expressing mouse on a LDLr knock-out background hade significantly decreased serum triglyceride levels[132]. These data indicate that the VLDLr indeed has a discrete role in the normal lipid metabolism, however in the stressed situation VLDLr is of great importance. What also needs to be taken into account when discussing the VLDLr in lipid metabolism is the difference in expression in different cell types and species[133]. Since VLDLr is primarily expressed in heart, adipose and muscle tissue it is likely to have a more prominent role in lipid metabolism in these tissues compared to low expressing tissues such as the liver and macrophages[133-135]. Exactly how the VLDLr affects the lipid metabolism has been under investigation, results indicates that the VLDLr works as a lipoprotein endocytosis receptor and other that it works as a docking point for lipoproteins so that LPL can function.

Lipoprotein Endocytosis

Early on it was suggested that the VLDLr has endocytotic functions. One indication to this is the close resemblance with the LDL receptor which functions as an endocytosis receptor. The receptor contains eight ligand binding repeats and certain head-to-tail combinations of these repeats are believed to specify ligand interaction where the LDLr with seven repeats is known to recognize apoB and apoE[136]. In the cytoplasmic region there are signals for receptor internalization via coated pits, containing the consensus tetrapeptide Asn-Pro-Xaa-Tyr (NPXY)[137]. These features indicate that the VLDLr should be able to bind and internalize lipoproteins, possibly of different kind than the LDLr. It has also been shown in *in vitro* experiments that the VLDLr binds and internalizes particles that are rich in apoE such as VLDL, IDL, and chylomicrons[119, 138] but not LDL with high affinity[96].

Cooperation with LPL

VLDLr has also been described to function in cooperation with LPL. Tacken and colleagues proposed a mechanism for this cooperation where VLDLr works as a docking point for triglyceride rich lipoproteins so that HSPG bound LPL or VLDLr bound LPL can come in close contact with the lipoprotein and can function to liberate FAs from the lipoproteins. The FAs are then free to be taken up by the cells by other transporters such as FAT/CD36[121] (Figure 3). In favour of this theory is the finding that the binding of lipoprotein particles to the VLDLr was stimulated by LPL[119, 138]. Further connection between the VLDLr and LPL is shown in studies describing that the LPL activity in plasma from VLDLr knock-out mice was significantly lower than in wild type mice[139, 140] however if this was dependent on an increased shedding of LPL from the endothelial wall or an increased activity is

not stated. VLDLr has also been described to be involved in LPL transcytosis of endothelial cells *in vitro*[141].

Coagulation

The VLDLr has also been implicated in blood coagulation by binding to coagulation factor VIII (FVIII). Blood coagulation is a highly conserved cascade reaction with many involved players. One of them is FVIII, which in its activated form (FVIIIa) serves as a cofactor for factor IXa (FIXa). Maintaining normal levels of FVIII in the circulation is critical for hemostasis: hereditary deficiency in FVIII results in a bleeding disorder hemophilia A, whereas elevated FVIII levels are associated with increased risk of thrombosis[142]. VLDLr binds and internalize FVIII in a similar fashion as LRP and LDLr which implies that VLDLr could be involved in FVIII clearance[143, 144]. However, experiments in knock-out mice do not support the in vitro experiments, instead VLDLr deficiency accelerated FVIII clearance[145].

Angiogenesis

In 2003 the VLDLr was found to be linked to retinal neovascularization, the results indicated a prominent inhibitory effect of VLDLr on retinal angiogenesis[146] and also to be significantly associated with age-related macular degeneration in humans (AMD)[147]. VLDLr was shown to be present in the two main retinal cell types, retinal vascular endothelial cells and retinal pigment epithelial cells[148] and in müller cells[148, 149]. Exactly how the VLDLr regulate retinal neovascularization is unclear. Furthermore, it has been shown that the VLDLr is involved in choroidal neovascularization in AMD[150]. VLDLr was also shown to be a negative regulator of the wnt signalling pathway in choroidal neovascularization, a pathway described to be involved in angiogenesis in skeletal muscle and cancer[151-153]. VLDLr knock-out mice showed an impaired phosphorylation of downstream targets in the wnt pathway, suggesting an increased activity of the pathway[150]. However, VLDLr has not been shown to influence other angiogenic factors such as VEGF and is yet to be shown to be important in angiogenesis in other tissues than the eye.

Metabolic Syndrome and Atherosclerosis

Metabolic syndrome is characterized by a cluster of obesity, insulin resistance, hypertension and atherogenic dyslipidemia and is associated with an increased risk of coronary heart disease. Results indicate that the VLDLr is involved in the mechanism of metabolic syndrome since VLDLr knock-out mice show a decrease in adipose mass[129] and the VLDLr bind remnant lipoproteins with high affinity[138].

Further on it has been shown that VLDLr knock-out animals challenged with a high caloric diet become less obese and thereby remain glucose tolerant unlike their wild type littermates[131]. These data indicate that inhibition of the VLDLr in adipose tissue might be therapeutic strategy for obesity. Results have also indicated a role for VLDLr in diabetic dyslipidemia. It has been shown that the VLDLr expression is decreased in the hearts, skeletal muscle and adipose tissue of streptozotocin treated rats, possibly increasing the hyperlipidemia[154] and that these effects can be reversed by injection of VLDLr adenovirus[155].

VLDLr have been described to be involved in the atherosclerosis progression both *in vivo* and *in vitro*. VLDLr expression is seen in macrophages as well on endothelial and smooth muscle cells[114-116, 156, 157], all important components in the formation of atherosclerotic plaques. *In vitro* VLDLr have been suggested to be involved in foam cell formation[111, 113, 158] and *in vivo* VLDLr mRNA and protein expression is present in both human and rabbit atherosclerotic lesions[114-116, 133]. In mice atherosclerotic lesions VLDLr expression have only been detected on mRNA level[133] but the involvement of the VLDLr protein have still been implicated by bone marrow transplantation studies with VLDLr knock-out bone marrow in wild type mice showing a reduction in atherosclerosis development[157]. However, the VLDLr protein expression was not confirmed[157]. Contrasting results have shown that there is no difference in the atherosclerosis development between VLDLr knock-out mice compared to wild types on a human apoB transgenic background[140] and that VLDLr deficiency increase intimal thickening after vascular injury[159]. These contrasting results indicate that there are differences in the mechanism of atherosclerotic lesion development between species that needs to be taken in to account.

Reelin Signalling in the Brain

In 1999 Trommsdorff and colleagues showed that the VLDLr and ApoE participate in transmitting extracellular Reelin signals to intracellular signalling processes initiated by Disabled 1 (Dab1)[7, 130, 80]. The binding of Dab1 to Reelin activates downstream signalling pathways, providing positional information that is essential for the migrating neurons along the radial glial in the early cortical development of the brain[160, 161]. Reelin is an extracellular glycoprotein important for the migration of purkinje cells[160]. Dab1 has been shown to work downstream from Reelin and interact specifically with the cytoplasmic tail of the VLDLr. A functional VLDLr-Dab1-Reelin interaction was shown to be required for the cortical layering, cerebellar foliation and the migration of purkinje cells[130, 162]. Both the VLDLr and the closely related ApoER2 receptor has been shown to bind and internalize Reelin in a way that seems to fine tune the Reelin signalling[137]. The VLDL receptor resides in the non-raft domain of the membrane and endocytosis by this pathway

destines Reelin for degradation, ApoER2 resides in membrane rafts and binding of Reelin leads to the degradation of the receptor complex by lysosomes generation signalling fragments. The action is roughly and on/off tuning of Reelin signalling[137].

Mutations in VLDLr

Reported mutations of the VLDLr have been associated with different phenotypes. In 1995 Bujo and colleagues described a naturally occurring mutation in the VLDLr in hens. They described a strain of mutant chickens termed restricted ovulators, characterized by female sterility via failure to lay eggs associated with severe hyperlipidemia and found that a single nucleotide substitution Cystein682Serine was responsible for the phenotype[127]. The mutation was located in the C-terminal cystein rich repeat and the corresponding mutation in the LDLr (Cys646) is responsible for familial hypercholesterolemia[127]. However contradiction evidence came in the form of the VLDLr knock-out mouse that was found to have normal fertility and no significant abnormalities in plasma lipoproteins[129].

Mutations in the human VLDLr gene have been described firstly in interrelated Hutterite families[163] and later in inbred Turkish[164] and Iranian[165] families. The mutations were located on chromosome 9p24, likely in exon 17 of the VLDLr gene encoding parts of the transmembrane helices and thereby generating loss of VLDLr protein[166]. The mutations were associated with cerebellar hypoplasia, mild cerebral gyral simplification, mental retardation, and disequilibrium. In the Turkish family quadrupedal locomotion was also seen in some cases, hypothesized to be part of the mutation phenotype[164], however Humphrey and colleagues suggested this was due to balance problems caused by the cerebellar hypoplasia rather than the mutation itself[167]. The phenotype of the mutation resembles that mutation seen in the *Reeler*-like mouse described by Trommsdorff and colleagues, which displays a double knock-out of the Reelin receptors VLDLr and ApoER2 displaying gait and cerebellar hypoplasia[130]. The VLDLr knock-out mouse with intact ApoER2 appear neurologically normal[129], however with a slightly smaller cerebellum with reduced foliation and heterotopic purkinje cells[130]. The mutation has also been found in patients born to nonconsanguineous parents[168]. The same mutation (on chromosome 9p24) has also been associated with gastric cancer by both homozygous deletion and epigenetic silencing[169].

Regulation

Promoter Regulation

Regulation of the VLDLr gene expression has been the subject of several studies, because regulation of expression reveals basal information about gene function. There are many potential regulatory elements present in the promoter of which only a few have been thoroughly investigated. Collectively so far the regulation has shown to be very heterogeneous. The expression of VLDLr varies between species and tissues and so does the effect of the regulatory elements. However results and the fact that the VLDLr gene is highly conserved over species have shown that the expression is likely more conserved over species than over tissues which are likely to depend on the difference in VLDLr tissue expression.

Several elements that are present in the VLDLr promoter region have previously been recognized to play a role in the regulation of genes implicated in lipid metabolism and maintenance of energy balance. The 5'-flanking region of the VLDLr promoter contains two potential sterol regulatory elements, which have previously been reported to mediate downregulation of the LDLr by sterols[11, 170]. However, these have not been shown to influence the VLDLr expression neither in human THP-1 macrophages nor in rabbit alveolar resident macrophages[11, 111, 171] when treated with sterols. The promoter region also contains regulatory elements binding CCAAT/enhancer-binding protein-β (C/EBP-β) and nuclear factor-Y (NF-Y)[172]. C/EBP-β is involved in regulation of energy homeostasis and in the differentiation of adipocytes[173-175], and NF-Y, an important regulator of LPL[172, 176]. These two sites have been shown to be active in human placenta cell line but not in human hepatoma cells. The VLDLr promoter also contains half a binding element for the oestrogen receptor[11, 110], and administration of oestradiol has been reported to increase VLDLr mRNA levels in rabbit cardiac ventricles[177]. VLDLr expression levels have further been described to be regulated by peroxisome proliferator-activated receptor gamma (PPARγ)[178] in mouse adipocytes and adipose tissue. PPARγ has previously been shown to regulate many target genes involved in FA metabolism and storage in adipocytes such as FAT/CD36, FATP and LPL[179, 180]. The findings have been confirmed in human cultured preadipocytes and functionally *in vivo* where it was shown that PPARγ upregulated VLDLr expression significantly increased the clearance of plasma TG in mice[181]. In mouse and human the VLDLr mRNA and protein expression was shown to be upregulated by bile acids in HepG2 via farnesoid X receptor (FXR), although without showing a promoter binding sequence[182]. Whether this regulation is important or not when the VLDLr expression in the liver is very low remains to be elucidated. The VLDLr has further been described to respond to granulocyte-macrophage colony stimulating factor[183], thyroid hormone[105], and 1a,25-dihydroxyvitamin D3[112] through promoter binding. Other sites in the 5'-flanking promoter region are AP-2,

E2A, NF-IL6, Sp1, SRE1 and PU.1[121]. The functional significance of these sites remains to be elucidated.

Post Translational and Nutritional Regulation

Apart from regulation of the promoter expression the VLDLr have been shown to be regulated post translationally as well as by nutritional status.

Mechanisms for post translational modulation of the VLDLr have been shown to include the E3 ubiquitin ligase IDOL (inducible degrader of the LDLr)[184] and PCSK9 (proprotein convertase subtilisin/kexin 9)[185] which have also been described as post translational regulators of LDLr[186, 187]. IDOL was shown to degrade VLDLr and ApoER2 in human HEK293 cells and that this was mediated by the liver X receptor (LXR) pathway. The effect of IDOL was supported by *in vivo* data showing that the level of VLDLr was increased in mouse brain lacking LXR[184] and that this caused disrupted neuronal migration[188]. PCSK9 has been shown to be important for VLDLr posttranslational regulation by enhancement of VLDLr degradation in HEK293 and NIH-3T3 cells both intracellularly and by re-uptake of the secreted protein[189, 190]. PCSK9 have been shown to be implicated in familial hypercholesterolemia through its effect on enhancement of LDLr degradation[186]. However the importance of posttranslational regulation of VLDLr expression has not been shown *in vivo* in any tissues but the brain.

Studies of the nutritional regulation of the VLDLr have shown inconsistent results. Jokinen et al. showed that fasting and refeeding in rats had no effect on VLDLr mRNA or protein levels in white adipose tissue, soleus muscle, brain and heart[105, 110]. In contrast, Tiebel et al. investigated the effect of prolonged feeding of an atherogenic diet on VLDLr expression in wild-type, LDLr$^{-/-}$, apoE$^{-/-}$ and LDLr$^{-/-}$/apoE$^{-/-}$ mice and found a downregulation of the VLDLr in heart and skeletal muscle upon atherogenic diet only in LDLr$^{-/-}$ mice and an upregulation of the VLDLr mRNA expression in adipose tissue[110]. Furthermore a study in mice by Kwok and colleagues[191] revealed an increase of VLDLr expression in heart together with LPL, FAT/CD36, FABP, acyl-Coenzyme A synthetase (ACS) and long chain acyl-CoA dehydrogenase (LCAD) together with a decrease in epididymal fat after fasting of Balb/c mice. These divergent results indicate that the nutritional regulation of the VLDLr depend on several factors including presence of other lipoprotein receptors, the tissue, the species and the strain.

In cell culture it was shown that rat L6 muscle cells treated with L-glucose, (the optical isomer of D-glucose not taken up by the cells) i.e. glucose starving the cells, increased the VLDLr expression together with an increased uptake of TG-rich lipoproteins and APMK activation. The results in L6 cells indicates a switch from glucose to FA as main energy source as seen in skeletal muscle cells[171]. It was also

shown that the VLDLr expression in skeletal muscle cells was not affected by either lipoprotein deprivation or oversupply as seen for the LDLr. It is possible that VLDL receptor induction by glucose deprivation is an adaptation for avoiding energy failure in the heart[171].

Hif-1α

A consequence of low oxygen pressure, for example during myocardial ischemia/hypoxia, is activation of different transcription factors. One example of such transcription factors are hypoxia inducible factors (Hifs). Hifs consists of three different proteins Hif-1, 2, and 3, of which Hif-1 is the most studied. Hif-1 is a sort of master switch that allows cells to respond to falling oxygen and over 70 genes are known to be directly regulated by Hif-1 in response to hypoxia. The genes controlled by the Hifs include those coding for proteins that regulates cell growth, division, survival, mobility, angiogenesis, formation of new blood cells, inflammation, as well as for glycolytic enzymes that can produce energy from glucose without the aid of oxygen, and has major roles in both embryonic development and in adults. Many kinds of cancers carry elevated levels of the proteins. This may contribute to the cancers' ability to grow and spread, partly because of it increase angiogenesis, which fuels the tumours' growth[192].

Hif-1 is a heterodimeric protein that consists of two subunits, Hif-1α and Hif-1β, Hif-1β is constitutively expressed and unaffected by oxygen concentration. Under normoxic conditions, Hif-1α has a very short half-life. Cells continuously synthesize and degrade Hif-1α protein. During normoxic conditions Hif-1α is hydroxylated on two prolyl residues (Pro402 and Pro564 in human Hif-1α) in the oxygen dependent degradation domain (ODDD). This oxygen-dependent hydroxylation regulates the interaction with the von Hippel–Lindau tumour suppressor protein (pVHL). pVHL is the recognition component of an E3 ubiquitin ligase complex that targets Hif-1α for proteolysis by the ubiquitin–proteasome pathway. Under hypoxic conditions, prolyl hydroxylation is suppressed and Hif-1α protein escapes proteasomal degradation and can translocate to the nucleus and dimerize with Hif-1β. The heterodimeric transactivating complex Hif then binds to the hypoxia responsive element (HRE) in promoter or enhancer sequences of target genes (Figure 8). The effects of hypoxia can be mimicked by disruption of pVHL[193], iron chelation, use of 2-oxoglutarate analogs such as dimethyloxalylglycine (DMOG) or substitution of Fe(II) by metal ions such as cobalt[194, 195]. Hif-1α has been described to be active in the myocardium. It has been suggested that Hif-1α is important in the development of cardiomyocytes by inducing the expression of cardiotrophin-1 which promotes cardiomyocyte differentiation from stem cells[196]. Hif-1α has also been suggested to upregulate

atrial natriuretic peptide (ANP)[197], VEGF[198], adrenomedullin[199] and endothelin-1[200] in the hypoxic myocardium. Many genes that are induced by Hif-1α are expressed at higher levels in cancer than in normal tissues, particularly angiogenic growth factors (such as VEGF) and enzymes of the glucose metabolism. One of the hallmarks of cancer metabolism which is influenced by Hif-1α is increased glucose uptake, lactate production and decreased respiration. Activation of Hif-1α is a common feature of many forms of cancer and can be used as an independent predictor of poor prognosis in certain types of cancer[194].

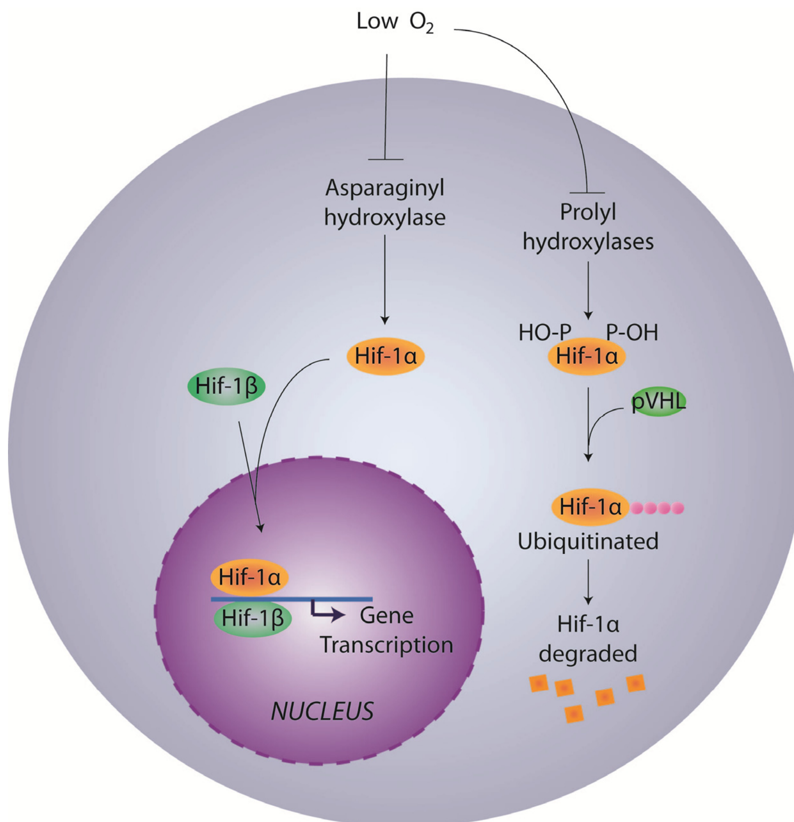

Figure 8. Schematic picture of Hif-1α activation.

Clear-Cell Renal Cell Carcinoma

Renal cell carcinoma (RCC) accounts for approximately 3% of all cancer diagnoses in the U.S. each year. In the U.S. in 2010, 58,000 individuals were diagnosed with RCC and approximately 13,000 died[201]. Today, RCC is recognized as a family of cancers in which each result from a distinct genetic abnormality with unique morphologic features, all are derived from renal tubular epithelium although if they originate from proximal or distal renal tubule is open to debate[202, 203]. There are five major subtypes currently recognized: clear-cell, papillary (type I and type II), chromophobe, collecting duct, and unclassified RCC[202], of these the clear-cell RCC is the most common kind (85%)[204].

On a molecular level clear-cell RCC can be characterized by a loss of function mutation in the von Hippel-Lindau protein (pVHL)[203, 205-207]. The loss of function mutation on pVHL was placed on chromosome 3p25 in the 1980s and authenticated in 1993, and is the same mutation as found in pVHL disease which is associated with several types of cancers[203, 208]. Loss of function in pVHL results in constitutively active Hif-1α witch can enter the nucleus, dimerize with Hif-1β and exert its transcriptional activity (See section Hif-1 above). As a consequence of increased HIF-1α activity clear-cell RCC overproduce angiogenic factors; vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) B, and transforming growth factor (TGF)-α along with their receptors, rendering the cancer notoriously rich in blood vessels[203]. HIF-1α also upregulates proteins involved in glucose uptake and glycolysis such as glucose transporter (GLUT) 1 which increases the glucose availability of the cancer as well as lactate dehydrogenase (LDH) which converts pyruvate to lactate. Lactate production promotes a malignant phenotype through induction of several oncogenes and the creation of an acidic environment that protects cancer cells from the immune system. Furthermore oxidative phosphorylation is inhibited in clear-cell RCC by Hif-1α targeting[209].

Morphologically clear-cell RCC is characterized by a distinctive pale, glassy cytoplasm, which results from intracellular storage of lipid and glycogen[210]. A majority of the stored lipid is cholesterol and esterified cholesterol (8-fold and 35-fold more than is found in normal kidneys, respectively). Although free cholesterol accumulation is toxic to cells, clear-cell RCC cells are protected against it due to an increase in activity of acetyl-CoA cholesterol acetyl transferase (ACAT)[211]. ACAT catalyzes the formation of cholesterol esters and it has been shown that specific inhibition of ACAT can inhibit the survival of glioma tumour cells[212], but there are currently no similar reports for clear-cell RCC[209]. Another cause of the excessive cholesterol accumulation, besides increased ACAT activity has not been elucidated. Potential explanations include defects in cholesterol synthesis, cholesterol efflux and/or cholesterol uptake. The issue of *de novo* synthesis of cholesterol being the cause of the cholesterol accumulation was shown to be unlikely by Gebhard and

colleagues who showed that HMG-CoA reductase (the rate-limiting enzyme in cholesterol synthesis) had a lower expression and activity in clear-cell RCC than in normal kidney cells[211, 213]. Work that investigated the uptake of cholesterol into clear-cell RCC compared accumulation of a radioactive cholesterol analogue in tumour tissue and normal renal parenchyma and showed no differences[214, 215]. The cholesterol efflux was also investigated and results are ambiguous[211, 215].

RCC is among the most resistant of tumours to therapy, they are resistant to conventional cytotoxic agents, possibly because of activation of pro-survival pathways and inactivation of apoptotic pathways[206], however treatments have been investigated. Possible pharmaceutical targets for treatment of clear-cell RCC has been sought in compounds that inhibit angiogenesis (VEGF, PDGF and TGF). In principle, interruption of the production of these growth and angiogenic factors would deprive the tumour of elements that contribute to its own survival. One example is bevacizumab, a humanized monoclonal antibody that targets VEGF which have been shown to inhibit tumour growth in human studies. Other drugs such as sunitinib and sorafenib also inhibit VEGF and PDGFβ pathways by acting on their receptors[216, 217]. Other investigated possible treatments are statins, metformin, diet[209], receptor tyrosine kinase inhibitors, and inhibitors of the mTOR pathway[206].

AIMS OF THE STUDY

I. To clarify the mechanisms behind the accumulation of lipids in the myocardium during ischemia and to determine the effect of lipid accumulation on survival following an acute myocardial infarction.

II. To map the regulation of the mouse and human VLDLr in hypoxic/ischemic condition.

III. To investigate if the VLDLr is involved in the intracellular lipid accumulation characteristic for clear-cell renal carcinoma and if this regulation is Hif-1α dependent.

METHODOLOGICAL CONSIDERATIONS

General descriptions of material and methods are given in each individual paper. In this section specific consideration and in some cases more detailed descriptions, of some of the methods are discussed.

In vitro studies

Cells

HL-1 (paper I and II)

The HL-1 cardiomyocytes is a spontaneously contracting cardiomyocyte cell line. It is derived from an atrial tumour from a female C57Bl/6 mouse. The cells have been extensively tested and express all cardiotypic phenotypes as primary adult cardiomyocytes[218, 219]. They have been tested for passage stability for up to 100 passages (Dr May Lam, Louisiana State University Medical Centre, New Orleans, personal communication). The HL-1 cardiomyocytes were a generous gift from Dr. William Claycomb of Louisiana State University Medical Centre, New Orleans[218]. The cells were maintained in Claycomb medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM norepinephrine in 30mM L-ascorbic acid and 10% fetal calf serum (FCS). Cells were sub-cultured when confluent in a split ratio of maximum 1:3. All culture flasks and dishes were pre-coated with a solution of 0.02% (wt./vol.) gelatine containing 5 µg/ml fibronectin. Beating HL-1 cardiomyocytes were maintained at standard cell culture conditions (37 °C, 5% CO2 and 95% relative humidity).

For hypoxia experiments cells were plated in the appropriate sized culture dish and let to reach confluency, thereafter the cells were incubated in 1% oxygen (37 °C, 5% CO_2, 1% O_2, 94% N_2 and 95% relative humidity) for 8 hours. As controls cells maintained in normal cell culture incubator (37 °C, 5% CO_2, 21% O_2, 74% N_2 and 95% relative humidity) were used. For hypoxia experiments in combination with transfections, HL-1 cells were plated at 60% confluency in appropriate size dishes. About 16 - 20 hours after plating the cells were transfected with siRNA or DNA using lipofetamine 2000 or LTX with PLUS reagent in medium without FCS, penicillin and streptomycin. Five hours after transfection complete media was added to the transfected cells and they were let to incubate for another 35 hours before they were put in hypoxic incubator as described above for the final 8 hours before harvest.

To be able to study the response of cardiomyocytes to hypoxia we needed to set up an *in vitro* model. We chose to use the HL-1 cardiomyocytes for several reasons.

Firstly we wanted to be able to study the lipid accumulation in both an *in vitro* and an *in vivo* model, since mice are more eligible for genetic manipulation the choice to use mice instead of for example rats was made. At the time of project initiation there were not many cardiomyocyte cell line available apart from the HL-1 cardiomyocytes, we considered using isolated primary cardiomyocytes, where protocol for isolation of rat primary cardiomyocytes are well evaluated, however transferring these protocols to mice showed to be inefficient. Furthermore isolation of primary cardiomyocytes was primarily performed with foetal animals generating foetal primary cardiomyocytes and it is known that foetal cardiomyocytes has a different metabolism compared to adult cardiomyocytes [30, 31, 35, 36, 38, 39]. In paper II were the studies were initiated in HL-1 cardiomyocytes we were able to confirm our data in human cardiomyocytes now available. These cells were isolated from adult cardiac tissue.

Primary kidney cells (paper III)

Renal tissue was obtained from nephrectomies performed due to malignancy. Areas for collection were chosen by an experienced urological pathologist. As reference material of healthy kidney tissue the cortical tissue farthest from the tumour of the same patient was selected. All specimens were collected after informed consent by the patient. Ethical permission was obtained from the ethical committee at Lund University, the ethical decision registration numbers are LU680-08 and LU 289-07. The tissue specimen was put in ice cold DMEM medium supplemented with 10% FCS with 2 mM glutamine (1%), 100 U/ml penicillin, 100 μg/ml streptomycin (1%). The tissues were rinsed, minced and subjected to overnight collagenase treatment at 37°C in a processing medium consisting of Ham´s F12/Dulbecco´s modified Eagle´s medium (F12:DMEM, 1:1, (v:v), supplemented with 5 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mg/ml Collagenase IV and 5 mg /ml deoxyribonuclease I type II. Following trituration by slow repeated pipetting through a 10 ml Steri Strip, the resulting tissue suspension was serially passed through tissue strainers with the mesh sizes 100 and 70 μm respectively, thereby excluding glomeruli from the preparation. The suspension was treated with 1X trypsin-EDTA for 5 minutes and passed through a 20 μm strainer, which resulted in single-cell suspension. Finally the cells were re-suspended, seeded and cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Tissue cultures were kept in T25 or T75 flasks, and maintained in normal cell culture incubator (37 °C, 5% CO_2, 21% O_2, 74% N_2 and 95% relative humidity) medium was changed every other day. For transfection experiments cells were

plated at 60% confluency in the appropriate sized culture dish. About 16 -20 hours after plating the cells were transfected with siRNA lipofetamine siRNAMAX in medium without FCS, penicillin and streptomycin. Five hours after transfection complete media was added to the transfected cells and they were let to incubate for another 43 hours before harvest.

In vivo studies

Animals (paper I)

VLDLr$^{-/-}$ animals[129] were purchased from Jackson Laboratories. They were kept in a temperature controlled environment on a 12 hour light cycle. VLDLr$^{+/+}$ littermates were used as controls. All animals were fed and watered *ad libitum* but fasted 4h before experiments, water was available *ad libitum* during the whole procedure. The experiments were approved by the Gothenburg Ethical Committee on Animal Experiments.

Induction of Myocardial Infarction (paper I)

The animals were anesthetized with isoflurane, intubated and connected to a ventilator for small animals. The animals were kept ventilated and maintained on 2% isofurane mixed with oxygen and room air, through the operation. Electrodes were placed to the extremities and coupled to an ECG device in order to observe the cardiac rhythm during the surgery. The chest was shaved using an electrical clipper. Left thoracotamy was performed between the 4th and the 5th rib to expose the left ventricle wall. The pericardium was removed and the branch of the left coronary artery was ligated proximally by positioning a suture between the pulmonary artery outflow tract and the left atrium. The efficacy of the procedure was directly assessed by characteristic changes in the ECG pattern, and akinesis of the left ventricular wall. If these chances were not seen an additional ligature was preformed. After induction of myocardial infarction (MI) the thorax was closed by the means of 3-4 ligatures. All animals received pre- and postoperative analgesia with buprenoprin 0,5mg/kg subcutaneously and were placed in cages with temperature control for spontaneous recovery. The sham operated mice were treated the exact same way except that the coronary artery was not ligated[220].

For antibody injections in combination with induction of MI the MI was performed as described above. For these experiments only VLDLr$^{+/+}$ control mice were used. The VLDLr antibodies used were generously provided by Dr Dudley K. Strickland,

University of Maryland School of Medicine, Baltimore. The antibodies were mouse monoclonal antibodies (1H10, 5F3 and 1H5) and were developed and tested in the Strickland laboratory. The antibodies were tested on western blot which showed that all but 1H5 reacted with VLDLr protein on SDS-PAGE, tests were also made with VLDLr binding assay showing that all but 5F3 could inhibit binding of binding of radiolabeled ApoE to the VLDLr *in vitro*[221]. Since none of these antibodies were evaluated *in vivo*, we used a mixture of the antibodies to block the VLDLr *in vivo* after an experimental MI. After the mice anesthetized, intubated, connected to a ventilator and shaved the antibody injection was made intraperitoneally according to Abbate and colleagues[222]. The mice were injected with 100µl antibody-saline (0,9%) or only saline solution, the antibody-saline solution had a concentration of 2,3µg/µl antibody.

Tissue Preparation (paper I)

For analysis of cardiac tissue post MI, mice were killed with an overdose of isoflurane. Swiftly whole blood was collected from the hearts before the hearts were removed, rinsed in cold PBS, weighed, embedded in OCT compound to protect from freeze drying and slowly frozen in liquid nitrogen to avoid tissue cracking. The samples were stored in -80°C until analysis.

For immunohistochemical analysis the hearts were cryosectioned into 8µm slices, transferred to polysine glass slides and let to air fix for 12 hours before storing them in -20°C until staining.

For mRNA and protein extraction the whole hearts were homogenized using Quiagen Tissuelyser using a 5mm steel bead with the settings 2 times 2min at 25Hz still kept frozen using dry ice. After homogenization extraction buffers were added and the solutions brought back to room temperature for mRNA and protein extraction respectively.

Analysis of Area at risk and Infarction size (paper I)

For analysis of infarct size and area at risk myocardial infarctions were performed as described above. 6 hours after occlusion the mice were sedated and coupled to a ventilator. The chest was opened and Evans blue (0.2ml, 2%) was injected into the right ventricle resulting in coloration of all perfused tissue, leaving the ischemic zone (the area at risk) uncoloured. Then the heart was excised and rinsed in saline. The hearts were then frozen in -20°C and thereafter sliced into 1mm slices. The slices were incubated in triphenyltetrazolium (1%) solution at 37°C for 15min. The tetrazolium stains the surviving tissue red and the infarcted tissue is tetrazolium negative and displays as a pale white/yellowish colour [223]. The infarct size is

calculated as a percentage of the area at risk. The calculations were made using the Image J software after staining. The results are presented as area at risk as percentage of left ventricle area and infarct size as percentage of area at risk. The measurement of area at risk is an indirect measurement of the reproducibility of the surgeon.

Human biopsies

Human heart biopsies

Ischemic heart biopsies were obtained from patients that were scheduled to undergo coronary artery bypass surgery due to atherosclerosis in the coronary vessels. All the patients gave informed and written consent and the experimental protocol was approved by the Gothenburg Regional Ethics Committee and performed according to the declaration of Helsinki, the ethical decision registration number is 187-01. 79 patients were enrolled in the study and angiograms of these patients revealed significant coronary atherosclerosis within the left anterior descending artery and/or the left coronary artery and/or branches from these vessels. The biopsies were taken from the left ventricle septum region, since it is the thickest part of the heart. The area chosen for biopsy collection was affected by ischemia but not fibrotic. The biopsies were collected using a fine needle for biopsy collection (1 mm diameter) and the collected sample was on average 100mg tissue. Of 79 biopsies, 27 were excluded because the biopsy was too small or because the RNA yield was insufficient or was of unacceptable quality. Therefore, 52 biopsies were analyzed for VLDLr expression and lipid content. A summary of patient characteristics are present paper I supplemental table 5.

Non-ischemic control biopsies were collected from the left ventricle of patients undergoing orthotopic heart transplantation. The aim of the study was to investigate tissue rejection after transplantation[224]. All the patients gave informed and written consent and the experimental protocol was approved by the Gothenburg Regional Ethics Committee and performed according to the declaration of Helsinki. Immunosuppressive treatment included cytolytic induction therapy followed by a triple-drug maintenance regimen with cyclosporine or tacrolimus, azathioprine or mycophenalate mofetil and corticosteroids. Routine endomyocardial biopsies were performed according to the following schedule: weekly during the first six weeks, every other week from week 6 to month 3 and monthly from month 3 to month 6. Biopsies were examined by experienced pathologists and graded according the 1990 working formulation of the International Society of Heart and Lung

Transplantation (ISHLT)[225]. Tissue samples and serum were stored at -70°C until analysis. Episodes of rejection grade 0 at 4 months after transplantation were used.

Non-ischemic heart biopsies from the left ventricle were also obtained from 5 patients undergoing aortic valve replacement. These subjects all had angiography-verified absence of coronary artery disease in any major myocardial coronary artery branch. The experimental protocol was approved by the Gothenburg Regional Ethics Committee and performed according to the declaration of Helsinki.

Human kidney biopsies

Renal tissue was obtained as described for the isolation of primary kidney cells described above.

For immunohistochemical analysis the biopsies were frozen and stored at -70°C degrees until analysis. The kidney biopsies were cryosectioned in 5µm slices and stored at room temperature until further analysis. For isolation of primary kidney cell cultures see below.

SUMMARY OF RESULTS

Paper I

In this paper we wanted to investigate the role of the VLDLr in ischemic/hypoxic cardiomyocytes.

In this paper we used *in vitro* and *in vivo* models to investigate the lipid accumulation in cardiomyocytes in response to hypoxia, we focused on the origin of the intracellular lipid and the consequences of the lipid accumulation. We could show, in line with previous studies that in response to hypoxia cardiomyocytes *in vivo* and *in vitro* accumulate intracellular lipid. The lipid accumulation primarily consists of an increase in the intracellular TG content. The TG did not seem to origin from an increased *de novo* lipogenesis, but rather from an increased uptake of extracellular triglyceride rich lipoproteins (TGL). The uptake of extracellular TGL was mediated by VLDLr which was significantly upregulated in response to hypoxia. Ablation of the VLDLr significantly reduced the intracellular TG levels both *in vitro* using siRNA knockdown in hypoxia-treated HL-1 cardiomyocytes and *in vivo* in VLDLr$^{+/+}$ and VLDLr$^{-/-}$ mice after a MI. We could also see that we had an increased expression of the VLDLr in human heart biopsies from ischemic patients compared to non ischemic patients and that the expression of VLDLr was correlated to the intracellular lipid content, indicating that the phenotype is important in human as well as in mice. We also investigated the regulation of the VLDLr during hypoxia and found indications that Hif-1α binding to a promoter HRE was responsible for the hypoxic upregulation. Aiming to evaluate the effect of the increased lipid accumulation during hypoxia we found that the VLDLr$^{-/-}$ mice had an improved survival compared to VLDLr$^{+/+}$ mice after MI and that the infarcted area was significantly smaller even though the area at risk was the same. To follow up on why the VLDLr$^{-/-}$ mice survived better we investigated several markers known to cause tissue damage and apoptosis. We found that the amount of very long chain ceramides known to be associated with reduced survival during hypoxia was decreased in the hearts of VLDLr$^{-/-}$ mice together with reduced markers for UPR compared to VLDLr$^{+/+}$ mice. In our experiments we also found that the toxic ceramides induced ER-stress and that VLDLr$^{-/-}$ mice had reduced apoptosis markers together with reduced apoptosis after MI. Finally we could show that blocking of the VLDLr *in vivo* reduced the lipid accumulation, the ER-stress and the apoptosis after MI in mice.

In summary we show in paper I that the VLDLr induce lipid accumulation after a MI and that lack of VLDLr reduce the lipid accumulation resulting in an improved survival possibly due to a reduced ER-stress and ceramide mediated apoptosis reducing the infarcted (non functional) part of the heart. Furthermore we could show that this phenotype is also present in humans and that the VLDLr is a possible drug target.

Paper II

In this paper we wanted to investigate the regulation of the VLDLr under hypoxic conditions and map the regulatory elements in the VLDLr promoter.

In this paper we thoroughly investigate the regulation of the VLDLr during hypoxia. We showed in paper I that the VLDLr is significantly upregulated during hypoxia in cardiomyocytes as well as in other cell types. We also showed that VLDLr is likely to be regulated by Hif-1α, but we could not see if the regulation was direct or indirect. In this paper we showed that the VLDLr expression in HL-1 cardiomyocytes in response to hypoxia is regulated not by a classical HRE (ACGTG) but by a variant HRE (TGCGTG) located at -162 to -158bp from transcription start. We could see that the binding of Hif-1α to this site was significantly increased during hypoxia and that mutation of this site muted the promoter response to hypoxia. We could also exclude several other transcription factors such as PPARγ and SP1 which have been suggested to regulate the VLDLr. We could also exclude other classical HRE elements located within the first intron and exon from being important for hypoxic VLDLr regulation. Finally we found the same HRE (TGCGTG) in the human VLDLr promoter and could show that likewise to the mice promoter the binding of Hif-1α to this site was significantly increased during hypoxia.

In summary we showed in paper II that the VLDLr is regulated by Hif-1α during hypoxia through binding to an HRE in the VLDLr promoter. We also showed that the same site was active in the human gene, however located further upstream.

Paper III

In this paper we wanted to investigate if the VLDLr is involved in the intracellular lipid accumulation characteristic for clear-cell renal carcinoma and if this regulation is Hif-1α dependent.

In this paper we wanted to extend our studies on the VLDLr beyond the heart. In paper I and II we showed that the VLDLr expression is upregulated due to hypoxia and that this upregulation is mediated through Hif-1α, we also showed that the receptor mediates uptake of extracellular lipoproteins causing accumulation of intracellular lipid. In this paper we studied clear-cell RCC using human kidney biopsies and isolated human primary kidney cells. Clear-cell RCC is interesting in the aspect of VLDLr expression since the cancer has a constitutive Hif-1α activity combined with a massive accumulation of intracellular lipid and being one of the most common kinds of kidney cancer. We wanted to investigate if an upregulation of VLDLr through Hif-1α was involved in the intracellular lipid accumulation. We showed that the lipid accumulation as well as the VLDLr expression was significantly increased both in biopsies from clear-cell RCC patients compared to healthy kidney as well as in isolated human primary kidney cells from clear-cell RCC patients. When investigating the expressional regulation we found that if the Hif-1α expression was knocked down using siRNA in the primary clear-cell RCC cells the VLDLr expression was significantly reduced as well as the intracellular lipid and the uptake of extracellular TGL. This indicates that the clear-cell RCC has a constant overexpression of VLDLr which cause an accumulation of intracellular lipid. We also investigated the intracellular lipid species and found that even though the amount of TG was significantly increased in the clear-cell RCC, as seen in the heart, the major lipid content consisted of cholesteryl esters as has been described previously. This explains why the reduction in intracellular lipid in response to Hif-1α and VLDLr siRNA treatment was significant but not complete.

In summary we showed in paper III that clear-cell RCC have a constant upregulation of the VLDLr expression and that the uptake of extracellular lipoproteins mediated through the VLDLr is partly involved in causing the massive lipid accumulation typical for clear-cell RCC. In humans these results could be of benefit by possibly using VLDLr as a biomarker for clear-cell RCC or as a target for specific drug uptake into the cancerous tissue.

DISCUSSION

In this thesis I have presented results regarding lipid accumulation in the myocardium. I have shown that the VLDLr is upregulated in response to hypoxia and that this upregulation is mediated by Hif-1α. The increased VLDLr expression leads to accumulation of ectopic lipid droplets in the cardiomyocytes and is accompanied by a reduction in survival, an increase in ER-stress and apoptosis. I have also shown that the ablation of the VLDLr counteracts the harmful effects and that the upregulation of VLDLr in response to Hif-1α is not a process that is isolated to cardiomyocytes.

The results raise the following questions that will be discussed. (1) Why is the VLDLr upregulated in response to hypoxia? (2) How does the VLDLr cause lipotoxicity and ER-stress? And finally (3) can the VLDLr be used as a drug target?

(1) Why is the VLDLr upregulated by hypoxia?

Our results have shown that hypoxic upregulation of the VLDLr mediate toxic lipid accumulation within the cardiomyocytes, then why the VLDLr is induced even though it is obviously harmful for the myocardium is not straight forward. One theory is that the VLDLr is upregulated to make lipids available for the heart after the ischemic attack is passed. Since the heart primarily relies on lipids as a fuel source it could be beneficial for the heart to have a limited store of lipid available to rapidly mobilize when oxygen returns. However this theory is unlikely, our studies have indicated that even though the $VLDLr^{-/-}$ mice have an improved survival acutely after MI there is no difference in the function of the heart long term after MI. If it was beneficial for the heart to have stored lipid for extra fuel boost, firstly the $VLDLr^{+/+}$ mice should have had an improved cardiac function compared to $VLDLr^{-/-}$ mice and secondly the lipid storage capacity of the heart should not have been so tightly regulated[31].

The upregulation of the VLDLr during hypoxia could also be a stress response due to the reduction in ATP production. Ischemia is a combination of oxygen and glucose deprivation and VLDLr have been shown to be upregulated during situations of glucose starvation[171] and hypoxia[113, 149, 226]. Hypoxia has been described to induce AMPK, which is a well-known energy sensor in skeletal muscle and the heart[227]. AMPK can be switched on by an increase in the AMP/ATP ratio due to interference with ATP production like hypoxia, glucose deprivation, ischemia and muscle contraction i.e. exercise[52, 227-229]. Upon activation by phosphorylation AMPK is a central component of a protein cascade which phosphorylates and inactivates key enzymes of TG synthesis and cholesterol synthesis but activates FA oxidation and glucose transport and uptake. AMPK has

been shown to induce FA transporter translocation[52] and also VLDLr expression[171]. So far no data show that AMPK regulate VLDLr expression during hypoxia, however there is an indirect relationship where AMPK upregulate p38 MAPK[229] which have been indicated to induce VLDLr expression[230]. Furthermore in the VLDLr promoter region there have yet not been isolated any regulatory elements known to respond to AMPK activation, like CREB and MEF2 and siRNA against AMPK did not influence the hypoxic VLDLr expression (Perman, unpublished results). However AMPK can activate peroxisome proliferator receptor associated protein 1α (PGC-1α)[231] which in turn can stabilize Hif-1α through decreasing the activity of prolyl hydroxylase[232] (Figure 8). Unpublished data show that PGC-1α mRNA expression is upregulated during hypoxia in HL-1 cardiomyocytes during hypoxia and that siRNA knockdown of PGC-1α reduced the VLDLr expression. Unfourtenately upregulation of PGC-1α also increase mitochondrial biogenesis leading to increased oxygen consumption, worsening the already hypoxic situation[232]. Another stress situation associated with hypoxia is ER-stress[233, 234] which we and others showed could upregulate VLDLr expression independent of hypoxia[235]. Two important mediators of ER-stress (XBP1 and ATF6) have been shown to contain ER-stress responsive elements (ERSE) in their promoter. The ERSE binds transcription factor nuclear factor Y (NF-Y)[235] and have been shown to be present in the VLDLr promoter as well[11, 172]. The presence of a NF-Y binding site (CCAAT) in the VLDLr promoter can explain why VLDLr is upregulated by ER-stress and hypoxia.

Another possible explanation could be that the VLDLr is important for internalizing molecules that could protect the heart during ischemia such as retinoic acid, which has been shown to be protective against hypoxic injury[80, 236]. Retinoic acid (vitamin A and its analogues) play critical roles in ensuring that normal cardiac development takes place during embryogenesis and in adult heart. The heart, like other tissues is incapable of *de novo* synthesis of retinoids, all of the retinoids present in the heart must be derived from the circulation. A significant portion of the retinoid present in heart tissue can be derived from retinyl ester transported in the core of chylomicrons[80]. We could show in unpublished results that the uptake of retinoic acid was induced in response to hypoxia and that this induction could be ablated by treatment with VLDLr knockdown. Since retinoids are important for cardiac development and the VLDLr is expressed already prenatally[110] the purpose of VLDLr might be retinoid uptake.

We could show in our data that the upregulation of the VLDLr during hypoxia was not confided to mouse cardiomyocytes but was also present in human cardiomyocytes and in many other cell types. The general induction of the VLDLr expression during hypoxia must mean that the upregulation is purposeful, otherwise it should have been evolutionary suppressed. To look at the Hif-1α regulation of the VLDLr expression in another tissue we used the clear-cell RCC model[204]. Clear-cell RCC is molecularly recognized by a loss of function mutation in pVHL[205-207].

pVHL is one of the players crucial for the degradation of Hif-1α in normoxic conditions. In accordance with our previous data on Hif-1α regulation we could see an upregulation of the VLDLr expression in these cells and by knocking down Hif-1α could see that the upregulation was indeed mediated by the proposed pathway. The functionality could also be shown through that we could reduce the uptake of extracellular lipoproteins by this treatment. These data underlines that the VLDLr is indeed regulated by Hif-1α and that this holds true not only for the myocardium.

(2) Cardiac lipotoxicity in relation to the VLDLr

Since lipid accumulation in the myocardium has been described to be harmful to the heart[237] and since hypoxia increase intramyocardial lipid we investigated how MI would affect the VLDLr$^{-/-}$ compared the VLDLr$^{+/+}$ mice. We found that not only the lipid content in the VLDLr$^{-/-}$ mice was reduced but they also survived significantly better after MI compared to the VLDLr$^{+/+}$ mice. The knockout mice also had a smaller infarct area even though the area at risk was the same. The infarct size has been shown to be an important determinant in the survival after MI[238, 239] and the smaller infarct size in the VLDLr$^{-/-}$ mice is likely of great importance to the improved survival. To elucidate the reason for the improved survival we investigated parameters previously suggested to cause tissue damage such as content of ROS, total ceramide content and ceramide species[240, 241].

Reduced oxygen pressure has for a long time been known to cause lipid accumulation in many different cell types[242] such as in cardiomyocytes[42] and also in intact hearts[49]. The increase in cardiomyocyte intracellular lipid seen in our studies was due to an increase in intracellular TG without any significant increase in any other lipid classes. TGs are inert lipids in its storage form and are not likely to harm the cells[243, 244]. Even so, lipid accumulation in the myocardium and in other non adipose tissue have been shown to be involved in tissue dysfunction[27, 56, 240, 245]. When the TG pool increases there is a concomitant increase in the TG hydrolysis and reestrification inducing the intracellular amount of intermediates such as DG and FAs[246] which together with ceramides that can also accumulate are toxic to the cell.

In situations of excessive intracellular FA accumulation, such as when FA oxidation is low or the uptake of lipid is increased FA can be shunted into synthesis pathways such as ceramide synthesis and TG synthesis. Accumulations like these can lead to lipotoxicity and any means at reducing accumulations like these have been shown to prevent lipotoxicity and reduce tissue damage[240]. Lipid accumulation in the myocardium has been shown to be followed by ceramide accumulation, apoptosis and impaired myocardial contractility[46, 80]. When analysing the ceramide content we found that even though there were no difference in the amount of total ceramides there was a significant differences in certain ceramide

species. Very long chain ceramides (C24-26 ceramide) have been shown to be associated with a reduced survival in relation to low oxygen pressure in *C. elegans*[85, 86]. We could see that the amount of very long chain ceramides was reduced in VLDLr$^{-/-}$ mice after MI. In line with these results are other studies in mice that have showed that not only the total pool of ceramides but also the ceramide species are important[247]. Previous studies have not isolated how the different ceramide species affect the cell even though ceramide accumulation in general has been associated with apoptosis[80].

Excessive ceramide accumulation as well as hypoxia as such is capable of inducing ER-stress[71, 248]. Our data show that hypoxia induce ER-stress, VLDLr upregulation and lipid droplet formation. Our data also indicate that the VLDLr upregulation induce ER-stress by causing lipid accumulation of toxic very long chain ceramides both *in vitro* and *in vivo*.

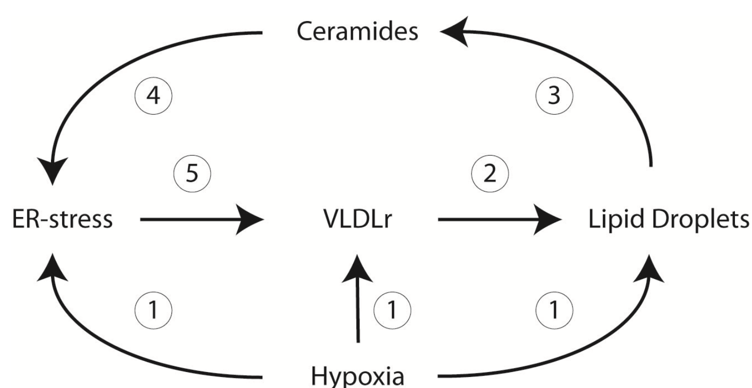

Figure 9. ER-stress results loop.

The results considering ER-stress and the VLDLr expression are confusing (Figure 9). Hypoxia induces VLDLr expression and ER-stress *(1)*, ER-stress induced by ER-stress stimulator in normoxia induces VLDLr expression *(5)* but ablation of VLDLr expression *in vivo* and *in vitro* reduces ER-stress *(2-4)*. This indicates the existence of a feedback loop where the effect of hypoxia on ER-stress can be amplified but it also renders us with a “chicken or the egg dilemma”, what comes first during hypoxia, the ER-stress or the VLDLr expression? It is possible that hypoxia independently instigate both ER-stress and VLDLr expression however our results show that, once started they are not independent of each other. Since the magnitude of the ER-stress in response to hypoxia is quite modest compared to the response seen when stimulating with ER-stress inducers it could be argued that the VLDLr expression drives the ER-stress *(2-4)*, this would also explain why ablation of the VLDLr expression renders the ER-stress at baseline (normoxic) level i.e. we have no ER-stress. However, ER-stress induced by the VLDLr is dependent on hypoxic conditions. When overexpressing VLDLr during normoxia ER-stress could

not be detected, this could depend on the β-oxidation being efficient enough during normoxia to oxidize the internalized lipids.

Hypoxia as well as myocardial infarction with ischemia/reperfusion has been shown to induce ER-stress both through the XBP-1 branch and increasing CHOP[92] and the ATF6 branch[94]. The ATF6 branch is initially a purely pro-survival pathway[89], which could explain why it is seen activated in both ours and other's models of ER-stress, however during prolonged ER-stress all branches of the ER-stress can trigger apoptosis as we and others detect[249]. In our studies we use permanent occlusion where we do see an ER-stress response however not very pronounced compared to chemical stimulation. Compared to other studies where a more pronounced response is seen is that they have used and ischemia/reperfusion model which showed to induce a more pronounced ER-stress compared to only occlusion[92]. One reason for the more pronounced response seen in an ischemia-reperfusion model is the additional effect of ROS which has been shown to be induced by ischemia-reperfusion[250, 251] and to induce ER-stress by altering Ca^{2+} homeostasis[252], and cardiac failure[241], however ROS is not pronounced in our model and does not show any difference in the presence or absence of the VLDLr. Apart from reduced ATP levels and distorted ER Ca^{2+} levels which are known to be caused by hypoxia and to cause ER-stress, hypoxia also inactivates prolyl hyrdoxylases (PHs), which are part of the hypoxic activation of Hif-1α (Figure 8). Inactivation of PH by DMOG for instance activates the ER-stress[95]. PH inhibition also increases the VLDLr expression. However this pathway primarily explains the hypoxic activation of both pathways rather than how ER-stress can induce VLDLr expression.

These results tells us that lacking the VLDLr during low oxygen pressure reduce the intracellular amount of very long chain ceramides which otherwise would have induced ER-stress which is associated with tissue damage[92-94]. We also found reduced caspase production as well as reduced apoptosis in $VLDLr^{-/-}$ mice after MI.

(3) The VLDLr as a drug target

Our research brings forward the VLDLr as a possible drug target both in the case of myocardial infarction and in the case of cancer. Firstly the results from our first study show that absence of VLDLr is beneficial in the case of a myocardial infarction. The effect of the protection of the VLDLr seems to lie in the reduced uptake of extracellular lipids and thereby the reduced accumulation of toxic ceramide species leading to pronounced ER-stress and cell death. Since the VLDLr expression is correlated to the amount of intracellular lipid also in humans, the possibility of finding a way to block the VLDLr is intriguing. Secondly in our second and third study we show that the VLDLr is regulated by Hif-1α which is constitutively upregulated in clear-cell RCC. In this case the VLDLr could be used

as for targeted drug delivery to the cancerous tissues or as a biomarker for the cancer.

In the first case, the obvious follow-up question out of a clinical perspective is whether VLDLr can be blocked to inhibit intracellular lipid accumulation post MI? Can we create a VLDLr-blocking drug and would such a drug create the same effects as removing the VLDLr in total?

Indeed we could show that by treating VLDLr expressing mice with antibodies against the VLDLr we could block the uptake of extracellular lipids and reduce the ER-stress response together with the apoptosis. Whether this effect was mediated by a reduction in lipotoxic ceramides remains to be clarified. The lipotoxicity of ceramides have for long been described[240], however more recently the importance of different ceramide species for toxicity have been shown to be important[86, 247]. Since efficient blocking of the VLDLr is possible using antibodies it is not unlikely that chemical compounds doing the same job would be possible to find. It is already known that VLDLr expression can be blocked by RAP a well known blocker of several receptors in the LDLr family[122]. However if VLDLr inhibition should be used as a pharmaceutical treatment the specificity is important, which is the case for the antibodies used[221] but not for RAP[122].

Since the VLDLr is not solely expressed in the heart but also in other tissues active in fatty acid oxidation (such as muscle and adipose tissue) systemic treatment with VLDLr blocker could disrupt lipid uptake in other tissues. However this problem could partly be overcome by the fact that the heart, in case of an MI, is hypoxic and therefore the VLDLr expression is significantly increased there, together with the heart having a big blood-demand to be properly supplied with nutrients. One could argue that much of the VLDLr blocker would end up blocking cardiac VLDLr. However, MI is not unknown to occur in patients with elevated plasma lipid levels as well as obese and atherosclerotic patients Goudriaan and colleagues described that VLDLr$^{-/-}$ animals are protected from diet induced obesity through a decrease in uptake of FAs in peripheral tissue[131]. They also saw an increased plasma TG, but not cholesterol nor FA in VLDLr$^{-/-}$, these observation was not shared by Frykman et. al. who described no difference in plasma lipoproteins in chow fed, high fat diet fed nor high carbohydrate fed VLDLr$^{-/-}$ mice[129]. Albeit the latter study was on a significantly shorter time span (2 weeks) compared to 17 weeks. Perhaps the possible side effects of blocking VLDLr would be to also block peripheral tissue lipid uptake which might not be altogether bad. Goudriaan's studies showed that even though plasma TG was increased, adipose was reduced and insulin sensitivity remained compared to VLDLr$^{+/+}$[131] perhaps treatment with VLDLr blocker would give similar effects. On the other hand increasing plasma TG is not necessarily a good idea since plasma TG is correlated to cardiovascular disease[253]. Even though VLDLr$^{-/-}$ animals was shown to have no difference in atherosclerotic lesion formation the intimal thickness was increased in the VLDLr$^{-/-}$ animals[159] and

intimal thickness has been shown to proceed atherosclerosis formation[254]. Unfortunately no studies have been made regarding the atherosclerosis development in VLDLr$^{-/-}$ mice on a high fat diet. However, if treatment with VLDLr blocker was not chronic but only for patients in high risk of getting MI, the slight increase in risk for induced atherosclerosis development might be worth taking. Nevertheless treating infarcted mice with VLDLr blocker most obviously improves survival.

In the second case, the follow-up question would be can the VLDLr be used as a delivery port for tissue specific drug delivery? Can such a drug be created so that it will not be taken up into other tissues as well?

We could show that the hypoxic upregulation of VLDLr is not cardiac restricted and that the regulation is mediated through transcription factor Hif-1α. Clear-cell renal carcinoma is the most common type of renal cancer[204]. It has gotten its name by the typical lipid filled cytoplasm appearing clear in immunohistochemical staining[210]. Another feature of clear-cell RCC is the loss of function mutation in pVHL giving rise to a constitutive activity of Hif-1α[205-207]. Clear-cell RCC is one of the most hard to treat cancer forms. There are very few approved treatments, one of them being high-dose interleukin-2 treatment which has shown to be quite toxic to the patients[217]. A possible way to overcome possibly harmful systemic treatment is tissue specific, or tumour specific treatment. It is not impossible that the VLDLr could be used for such a purpose. However even though clear-cell RCC is characterized among other kidney cancers as being pVHL deficient, this malfunction is not specific for clear-cell RCC among other cancers. The loss of function in pVHL can be sporadic / nonhereditary or depend on an inherited pVHL deficiency called von Hippel-Lindau disease. The inherited pVHL disease is associated with not only kidney cancer but tumours in retina, cerebellum, spinal cord and pancreas just to mention some. If the upregulation of VLDLr by Hif-1α would occur in all tissues overexpressing Hif-1α, the aimed for kidney specific treatment would be hard to produce. Another problem would of course be if there were hypoxia mediated upregulation of the VLDLr in other tissues such as the heart, toxic compounds aimed at destroying cancerous tissues would end up where you absolutely did not want them.

As a drug target, the VLDLr is likely most suitable as a treatment for post MI fatality.

CONCLUSION

We have for the first time identified the VLDLr as a key receptor in the pathological hypoxic lipid accumulation.

We have shown that the VLDLr is upregulated in response to Hif-1α activity. Hif-1α activity can be induced by hypoxia, stabilizing agents such as DMOG or mutations in pVHL, a protein important for Hif-1α degradation. The most prominent effect of VLDLr upregulation that we have seen in these studies is the effect of the VLDLr in the hypoxic heart. The VLDLr is upregulated after a myocardial infarction and mediates increased uptake of extracellular lipoproteins into the cardiomyocytes. The accumulation gives rise to an increase in cardiotoxic ceramides which cause ER-stress in the cardiomyocytes inducing ER-stress with following apoptosis, tissue death and reduced survival.

On the bright side, the VLDLr can be blocked *in vivo* reducing the lipid accumulation, ER-stress and apoptosis similar to a knockout model. And since the VLDLr seems to be active in the human ischemic myocardium as well, it could possibly be used as a drug target.

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