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LIPID MICRODOMAINS IN INSULIN RECEPTOR SIGNALLING

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ACADEMIC DISSERTATION

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To my Dad

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ORIGINAL PUBLICATIONS

I Vainio S, Heino S, Månsson J-E, Fredman P, Kuismanen E, Vaarala O, and Ikonen E. Dynamic association of human insulin receptor with lipid rafts in cells lacking caveolae. EMBO Reports, 2002, 3(1):95-100

II Vainio S, Bykov I, Hermansson M, Jokitalo E, Somerharju P, and Ikonen E. Defective insulin receptor activation and altered lipid rafts in Niemann-Pick type C disease hepatocytes. Biochemical Journal, 2005, 1;391(Pt 3):465-72

III Vainio S, Jansen M, Koivusalo M, Róg T, Karttunen M, Vattulainen I, and Ikonen E. Significance of sterol structural specificity: Desmosterol cannot replace cholesterol in lipid rafts. Journal of Biological Chemistry, 2006, 281(1):348-55

ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	acyl-coenzyme A:cholesterol acyl transferase
BCR	B-cell receptor
CHO	Chinese hamster ovary
DM	diabetes mellitus
DPH	1,6-diphenyl-1,3,5-hexatriene
DRM	detergent-resistant membrane
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FFA	free fatty acid
FRAP	fluorescent recovery after photobleaching
FRET	fluorescent-resonance energy transfer
GLUT	glucose transporter
GPI	glycosyl phosphatidylinositol
GSL	glycosphingolipid
HDL	high-density lipoprotein
HMG	3-hydroxy-3-methylglutaryl
IR	insulin receptor
IRS	insulin receptor substrate
Ld	liquid-disordered
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
Lo	liquid-ordered
NPC	Niemann-Pick type C
OD	optical density
PC	phosphatidylcholine
PE	phosphatidylethanolamide
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3-phosphate
PKC	protein kinase C
PPARγ	peroxixome proliferator-activated receptor γ

So	solid-ordered
SM	sphingomyelin
SR-B1	scavenger receptor B1
TNFα	tumor necrosis factor alpha
TCR	T-cell receptor
VLDL	very low-density lipoprotein

ABSTRACT

Obesity, metabolic syndrome, insulin resistance and type 2 diabetes, are ever growing health problems worldwide. The links between high fat diet, disorders of lipid metabolism and insulin resistance are well established, but the underlying mechanisms are multiple and currently only partially understood. The liver plays a major role in controlling both the glucose and lipid – especially cholesterol – homeostasis of the body, and is thus also central to the development of the aforementioned disease states.

Cholesterol is an essential molecule for mammalian cells, and its most important role is to function as a membrane component. Recent studies suggest that cholesterol can induce lateral segregation of membrane components into microdomains, which are commonly called lipid rafts [1]. These microdomains are thought to form because of favorable interactions between cholesterol and certain other membrane lipids (mostly sphingolipids) and to have a more ordered structure than the rest of the membrane. The domains include a subset of proteins based on how well they fit into this ordered environment, while excluding others. They have been suggested to function in several cellular processes, e.g. signal transduction [1, 2].

The insulin signaling cascade is one of the pathways proposed to function via lipid rafts [3, 4]. Thus far, research in the field has concentrated on adipocytes. In the current study, we aimed at characterizing the role of lipid rafts in insulin signaling in hepatocytes. First, by using a cell line of hepatic origin, we demonstrated that the insulin receptor (IR) associated with detergent-resistant membranes (DRMs; operational definition for lipid rafts) in its active form and the activation of the receptor was sensitive to treatments affecting lipid raft constituents. Decreased activation of IR was associated with decreased DRM-recovery of the receptor.

Second, we studied the function of IR in primary hepatocytes from control and Niemann-Pick type C (NPC) disease mice. The NPC disease is an inherited late endosomal/lysosomal cholesterol storage disorder [5], and we used the NPC cells as a model for intracellular cholesterol imbalance. We found that in NPC hepatocytes, the levels of IR were up-regulated but its activation compromised. The activation defect

was present also in isolated plasma membranes from NPC mouse livers. Lipid compositional analysis revealed that the NPC plasma membranes were enriched in cholesterol and saturated phospho- and sphingolipid species, and accordingly, their fluidity was decreased. IR exhibited increased DRM-association in NPC cells, and removal of cholesterol from the NPC plasma membranes significantly improved IR activation.

Third, we addressed the special structural characteristics of cholesterol by comparing it with its immediate biosynthetic precursor, desmosterol. These two sterols differ only by the presence of an addition double bond in the tail of desmosterol, but experiments in liposomes revealed that desmosterol had a lower capacity for ordered domain formation than cholesterol. In cells, desmosterol was present at the plasma membrane but almost completely excluded from DRMs. When the majority of plasma membrane cholesterol was exchanged for desmosterol, IR activation became severely compromised and its DRM-association was abolished. Atomic-scale molecular dynamics simulations revealed that the double bond in the tail of desmosterol makes the molecule significantly more tilted than cholesterol and decreases its ordering potential. Together, the data shows that the function of IR in hepatocytes is highly sensitive to changes in its membrane microenvironment and that intracellular lipid accumulation can result in changes in the lipid composition of the plasma membrane. These findings may have implications for the pathogenesis of insulin resistance, especially in the light of the so-called “membrane hypothesis of insulin resistance”, formulated already twenty years ago [6].

REVIEW OF THE LITERATURE

1. Cell membranes and cholestesterol

Cholesterol is essential for the growth and viability of all mammalian cells. In addition, all other eukaryotic cells contain a sterol of some kind, like ergosterol in yeast [7], indicating that sterols are necessary for higher forms of life in general. In the human body, cholesterol is needed for such things as an antecedent for steroid hormones, bile acids, vitamin D and oxysterols, i.e. oxidated derivatives of cholesterol that have important regulatory roles in many cellular processes [8]. Cholesterol is also a central molecule in embryonic development, and genetic errors in cholesterol biosynthesis result in devastating phenotypes [9]. One of the underlying reasons appears to be that the key morphogen Hedgehog contains a cholesterol modification which is essential for its function [10]. However, probably the most important role of cholesterol (and other sterols) is to function as a component of cellular membranes [11, 12]. This may indeed be the reason why sterols have evolved, but the membrane effects also make cholesterol harmful to cells when it is present in excessive amounts [13].

1.1 Cellular membranes

1.1.1 Basic membrane structure and components

Cell membranes are bilayers with an asymmetrical distribution of constituents in their two leaflets. The basic types of lipids in the membranes are glycerophopholipids, sphingolipids and cholesterol (Figure 1). Glycerophospholipids consist of a glycerol backbone with two fatty acids and a polar phosphate-containing head group linked to it. Glycerophospholipids are classified according to their head group structure, the most abundant class in mammalian cells being phosphatidylcholine (PC). Other classes include aminophospholipids (e.g. phosphatidylethanolamide, PE, and phosphatidylserine) and phosphapatidylinositide, and within each class, there are species with varying chain lengths and degrees of unsaturation. In most naturally occurring PCs, the two hydrocarbon chains are nearly similar in length. The acyl chain in the *sn1*-position is usually saturated, while the *sn2* fatty acid is unsaturated [14, 15]. The unsaturated fatty acid typically contains 1 – 6 double bonds in the *cis*-configuration.

Sphingolipids consist of a long-chain sphingoid base (sphingosine in most naturally occurring sphingolipids) with an amide-linked fatty acyl side chain, and a polar head group. A sphingolipid with a phosphatidylcholine head group is called sphingomyelin (SM; the only sphingolipid belonging to the group of phospholipids), whereas sphingolipids with carbohydrate-containing head groups are collectively called glycosphingolipids (GSLs, including e.g. gangliosides and cerebrosides). Although the basic hydrophobic-hydrophilic structure of glycerophospholipids and sphingolipids is similar, there are important differences. The fatty acid chain in sphingolipids is often very long (20 – 24 carbons), longer than the hydrocarbon chain of the sphingoid base, which leads to an intramolecular chain-length mismatch [16, 17]. The fatty acyl is also normally saturated or monounsaturated, as is the sphingoid base itself, and thus, natural sphingolipids as a group are more saturated than glycerophospholipids [18]. Further, the interfacial region in sphingolipids is more polar than in glycerophospholipids and contains both donor and acceptor groups for hydrogen bonding [19, 20].

Cholesterol, in turn, differs structurally from the other two types of lipids. It consists of four hydrocarbon rings – three six- and one five-membered ring – which form the so-called sterol backbone, and an iso-octyl side chain. The rings of cholesterol are joined by single bonds whose substituents are in the *trans*-configuration, allowing the ring structure to adopt a planar conformation. A double bond between C5 and C6 also affects the conformation and appears to be one of the essential structural features of cholesterol [21]. The 3-β-hydroxyl group in cholesterol converts the otherwise highly hydrophobic molecule into an amphiphile, and orients it in the membranes. In addition, it can mediate hydrogen bonding with water and possibly other membrane lipids [20, 22]. When introduced into lipid bilayers, cholesterol intercalates between the hydrocarbon parts of the other lipids, filling in the flickering spaces between the acyl chains. Because of its rigid planar structure, cholesterol increases the order of the neighbouring acyl chains, making the membranes laterally more condensed and more densely packed [23-25]. Thus, the physicochemical properties of the membrane are altered; in particular, permeability is decreased and mechanical strength and rigidity increased [11, 12]. Cholesterol also affects the phase behaviour of membrane lipids

(discussed in Section 2.1.2). The amount of cholesterol in a membrane is therefore one of the main determinants of the membrane's physical characteristics, however the degree of unsaturation of the constituent glycerophospho- and sphingolipids also affects these properties [15].

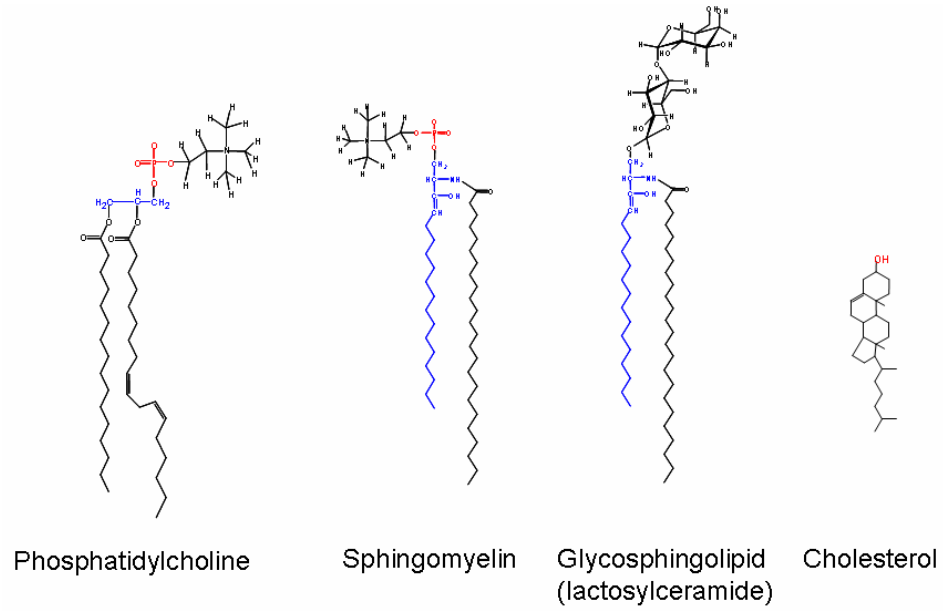

Figure 1. Basic structures of the membrane lipids of mammalian cells. The glycerol and sphingosine backbones are shown in blue and the phosphate groups in phospholipids and the 3-β-hydroxyl group in cholesterol in red.

In addition to lipids, cell membranes contain a variety of proteins. Membrane proteins can be integral (embedded in or traversing the membrane), or peripheral (connected to the membrane via an anchor that can consist of lipids, motifs in the protein structure, or both). Lipid-anchored molecules include glycosyl phosphoinositide (GPI) anchored proteins on the exoplasmic leaflet, and doubly acylated signaling kinases on the cytosolic side. Integral membrane proteins can also be lipidated (e.g. palmitoylated on cysteine residues), and these modifications can affect the protein-membrane interaction and/or serve to regulate the function of the proteins [26]. A special part of the actin cytoskeleton, called the membrane cytoskeleton, localizes under the membrane bilayer. It has many important roles, such as offering mechanical support for the membrane, participation in membrane remodelling events and connecting the

cell interior into the extracellular matrix via transmembrane proteins. The basic structure of cell membrane is shown in Figure 2.

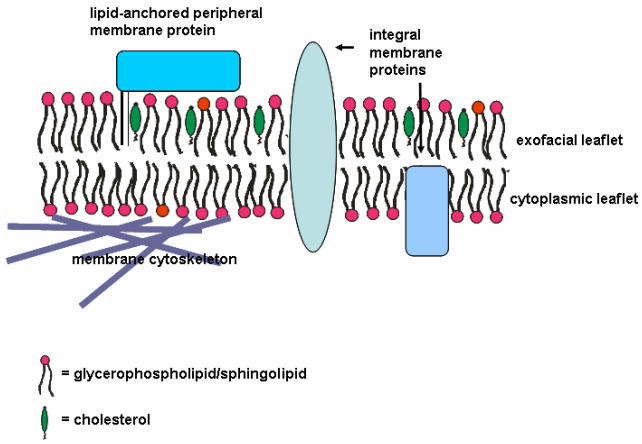

Figure 2. The basic structure of the plasma membrane of mammalian cells. The lipid bilayer, the different types of membrane proteins and the membrane cytoskeleton are depicted. Glycerophospholipids, and to a lesser extent, sphingolipids, localize to both leaflets of the bilayer, whereas cholesterol is believed to mostly reside in the exofacial leaflet of the membrane (see section 1.1.3).

1.1.2 Cholesterol-sphingolipid interactions

The rigid ring structure of the sterol backbone best fits next to relatively long and straight hydrocarbon chains, thus cholesterol interacts more strongly with saturated than unsaturated lipid species [23, 27-32]. The *cis*-double bonds in the *sn2*-fatty acids of glycerophospholipids make these chains kinked, often at a location where this kinking will interfere with the interactions between the glycerophospolipid and cholesterol. In contrast, the *trans*-double bond often present in the sphingoid base does not form kinks and is at a position (close to the interfactial region) where it is not in contact with the steroid backbone [33-35]. In addition, the fatty acyl moiety of sphingolipids is mostly saturated, thus the favorite interaction partners for cholesterol in mammalian cells are thought to be sphingolipids. A considerable amount of data from model membrane studies supports this idea: the rate of cholesterol absorption from SM-containing membranes is lower than that from PC-containing membranes, and the water permeability of SM-cholesterol mixtures is lower than that of PC-

cholesterol mixtures [15, 27, 36, 37]. Some studies have found no specific interaction between cholesterol and SM [24, 38, 39], but on the other hand, several cellular studies are again in favour of the aforementioned conclusion (see Sections 1.1.3 and 2.).

In general, the interaction of cholesterol with the acyl chains of polar lipids is mediated by van der Waals and hydrophobic forces [15]. However, while a mismatch in the hydrophobic chain length interferes with cholesterol-PC interactions, the strength of associaton between SM and cholesterol is not affected [30, 40]. Thus, it has been suggested that an additional force may play a role in strengthening the cholesterol-SM interaction. Such a force could be hydrogen bonding between the interfacial region of SM and the hydroxyl group of cholesterol [22, 30, 41-43], but whether this hydrogen bonding is indeed physiologically relevant is currently not known.

1.1.3 Distribution of cholesterol and other lipids in cell membranes

Most cellular cholesterol resides in the plasma membrane, but estimates vary from 40 to 90 %, mainly depending on cell types and research methodology used [44-46]. The relative amount of cholesterol in mammalian plasma membranes is approximately 30 mol%. The early and recycling endosomal compartments also have a relatively high cholesterol content [47-49], whereas the late endosomal and lysosomal membranes are normally cholesterol-poor [49, 50]. On the secretory route, the ER contains little cholesterol [51, 52], but this small amount plays an important regulatory role in cholesterol homeostasis (discussed in Section 1.2.3). In the Golgi apparatus, the amount of cholesterol increases in the *cis* to *trans* direction [53-55].

Cholesterol and SM have a similar distribution in cellular membranes in that their amount increases along the secretory pathway and the highest amounts are found at the plasma membrane [45, 52, 54, 56]. A positive correlation between the amounts of these two lipids was already noticed in the early 1970s [57] and subsequently, it has been observed in several studies that modulating the amount of SM results in accompanying changes in the amount and/or distribution of unesterified cholesterol [58-62]. The effects of altered cholesterol levels on the amount of SM are less clear [63-65], but nevertheless, the general consensus is that SM and cholesterol levels in

cells are co-ordinately regulated [66]. It has been suggested that owing to the favourable interaction of these lipids, the plasma membrane's capacity to solubilise cholesterol is a function of its SM content [61, 64, 67].

GSLs in cells localise to the plasma membrane and the Golgi apparatus, where they are synthesised [54, 68]. Normally, the amount of GSLs is small in comparison to SM and glycerophospholipids, but in certain cells, such as polarised epithelial cells, GSLs constitute a significant part of the apical membrane [68-70]. In these cells, GSLs at the exofacial leaflet of the plasma membrane can serve a special protective role, e.g. as the apical compartment faces the bile acid containing intestinal fluids.

Glycerophospholipids display a more even distribution among cellular membrane compartments than sphingolipids, but they localise to the different leaflets of the plasma membrane in a highly asymmetric fashion. For example, PC is enriched at the exoplasmic leaflet, whereas phosphatidylserine localizes almost exclusively to the inner leaflet [15, 71]. Sphingolipids are mostly found at the exoplasmic plasma membrane leaflet, but small amounts of SM have been reported in the cytoplasmic leaflet as well [72, 73]. The differential leaflet composition has several critical roles in cells and thus, it is actively maintained [74, 75]. In contrast to other membrane lipids, cholesterol seems to flip-flop between membrane leaflets with fast kinetics ($T_{1/2}$~1 s) [76].

1.2 Cholesterol homeostasis in mammalian cells

1.2.1 Cholesterol biosynthesis, lipoprotein uptake and cholesterol efflux

All mammalian cells, with the exception of mature erythrocytes, are capable of synthesising cholesterol to fulfill their needs. Cholesterol biosynthesis proceeds from acetyl-coenzyme A (acetyl-CoA) through a series of over 30 enzymatic reactions. The rate-limiting enzyme in this series is 3-hydroxy-3-methylglutaryl CoA-reductase (HMG-CoA reductase), which converts HMG-CoA into mevalonate and is inhibited by the statin class of cholesterol-lowering drugs. The enzymes of cholesterol biosynthesis localise predominantly in the ER, but peroxisomes also contain considerable amounts of enzyme activity [77, 78]. The first intermediate of cholesterol biosynthesis with a steroid backbone structure is lanosterol. From

lanosterol, the synthesis can progress via two intersecting routes, the Kandutsch-Russell or Bloch pathways. The choice of pathway is determined by the stage at which the double bond at position C24 in the sterol side chain is reduced. If this double bond is retained until the last reaction, cholesterol synthesis proceeds via desmosterol (Bloch pathway), whereas early reduction leads to the formation of lathosterol and 7-dehydrocholesterol (Kandutsch-Russell pathway) [79]. The significance of these alternative routes is not known.

In addition to *de novo* synthesis, cells take up cholesterol from circulation in lipoprotein particles, mainly in the form of low-density lipoprotein (LDL). LDL binds to its receptor, the LDL receptor (LDLR) at the cell surface, and is subsequently internalised via receptor-mediated endocytosis [80]. Along the endocytic route, the LDL particles are separated from the receptor and metabolised. Cholesterol in LDL is mainly in the form of cholesterol ester, which is hydrolysed by the enzyme acid hydrolase into free cholesterol and fatty acids [81, 82]. Traditionally, the hydrolysis has been thought to occur mostly in the late endosomes/lysosomes [82], but recent work has shown that the enzyme can act on earlier compartments as well [83, 84]. The metabolites are subsequently transported out of the endosomal system. The free cholesterol derived from LDL is mostly transported to the ER for re-esterification (discussed in Section 1.2.2). The main transport route from late endosomes to the ER appears to involve the plasma membrane [85, 86], but plasma membrane-independent movement has also been reported [86, 87].

Specialised cell types, like hepatocytes (discussed in Section 1.2.5) and steroidogenic cells also take up cholesterol (free cholesterol and cholesterol esters) from high-density lipoprotein (HDL) particles via pathways mediated by the scavenger receptor B1 (SR-B1) [88]. Macrophages, in turn, internalise modified (e.g. oxidised) lipoproteins via pathways mediated by other scavenger receptors, such as SR-A- and CD36- mediated routes [13]. Unlike the uptake via the LDLR, these routes are not under the normal control of cholesterol homeostasis, but can proceed unlimited and lead to the formation of lipid-laden foam cells. Such foam cells play a well-know role in the pathogenesis of atherosclerosis [13].

While the ways to provide mammalian cells with cholesterol are plentiful, there are less available means for removing excess cellular cholesterol. HDL carries cholesterol from peripheral cells into the liver, the only organ capable of excreting cholesterol out of the body. It has been known for some years that the ATP-binding cassette (ABC) transporter A1 plays a role in cholesterol efflux to HDL, or its lipid-poor apoprotein component ApoAI [89], and recently, transporters ABCG1 and G4 have also been implicated in the process [90]. In addition, plasma membrane cholesterol-enriched invaginations, caveolae and their main protein components, caveolins, have been suggested to participate in cholesterol egress [91, 92]. SR-B1 mediated cholesterol efflux has also been described [93], but the details of these processes remain elusive.

1.2.2 Cholesterol esterification and lipid droplets

Cholesterol esterification serves both as a means to store cholesterol for later use and as a detoxification reaction to protect cells from excess free cholesterol [94]. The esterification reaction is carried out by the enzyme acyl-coenzyme A:cholesterol acyl transferase (ACAT), which resides in the ER. In mammalian cells, there are two isoforms of ACAT, ACAT1 and 2 [95, 96]. ACAT1 appears to be ubiquitously expressed [96], whereas ACAT2 expression is restricted to the liver and the intestine [95, 97]. Cholesterol functions as an allosteric activator of ACAT [98], thus cholesterol arriving at the ER becomes esterified rapidly. Cholesterol esters are stored in lipid droplets, which also contain other storage forms of lipids, such as triacylglycerols. The neutral lipid core of the droplets is surrounded by a monolayer of phospholipids, which is thought to originate from the leaflets of the ER as the droplets form in between them [99]. In addition, certain proteins localise to the limiting membrane of the droplets and at least in adipocytes, the droplets are surrounded by a layer of free cholesterol [99, 100]. Cholesterol ester in the droplets can be hydrolysed by the putative enzyme cholesterol ester hydrolase, and cholesterol in the lipid droplets appears to undergo continuous cycles of hydrolysis and re-esterification [101, 102].

1.2.3 Regulation of cholesterol homeostasis

Mammalian cells exert strict control over their cholesterol content, and this regulation is mainly governed by transcription factors called the sterol-response element binding proteins (SREBPs) and by the cholesterol content of the ER [103]. When cholesterol

in the ER is abundant, the SREBPs are retained in this compartment together with a protein binding to them, called the SREBP-cleavage activating protein (SCAP). Cholesterol in the ER induces a conformational change in the SCAP that favors an interaction with ER resident proteins called Insigs (Insig-1 and Insig-2; for insulin-induced gene-1 and -2), and this interaction prevents the SCAP-SREBP complex from entering the transport vesicles for Golgi delivery [104]. When sterols are scarce, SCAP dissociates from Insigs and moves together with the SREBPs to the Golgi apparatus, where the SREBPs are then cleaved by two enzymes (site-1 and site-2 proteases) [103]. These reactions release the transcription factor parts of the SREBPs, which then move into the nucleus and activate the transcription of genes containing a sterol-response element [103]. These genes include HMG-CoA reductase, LDLR and many others. Also genes involved in the metabolism of other lipids, especially the synthesis of unsaturated fatty acids, are regulated by the SREBPs [105].

SCAP contains a structural motif called the sterol-sensing domain, which plays a central role in the Insig-SCAP interaction [104, 106, 107]. How the sterol-sensing domain actually functions is not known, but research suggests that it may trigger the conformational change induced by the membrane sterols [107]. This domain is found in many key proteins in cholesterol homeostasis, such as HMG-CoA reductase [108] and Niemann-Pick type C1 (NPC1, see Section 1.3). The sterol-sensing domain of HMG-CoA also binds Insigs, and this binding appears to mediate the sterol-dependent degradation of the enzyme [109].

The components of the SREBP-SCAP pathway have also been identified in *Drosophila melanogaster*, but the pathway appear to exclusively regulate the enzymes involved in fatty acid biosynthesis [110]. Moreover, SREBP processing is regulated by palmitatic acid, not by sterols, suggesting that the role of the ancestral SREBP-SCAP pathway is to control membrane integrity rather than sterol synthesis [110]. This function has in fact been conserved in evolution, as the amount of cholesterol and degree of fatty acyl unsaturation are the major determinants of membrane fluidity in mammalian cells,

1.2.4 Intracellular cholesterol transport

In order to maintain the cholesterol gradient among cellular compartments, cells have a constant need to transport cholesterol towards the high sterol-content organelles, most importantly the plasma membrane. As discussed in Section 1.2.1, LDL-derived cholesterol is transported out of the endosomal system to the plasma membrane and to the ER for esterification. Cholesterol from other sources (e.g. from the plasma membrane) entering the endosomes is largely recycled back to the plasma membrane, and the recycling endosomal compartment appears to be central to this process [111, 112]. Biosynthetic cholesterol is rapidly transported out of the ER and becomes available for extracellular acceptors in ~30 min after a pulse of radio-labeled acetate [113]. There seem to be many alternative routes for cholesterol transport from one intracellular compartment to another, and many of them still await proper characterisation [114].

The intracellular machineries for cholesterol transport are also somewhat poorly understood, but in principle, cholesterol can be transported within cells by at least three different mechanisms: 1) as part of the vesicular membrane trafficking; 2) via direct membrane-to-membrane contact and 3) with the aid of carrier proteins [114]. Because of its highly hydrophobic nature, cholesterol is unlikely to move around in the aqueous cytoplasma by simple diffusion, although evidence for fast, ATP-independent transport processes for sterols has been presented [115]. Some cholesterol is known to be transported as part of the vesicular trafficking, and the master regulators of vesicular transport, small GTPases of the Rab-family, also partly control cellular cholesterol trafficking [111, 116-118]. A cholesterol carrier protein has been identified in some special cases, like in the transport of cholesterol across the mitochondrial membranes by the steroidogenic-acute regulatory protein (STAR), [119]. The cholesterol content of cellular compartments and the key molecules in intracellular cholesterol processing are outlined in Figure 3.

1.2.5 Cholesterol metabolism in hepatocytes

The liver plays a key role in regulating whole-body cholesterol (and other lipid) metabolism, and hepatocytes are a special cell type in terms of cholesterol processing. The liver is to a large extent responsible for removing dietary cholesterol (in the form of chylomicron remnants) from the circulation [120, 121] and with its high LDLR

content, the main tissue for LDL uptake as well [121]. In addition, the liver is central to the process of reverse cholesterol transport. It is the end-station of the pathway, with HDL-derived cholesterol entering the hepatocytes via SR-B1-mediated transport [122]. In addition, the liver is the main source of lipid-poor ApoAI and recently, has also been shown to be the major organ effluxing cholesterol to HDL [123]

Cholesterol delivered to the liver can be processed in several ways. Hepatocytes contain cholesterol-modifying enzymes (e.g. cholesterol 7-hydroxylase, only expressed in hepatocytes), which convert the highly hydrophobic cholesterol molecule into bile acids that can be readily secreted [124]. Cholesterol in the liver thus functions as a precursor for bile acid synthesis, but this synthesis and secretion into bile at the same time constitute the only way for removing cholesterol from the body. Hepatic cholesterol can also become incorporated into very high-density lipoproteins (VLDL), which are assembled and secreted by the liver as a means for delivering lipids to other tissues [125]. The main lipid constituents of VLDL-particles are triacylglycerols, thus cholesterol, fatty acid, and triacylglycerol syntheses, as well as the influx of these molecules, are interconnected phenomena in hepatocytes [126]. Accordingly, hepatocytes are also highly active in cholesterol biosynthesis [127].

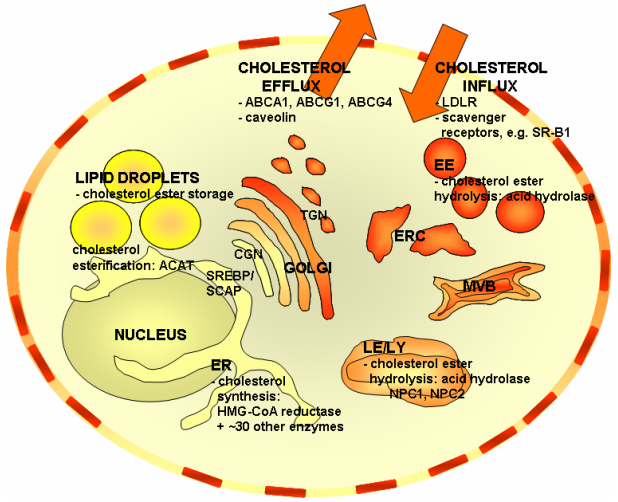

Figure 3. A schematic presentation of the cholesterol content of cellular compartments and the key molecules in cholesterol homeostasis. The orange and yellow colors indicate high and low free cholesterol content, respectively. The

variable color of the plasma membrane represents cholesterol-enriched domains, the so-called lipid rafts (see Section 2.). LDLR and SR-B1 are known mediators of cholesterol influx, whereas ABC-transporters A1, G1 and G4 are implicated in cholesterol efflux. A role for caveolar proteins, especially caveolin-1, in this process has also been suggested. The enzyme acid hydrolase localises to both late endosomes/lysosomes and earlier endosomal compartments. Niemann-Pick type C (NPC) proteins 1 and 2 regulate the egress of cholesterol from late endosomes. EE, early endosomes; ERC, endosomal recycling compartment; LE/LY, late endosomes, lysosomes; ER, endoplasmic reticulum; CGN, *cis*-Golgi network; TGN, *trans*-Golgi network.

1.3 Niemann-Pick type C (NPC) proteins and disease

Niemann-Pick type C (NPC) disease, caused by mutations in the *NPC1* or *NPC2* genes, is an autosomally inherited, rare (prevalence ~1/150 000 for both disease forms together) neurovisceral cholesterol storage disease [5, 128]. Clinically, the NPC disease manifests in hepato- and spleno- megaly and progressive neurological symptoms, which typically appear at the age of 4 – 5 years and lead to death in the early teens [5, 128]. The two types of NPC disease are phenotypically indistinguishable [129]. At the cellular level, NPC disease is characterised by late endosomal/lysosomal accumulation of free cholesterol and impaired homeostatic responses to LDL uptake (i.e. an increase in cholesterol esterification, down-regulation of LDLR expression, and cholesterol biosynthesis) [130, 131]. In addition to cholesterol, the storage organelles contain other lipids, especially sphingolipids. In contrast to many other lysosomal storage diseases (such as Niemann-Pick type A and B sphingolipidoses), NPC is not caused by the impaired degradation of lipids, but appears to be a primary lipid transport disorder [5, 132]. Traditionally, the principle defect in NPC has been thought to be in cholesterol transport, but recent research suggests that the accumulation of cholesterol could be secondary to impaired sphingolipid transport [133-135]. It has also been proposed that the substrates for NPC1-mediated transport could actually be fatty acids [136]. The lipid imbalance and transport defects in NPC cells are reflected in the composition of other membrane compartments, including the Golgi, early endosomes and the plasma membrane [137-140], in addition to the late endosomes/lysosomes .

NPC1 is a large, polytopic membrane protein with 13 membrane-spanning domains, including the so-called sterol-sensing domain [141]. The exact function of NPC1 is not known, but the sterol-sensing domain appears to have a critical role, since mutations in this part of the protein result in severe phenotypes [5]. In normal cells, NPC1 is localised to late endosomes and the tubular structures emanating from them [142-144]. Thus, there is evidence to suggest that NPC1 regulates vesicular transport. Recently, it was shown that the protein binds to cholesterol [145]. NPC2 (originally called HE1), in turn, is a soluble protein localising to the luminal side of late endosomes/lysosomes [146, 147]. It reaches this compartment by mannose-6-phosphate receptor-mediated transport [147]. It contains a cholesterol-binding domain, and has been shown to bind cholesterol with high affinity [148]. This cholesterol binding function of NPC2 appears critical for the control of late endosomal cholesterol levels [149]. NPC2 can also be secreted by cells, and is found in abundance in certain biological liquids, such as milk and epindidymal fluid [146]. The function of NPC2 in these fluids is not known. Both NPC proteins are evolutionary conserved, and of NPC1, there are several models from yeast to mammals [150, 151]. Interestingly, the mammalian models represent naturally-occurring mutations in the NPC1 gene.

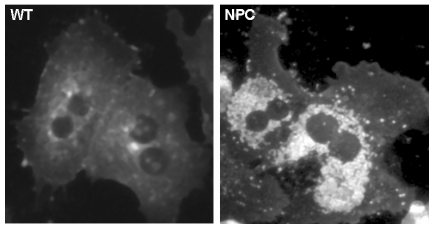

Figure 4. Filipin staining of control (wild type, WT) and NPC1 mouse primary hepatocytes. Filipin is a fluorescent polyene antibiotic that binds to free cholesterol. The perinuclear cholesterol-laden storage organelles in NPC cells are clearly visible.

1.4 Other sterols in mammalian cells

In contrast to the high diversity among the other membrane lipids, mammalian cells contain only one principal sterol, cholesterol. Other sterols, mainly of dietary origin, such as plant-derived phytosterols, enter the human body in relatively high amounts

but are very efficiently secreted out of the body [152-154]. Cell biological studies suggest that other sterols cannot substitute for cholesterol in mammalian cells [155], and inborn errors in cholesterol biosynthesis in humans result in severe malformations and mental retardation [9]. The biological basis for this sterol selectivity is not known, but cholesterol appears to be the most efficient sterol in modulating the physicochemical properties of membranes [11]. In a very recent study, it was shown that, in fact, many different sterols can fulfill the bulk requirements in mammalian cells, when present together with tiny amounts of cholesterol [21]. Interestingly, the enantioisomer of cholesterol could overcome the need for even minute amounts of cholesterol, indicating that the specific requirements for cholesterol are not stereoselective, and could thus be lipid-lipid, rather than lipid-protein -based interactions [21].

Cholesterol biosynthesis is an endogenous source for non-cholesterol sterols, as it produces a wealth of ring-structured cholesterol precursors that can be relatively abundant in cells, and get incorporated in the cellular membranes [156-158]. The precursors were thought to reside mostly in ER, where cholesterol biosynthesis takes place, but it has now been shown that they are also present e.g. at the plasma membrane, can be transported there even faster than cholesterol, and are avidly available for extracellular acceptors [158, 159]. One of the most abundant sterol precursors is desmosterol, which differs from cholesterol only by the presence of an additional double bond at C24. Desmosterol has been shown to have a membrane distribution similar to cholesterol [159], and is a major membrane component in certain cell types, such as astrocytes [160] and spermatozoa [157, 161, 162]. At the whole body level in humans, however, desmosterol does not appear equivalent to cholesterol, as mutations in the gene coding for 24-dehydroxy cholesterol reductase (the enzyme converting desmosterol into cholesterol) have devastating outcomes [161, 162].

2. Cholesterol-rich membrane domains, “lipid rafts”

Based on the biophysical properties of the constituent lipids, the concept of lateral inhomogenities (domains) in cellular membranes was outlined already more than thirty years ago [163, 164]. In recent years, increasing knowledge about the phase behaviour of membrane lipids, as well as cell biological observations, has led to the

formation of the lipid raft hypothesis, i.e. the presence of functional cholesterol-sphingolipid domains in cell membranes [1, 165]. Since its appearance, the lipid raft hypothesis has aroused great interest among both cell biologists and membrane biophysics, but the exact nature of these domains still awaits characterisation.

2.1 Biophysical basis for domain formation

2.1.1 Phase behaviour of membrane lipids

Polar membrane-forming lipids exist in different phases depending on temperature and other physical parameters. In the solid-like (gel, solid-ordered or So) phase, the lipid acyl chains are ordered in an all-*trans* conformation, the molecules tightly packed, and they undergo little lateral or rotational motion. In contrast, in the liquid-like (liquid-disordered, Ld) state, the lipids are loosely packed and diffuse relatively freely. The temperature at which the phase transition takes place is called the melting temperature (Tm), and is characteristic for each type of lipid: lipids with long and saturated acyl chains have high Tms, whereas the Tms of lipids with unsaturated acyl chains are lower [166, 167]. In addition, the polar head group of the lipid affects the Tm, with e.g. neutral GSLs having higher Tms than SM or charged GSLs [168]. Even in membranes containing only one lipid component, the So and Ld phases can co-exist within a certain temperature range, and this phenomenon is especially well-known for mixtures of high- and low-Tm lipids [166, 167].

Eukaryotic cell membranes have both high- and low-Tm components, thus it was thought that sphingolipid domains of some sort might exist in them [163, 164]. The observation that detergent treatment of erythrocyte ghosts leaves behind sphingolipid-rich membrane fragments [169] supported this idea. Further, it was shown that GSLs have a high tendency for hydrogen bonding [20], and that they are found in small clusters (~15 molecules) in native and model membranes [164]. A functional role for such sphingolipid domains in the apical sorting of lipids and proteins in epithelial cells was also suggested [69, 170, 171]. However, the rigid So phase of the pure sphingolipid domains did not seem compatible with the dynamic functions of cell membranes [172]. Moreover, the presence of cholesterol in cell membranes also had to be taken into consideration (see below).

2.1.2 Domain formation in cholesterol-containing membranes

The presence of cholesterol in membranes changes their phase behaviour considerably. When introduced to an So-state membrane, cholesterol decreases the ordering of acyl chains, but in an Ld-state membrane, it has the opposite effect: it creates more order in the acyl chains, decreasing the area per molecule [43, 173, 174]. This property of cholesterol is accounted for by its rigid and planar ring structure, which is in contact with and affects the shape of its neighbouring acyl chains. The addition of cholesterol into a bilayer of saturated PC species abolishes the formation of co-existing So and Lo domains and instead, the whole membrane adopts an intermediate state [175-177], called the liquid-ordered (Lo) phase [178]. The Lo state forms to balance the unfavourable free energy of cholesterol mixing with either the Ld or So phase. In the Lo phase, the ordering of acyl chains present in the So phase is maintained, but decoupled from the lateral and rotational diffusion restrictions, and thus, the Lo phase lipids are highly mobile [178-181].

Phase diaphragms of binary mixtures of cholesterol and saturated PC also suggested that under certain conditions, the Ld and Lo phases could actually co-exist [173, 176, 178]. These phase diaphragms have other interpretations as well [182-184], but further evidence for the co-existing liquid phases has come from studies using 1:1:1 ternary mixtures of a low Tm lipid (unsaturated phospholipid), a high Tm lipid (SM or saturated phospholipid) and cholesterol. It is a crude model of biological membranes, especially the outer leaflet of the eukaryotic plasma membrane, and the inspiration to use such a mixture came from cell biological studies [185]. In these membranes, the formation of large (micronscale), immiscible, liquid-liquid domains has been observed by several investigators and through a number of different methods [186-190]. The Lo domains are generally enriched in cholesterol and the high Tm component(s) of the membrane, although a rather equal distribution of cholesterol among the two phases has also been reported [190]. However, the presence of cholesterol (or some other sterol, see Section 2.1.3) seems to be a prerequisite for the formation of the Lo phase [191, 192].

2.1.3 Detergent-resistant domains in cells and model membranes

As mentioned above, the isolation of detergent-resistant sphingolipid-rich membrane fragments from erythrocytes (and subsequently from several other cell types) led to

the suggestion that these membranes would represent sphingolipid clusters in cell membranes [169]. The connection with detergent resistance and lipid domains was reinforced by studies demonstrating the detergent resistance of apically targeted GPI-anchored proteins in epithelial cells [185, 193]. Moreover, in favour of the functional role of these domains, it was shown that the GPI-anchored proteins gain detergent-resistance *en route* through the Golgi apparatus [185]. The detergent-resistant membranes (DRMs) isolated in this study were big vesicles, around 100 nm in diameter, and contained not only sphingolipids, but an approximately 1:1:1 mixture of glycerophospholipids, sphingolipids (both GSLs and SM) and cholesterol. The detergent used was Triton X-100 and the extraction was performed at 4˚C.

Model membrane studies progressed in parallel to show a connection between the physical state of the membrane and detergent resistance [194]. The detergent resistance of cellular components had been taken as an indication of cytoskeletal association, but it subsequently became clear that lipids as such can form detergent-resistant complexes. Lipids in the Ld phase are generally soluble in cold, non-ionic detergents, whereas lipids in the Lo or So phases are resistant to the treatment, owing to a tighter packing of the acyl chains, and possibly to hydrogen bonding between the constituent lipids [195]. In a series of model membrane studies, Brown and London showed a good correlation between the extent of Lo phase formation and the detergent-resistance of the lipid mixtures. They also showed that the components of the model membrane Lo domains closely resembled that of DRMs, i.e. both were enriched in cholesterol and high-Tm lipids [191, 194, 196]. They strongly argue that DRMs represent pre-existing Lo domains in membranes, even though the size of these domains can be misjudged by the method [172]. Other studiess have also found that the physical properties of DRMs from cell membranes are similar to those of model membrane Lo domains [197, 198]. In addition to the sterol-dependent Lo phase, high sphingolipid content can confer detergent resistance to membranes [199, 200].

2.2 The lipid raft hypothesis

2.2.1 Classical views and methods for studying rafts

The hypothesis of functional lipid rafts in cell membranes suggests that Lo phase cholesterol-sphingolipid domains exist in the bulk Ld phase of glycerophospholipids

[1, 165]. These domains include a subset of proteins based on how well they fit within this organised lipid environment, while excluding others. Lipid rafts thus provide a mechanism for the lateral segregation of membrane components, and in addition to apical sorting, they have been suggested to function in various cellular processes, most notably signal transduction (see Section 2.3). Notifications that the domains are in the Lo phase and enriched in cholesterol have yielded the two operational definitions for lipid rafts: resistance to cold non-ionic detergents, especially Triton X-100, and the sensitivity to cholesterol depletion.

Extraction with cold Triton X-100 and the recovery of DRMs as low buoyant-density fractions has become a widely used method for identifying putative lipid raft components. The majority of cholesterol, SM, GSLs and GPI-anchored proteins are regularly found in DRMs, whereas other proteins typically exhibit only partial (10-30%) recovery in the DRMs [201-203]. These other DRM-associated molecules include proteins attached to the membrane via a saturated lipid moiety, such as palmitoylated and/or myristoylated cytosolic signalling proteins, and some transmembrane proteins, especially palmitoylated ones. Most transmembrane proteins as well as prenylated peripheral membrane proteins tend to be excluded from DRMs [1, 203]. Model membrane studies with purified proteins and peptides support these cell biological observations [194, 196, 204, 205]. The use of other mild detergents, such as agents from the Brij- and Lubrol-series or CHAPS, yields DRMs differing in both their lipid and protein components, which has been interpreted as an indication of the existence of different raft subtypes, or of weaker and stronger raft-associations of molecules [206, 207]. Because of the possible artifacts related to the treatment of cells with detergents (see Section 2.2.6), attemps for developing detergent-free methods for raft isolation have been made [206, 208].

Many cellular processes mediated by DRM-associated proteins have been shown to be sensitive to cholesterol depletion, or to the modulation of other lipid raft constituents [1, 2, 201]. Cholesterol depletion can be achieved by several means, e.g. by using cholesterol binding or chelating compounds such as filipin or saponin, inhibiting cholesterol biosynthesis with statins, or removing cholesterol from the plasma membrane with cyclodextring. The specifity of the treatment can be confirmed by adding back cholesterol. Other methods for disrupting rafts include the addition of

exogenous cholesterol or sphingolipids, the inhibition of GSL synthesis, and the crosslinking of raft components [2, 209, 210].

2.2.2 Caveolae and caveolins

Caveolae are small, flask-shaped invaginations observed at the plasma membrane of many, but not all cell types. These structures were first observed in electron micrographic studies in the 1950's. They appeared to have a distinct sterol composition [211, 212] and were found to be sensitive to cholesterol depletion [213]. The main protein component of caveolae, called caveolin-1, was identified [213] and subsequently shown to be a cholesterol-binding protein [214, 215]. Caveolin-1 was found to be highly enriched in DRMs obtained from both plasma membranes and the apical transport vesicles of epithelial cells [216-218]. In addition to cholesterol, other lipid raft constituents (i.e. GPI-anchored proteins and sphingolipids) were detected in caveolin-containing membrane fractions [218, 219]. Thus, there has been some confusion between DRMs, caveolin-enriched membrane fractions and caveolae. However, DRMs can be isolated from cells with no morphological caveolae, and currently caveolae are considered to be a subtype of lipid rafts [1, 220]. Moreover, many investigators agree that true caveolar localisation can only be observed by immuno-electron microscopy [221-223].

Mammalian cells express three caveolin isoforms: caveolin-1, -2 and -3 [222]. Of these, caveolin-1 and caveolin-3 form homo-oligomers at the cell surface and when expressed in caveolin-negative cells, are capable of creating caveolae *de novo* [99, 224, 225]. Cell types with the most abundant caveolae express different caveolin isoforms, with caveolin-1 being the principal form in adipocytes and endothelial cells [213], whereas caveolin-3 is expressed in striated and cardiac muscle [226, 227]. Cell types expressing no or only low levels of caveolins include lymphocytes, neurons and hepatocytes [228]. It has been suggested that caveolae function in such processes as transcytosis, clathrin-independent endocytosis, cholesterol efflux [222, 228, 229] and more recently, viral entry [229]. Several signalling molecules have been localised to caveolea and many of them interact with caveolin-1 [230]. This interaction seems to serve a regulatory – usually inhibitory – role in signalling [230, 231].

2.2.3 Lipid rafts and other sterols

Since the concept of cholesterol-dependent Lo phase domains has gained considerable attention, the domain-forming capacity of other sterols has also been addressed. These studies are of interest for at least two reasons: first, they describe the domain-forming capacity of the structural sterols in other eukaryotic cells, and second, they can help to define the special characteristics of cholesterol that make it indispensable for mammalian cells [7]. Ergosterol, the structural sterol in yeast, has been shown to promote domain formation in a similar way to cholesterol [232-234]. In studies using a variety of natural and unnatural sterols, a close correlation between domain-forming capacity and the ability of the sterol to pack closely with dipalmitoyl-PC or SM has been demonstrated [234-237]. The cholesterol precursors, lathosterol and 7-dehydrocholesterol exhibit stronger domain-forming capacities than cholesterol [158, 238], whereas desmosterol has been found to have a similar [239] or reduced [240] ordering potential in comparison to cholesterol.

2.2.4 The size and properties of lipid rafts in cells

Whereas the Lo domains in model membranes are large and with time, tend to coalesce to minimise the line tension between the two phases [189], lipid domains in cell membranes have proven extremely difficult to visualise. Both GPI-anchored proteins and GSLs exhibit an even distribution on the plasma membrane when viewed by light microscopy [209], which has led to the conclusion that lipid rafts must be at least smaller than the resolution of light microscopy (i.e. <300 nm). However, even conventional electron microscopy has failed to reveal a significant clustering of raft components [221, 241]. Studies using fluorescent-resonance energy transfer (FRET), a method sensitive to nanoscale (~10 nm) clustering [242, 243], or chemical short-range cross-kinking [244] suggest that at least a fraction of GPI-anchored proteins exist in small (~10 molecules) clusters at the plasma membrane, and these clusters are sensitive to cholesterol depletion. A recent study reduced the size of these clusters to approximately 4 nm, with about 20-40 per cent of GPI-anchored proteins at the plasma membrane present in these complexes [245]. New electron microscopic observations combined with computational modelling suggest a similar clustering pattern for inner leaflet raft molecules [246, 247]. However, some FRET studies have identified no specific clustering of GPI-anchored proteins [248, 249] or GSLs [250], and clustering behaviour similar to raft-associated molecules has been reported for

non-raft proteins as well [246, 247], although these clusters were cholesterol-independent.

When crosslinked with e.g. antibodies, raft-associated molecules form clusters [209, 251], and simultaneously crosslinked raft components co-localise, although the crosslinking of one component does not induce the co-clustering of another [209]. These results have contributed to the hypothesis that in resting cells, lipid rafts are small and unstable, but crosslinking can induce the formation of more stable structures. The ligation of signalling molecules is an example of a natural crosslinking event, and it has been suggested that this would trigger the creation of lipid raft signalling platforms [2]. It was recently demonstrated in model membranes that the crosslinking of GM1 molecules can also induce phase separation in previously uniform, cholesterol-containing membranes [252].

If partitioning into lipid rafts is a major determinant of the diffusion rate of a molecule, all raft-associated molecules would be expected to have a similar diffusion coefficient, and these might differ from those of non-raft molecules. The diffusion of raft-constituent molecules should also be sensitive to cholesterol depletion. A study using photonic force microscopy indeed reported results in line with these expectations [253], but other studies using such methods as fluorescent recovery after photobleaching (FRAP) have failed to do so [254, 255]. Kusumi and co-workers analysed the diffusion behaviour of several raft and non-raft proteins and lipids by combining single-particle tracking and extremely high-resolution video recording [256-258]. According to their data, all of these molecules underwent so-called hop-diffusion, with a transient confinement in a small area (~30 – 250 nm in diameter, depending on cell type) and repeatedly hopping into new areas of confinement. They claimed that the confinement areas were formed by the membrane cytoskeleton and transmembrane proteins attached to it (the so-called “picket-fence” model of plasma membrane) and hopping occurred when breaks in these structures allowed molecules to diffuse further. No difference in the diffusion speed or pattern has been observed between raft- and non-raft components.

2.2.5 Current view of lipid rafts

The current understanding of lipid rafts under basal conditions sees them as extremely small and/or dynamic assemblies of lipids and proteins, with their components changing very quickly with the surrounding membrane [2, 256, 259]. Such nanoscale clusters of lipids and proteins hardly represent true, thermodynamically equilibrated phases [259, 260] and indeed, it appears that this organisation is actively maintained and does not result from passive segregation into pre-existing domains or phases [245]. Notably, work in model membranes has also addressed the existence of small domains that could represent fluctuations or complexes within a given phase [261-263]. These features of the lipid raft hypothesis in fact bring it closer to the concept of boundary lipids, which describes the dynamic association of a small number of lipid molecules with the transmembrane parts of membrane proteins [256].

Even though the exact nature of lipid micro- (or nano-) domains still awaits characterisation, the concept of functional lipid-lipid and/or lipid-protein clusters in cellular membranes is supported by a wealth of evidence [201, 203, 256, 259]. The term “lipid raft” to describe these complexes has been established in the nomenclature and will also be used in the subsequent sections of this thesis.

2.2.6 Pitfalls in the traditional methods for studying rafts

Since the early days of the lipid microdomain studies, the validity of the detergent resistance method has been debated. In most cases, DRMs are not observed if the solubilisation is performed at 37˚C, and as temperature is one of the main parameters affecting the phase behaviour of lipids, it can be argued that detergent resistance at low temperatures does not describe the phases existing at higher temperatures [202]. Furthermore, the interaction of detergent with membrane is a complex event, strongly affected by such factors as lipid to detergent ratios [201, 202]. Detergent incorporation can induce several nonphysiological rearrangements in the membrane, such as the mixing of outer and inner leaflet constituents, or even the creation of Lo domains in a previously homogenous membrane [2, 202, 264]. When live cells were treated with Triton X-100 and analysed by fluorescent microscopy thereafter, the detergent created a few large holes, leaving the rest of the plasma membrane intact [265]. This does not agree with the current view of small, dynamic rafts, but more closely resembles the large DRM vesicles reported by Brown and Rose [185]. Indeed,

as the current view of lipid rafts no longer assumes that they correspond to true Lo phase domains, the origin of DRMs in cell membranes remains somewhat enigmatic [266].

Nevertheless, the detergent resistance method can be useful on certain occasions, such as when studying the behaviour of a given molecule under differing cellular conditions [202, 203]. Observed changes in detergent resistance are likely to reflect alterations in the physical state of the surroundings of the molecule.

The problem with cholesterol depletion – or the modulation of any other raft component – is that it does not prove the existence of lipid domains. Any other role cholesterol or sphingolipids might have in the membrane would be equally affected by the treatments [202] and many membrane proteins are known to be sensitive to the physical properties of the membrane *per se* [267]. Especially cholesterol depletion alters these properties, and it has even been suggested that the presence of cholesterol actually prevents the formation of gel-phase domains in cell membranes [15, 202]. In support of this view, it has been recently shown that cholesterol removal from the plasma membrane induces ordered-phase domain formation [268]. Cholesterol depletion has also been seen to induce global changes in the organisation of the plasma membrane, including the membrane cytoskeleton [269]. Thus, the results from cholesterol depletion studies should be carefully controlled and interpreted with caution.

2.3 Lipid rafts in signal transduction

Activation of immune cells is signalled via kinases attached to the cytosolic leaflet of the plasma membrane but curiously, crosslinking of GPI-anchored proteins at the exoplasmic leaflet can induce this activation [201]. A solution to this puzzling topological problem appeared when GPI-anchored proteins and kinases of the src-family were both detected in DRMs from activated lymphocytes [270-272]. It was suggested that crosslinking GPI-anchored proteins would bring together lipid rafts at both membrane leaflets and thus enable the initiation of signalling cascades. Subsequently, the association of several signalling receptors, cytosolic non-receptor kinases and the Gα-subunits of trimeric G-proteins with DRMs were described (263-

266). In addition to the trans-bilayer contacts, the lipid raft concept has been used to explain other seminal questions in signal transduction, like the spatial separation of different pathways and the ligand-induced clustering of pathway components [2, 273-276].

Immune cell receptors, especially T-cell receptors (TCR), B-cell receptors (BCR) and the IgE receptors of mast cells, are one of the best-studied examples of raft-associated signalling molecules [273-276]. Studies have demonstrated that the key components of TCR-signalling machinery are recovered in DRMs in active but not inactive T-cells [277, 278]; that signalling molecules do not exhibit DRM association in activation-incompetent, immature B-cells, but they do in mature B-cells [279]; and that inner-leaflet raft-associated molecules cluster together with outer-leaflet components when receptors are ligated [251]. However, the general markers of lipid rafts, such as the ganglioside GM1 or GPI-anchored green fluorescent protein, display a random distribution in active T-cells [249, 280]. Recently, it was shown using single-molecule confocal imaging that the formation of TCR-signalling complex is based on protein-protein interactions, and does not require lipids, or the actin cytoskeleton, for maintenance [281]. However, the authors speculate that small-scale lipid domains might function in initiation of the complex formation.

Immune cells do not contain caveolae, thus the concept of caveolar involvement in signalling events can not be addressed in them. On the contrary, a role for caveolae/caveolins in regulating the function of many growth factor receptors, including the epidermal-growth factor receptor (EGFR), platelet-derived growth factor receptor and several neurokine receptors, has been frequently reported [275, 276]. Yet, despite direct interaction with caveolin-1 [282], receptors such as EGFR have been shown to associate with DRMs different from the caveolin-containing vesicles [283]. In most cases, the interaction with caveolin-1 inhibits growth factor receptor signalling. Therefore, it has been suggested that the inactive receptors would be sequestered to caveolae [276, 284]. However, the opposite view, with active receptors partitioning in lipid rafts has also been proposed [2]. Many of these receptors dimerise upon ligand binding, which could serve as a driving force for the lipid raft association of the active receptors [2].

3. Insulin receptor signalling and insulin resistance

Insulin is a powerful anabolic hormone and the master regulator of whole-body energy homeostasis: it governs not only glucose but also protein and lipid metabolism. Insulin is secreted by the beta cells of the Langerhans islets in the pancreas, and the destruction of these cells leads to the disease type 1 diabetes mellitus (DM). Resistance to the effects of insulin, in turn, leads to type 2 DM that constitutes an ever-growing health problem all over the world. Thus, the mechanisms of insulin action are the subjects of intense research and currently understood in considerable detail. The insulin-signalling cascade is initiated at the surface of its target cells, where insulin binds its cognate receptor, the insulin receptor (IR).

3.1 Insulin target tissues

Insulin is a key growth factor to many cell types, especially during embryonic development [285], thus IR is expressed in virtually all cells in the human body, but for the metabolic effects of the hormone, the most important target tissues are adipose tissue, striated muscle, and the liver. In these tissues, IR is expressed at high levels, with over 10^5 receptors per hepatocyte [286], and they each have their special roles in the body's energy homeostasis. The adipose tissue is most of all involved in the storage and release of energy in the form of fatty acids. In the presence of insulin, adipocytes take up glucose and lipids from the blood and store them as triacylglycerols. The adipose tissue is the major site for the uptake of dietary fatty acids (circulating in the form of chylomicron particles) [287]. Insulin also prevents the hydrolysis of the stored triacylglycerols, and thus the release of free fatty acid (FFAs), in adipocytes. In the fasted state – in the absence of insulin – the adipose tissue then functions supplies energy for other tissues by releasing FFAs into circulation [287].

Striated muscle cells take up glucose from the circulation in response to insulin, and are largely responsible for clearing post-prandial hyperglycemia [288]. Insulin also increases glucose storage in muscle cells in the form of glycogen. The increased glucose uptake by both of muscle cells and adipocytes is mediated by a type 4 glucose transporter (GLUT4), which is stored in intracellular vesicles in the absence of insulin [289]. Insulin signalling increases the exocytosis of these vesicles and consequently,

GLUT4 translocates to the plasma membrane. Insulin may also increase the activity of the transporter, and to some extent, reduce its endocytosis [290].

In contrast, the liver functions as a whole body "glucostat" by sensing the blood glucose levels via the glucose transporter GLUT1, which is constitutively localised at the plasma membrane [291]. Hepatocytes contain the enzyme glucose-6-phosphatase, which allows them to release glucose for transport out of the cell in response to dropping blood glucose levels [288]. Insulin decreases the activity of this enzyme, and thus glucose output by the liver. In addition, insulin e.g. decreases gluconeogenesis and VLDL secretion and stimulates lipogenesis and glycogen synthesis by the liver [288].

3.2 The insulin receptor and its signalling system

3.2.1 The insulin receptor (IR)

The IR belongs to the family of receptor tyrosine kinases, together with many other growth factor receptors. It is a disulphide-bonded heterotetramer of two alpha and two beta subunits, of which the alpha subunits form the extracellular, insulin-binding domain and the beta subunits correspond to the transmembrane and cytosolic parts, including the kinase domains [292-294]. Upon insulin binding, the IR undergoes a conformational change, which brings the beta subunits into close apposition and allows for *trans*-autophosphorylation of several tyrosine residues in the beta-subunits [295, 296]. In the inactive state, so-called activation loops occupy the active sites of the IR kinase, but as a result of autophosphorylation at residues 1158, 1162 and 1163, the positions of the loops change, and ATP and other substrates can access the active sites [295, 297] Most of the other growth factor receptors with intrinsic tyrosine kinase activity, such as the EGFR, consist of monomers that are activated by ligand-induced dimerisation. Even though a single IR molecule is functional by itself, it has been shown that IR micro-aggregation at the cell surface is important for insulin action [298]. IR has been shown to contain palmitoyl and myristoyl residues [299, 300], but the significance of these modifications is unknown.

At the hepatocyte plasma membrane, single IR molecules are concentrated on the microvilli, whereas in adipocytes, the receptors are mostly found in clusters [301].

Some studies have also reported caveolar localisation of IR in adipocytes, but others have not confirmed these findings (discussed in Section 3.2.3). The unoccupied receptor is tethered onto the plasma membrane, possibly through interaction with cytoskeletal elements, but insulin binding releases this constraint, allowing the receptors to diffuse more freely [302-304]. This release is necessary for IR internalisation. The IR contains an internalisation motif, which is found in several other receptor tyrosine kinases that are internalised in a ligand-dependent fashion via the clathrin-coated pits [305-307]. The clathrin-coated pit pathway has been reported to be the major internalisation route for the IR [305, 308-310], although caveolar endocytosis has also been suggested [311].

Insulin dissociates from the IR in the acidic environment of the early endosomes and the IR is recycled back to the plasma membrane, whereas insulin is degraded in the endosomal compartments [308]. Endocytosis of the receptor-ligand complex is a way to down-regulate receptor activity and in the case of hepatocytes, it also serves to remove insulin from circulation [312]. On the other hand, part of IR signalling may take place in the endosomes (see Section 3.2.2). Protein tyrosine phosphatases, especially type B1 (PTPB1), also play a role in terminating IR signalling [313].

3.2.2 Downstream targets of IR

Downstream to the IR, a complex network of effectors convey the signal forward to regulate the ultimate targets of insulin, such as the activities of metabolic enzymes, translocation of GLUT4 to the plasma membrane and gene transcription. In the first line, there is a group of adaptor molecules called the IR substrates (IRS) that are phosphorylated by the IR itself. These molecules include IRS-1, -2, -3 and -4, which are considered to be most specific for insulin signalling, as well as Gab-1 and Shc [314-316]. The IRS molecules often contain a pleckstrin-homology (PH) domain for attachment to the membrane in the vicinity of the IR, a phosphotyrosine binding domain for phospho-IR recognition and an src-homology (SH) 2 domain for interaction with downstream effectors [314, 317]. Especially for IRS-1 and -2, there are tissue-specific differences in their relative importance as IR targets [316], and in some cell types like hepatocytes, they appear to have complementary roles in the signalling cascade [318]. The IRSs are believed to associate with the IR at the plasma membrane only transiently, after which they translocate to different cellular

compartments for further activity [314]. It has also been suggested that IR activity could be partly mediated by IR localised to endosomes, as the receptor is rapidly internalised after insulin binding [319].

IRSs are normally not enzymatically active themselves, but rather convey the signal to further target molecules. Phosphorylated IRS molecules function as docking sites for a number of SH2-domain-containing adapters and enzymes. For the metabolic actions of insulin, the most important of these molecules appears to be the regulatory subunit of the type 1A phophatidylinositol 3-kinase (PI3K), p85, which then binds to the actual enzymatic part of the molecule, p110 [320, 321]. If PI3K activity is inhibited pharmacologically or using a dominant-negative mutant, most of the metabolic actions of insulin are repressed, including GLUT4 translocation and glucose uptake [320]. As a result of PI3K activity, phosphatidylinositol-3,4,-phosphate is created in cell membranes, and these in turn are recognised by the PH domains of other proteins, such as phosphatidyl inositiol-dependent kinase (PKD) 1 [322]. PKD1 activates the protein kinase B (PKB/Akt) serine/threonine kinase pathway and the apitypical proteins kinase C (PKC) isoforms ξ/λ, which both appear critical for increased uptake of glucose in response to insulin [322-325].

Other IRS-binding adapters include Grb2 and Crk, which can e.g. recruit Sos, a guanine-nucleotide exchange factor for the small GTPase Ras. This leads to the subsequent activation of the mitogen-activated protein kinase (MAPK) pathway and the mitogenic effects of insulin [326-328]. The insulin signalling system is outlined in Figure 5.

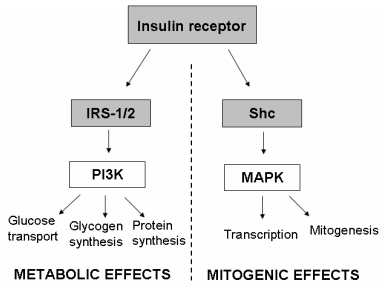

Figure 5. A schematic presentation of the insulin signalling system and its components. The cascade is initiated at the plasma membrane, where insulin binds to its receptor. Intracellular signalling molecules then convey the signal further, ultimately leading to the metabolic and mitogenic effects of the hormone. Only a few key molecules of the signalling system are depicted.

3.2.3 Caveolae and lipid rafts in IR signalling

The insulin signalling machinery downstream from the IR is not unique to insulin, and enzymes such as PI3K can be activated by other growth factors as well. However, the action of these other factors does not lead to the metabolic effects of insulin, most notably GLUT4 translocation and increased glucose uptake. Thus, even though PI3K signalling is necessary for this action of insulin, researchers have long been looking for one or more other insulin-activated pathways needed for the translocation of GLUT4. Caveolae and compartmentalisation of signalling events appeared in this context when it was found that caveolin-1 is phosphorylated in response to insulin [329-331]. Subsequently, an insulin-activated pathway localising to membrane microdomains has been identified.

First, the proto-oncogene product c-Cbl was found to be phosphorylated by insulin only in cell types capable of GLUT4 translocation [332]. Cbl was shown to be recruited to the IR by an adapter protein APS [333] and upon Cbl phosphorylation, this complex dissociated from the receptor and phospho-Cbl accumulated in DRMs [330, 332]. This accumulation was found to be mediated by a Cbl-associated protein (CAP), which binds a caveolar/lipid raft-associated protein called flotillin [334, 335]. Phosphorylated Cbl then functions to recruit the adapter protein CrkII, together with the guanine-nucleotide exchange factor C3G, into the microdomains. C3G, in turn,

activates the constitutively lipid raft-associated small GTPase TC10 [334, 336]. Inhibiting the Cbl-CAP-TC10 pathway appears to abolish GLUT4 translocation, in a similar way to PI3K-inhibition [334, 337], and the lipid raft localisation of this pathway is necessary for its function [338]. It has been suggested that PI3K-dependent signalling would release the intracellular GLUT4-containing vesicles for trafficking to the plasma membrane, whereas the Cbl-CAP-TC10 pathway might regulate trafficking, docking or fusion of these vesicles [4]. Indeed, the exocyst complex appears to be a downstream target of TC10 [339]. GLUT4 has been shown to localise to caveolae, which might explain the need for the TC10 pathway to act on these microdomains [340, 341]. It has also been reported that both the Cbl-CAP-TC10 and PI3K-pathways regulate the activity of PKCξ/λ, but TC10 functions to localise this activity to lipid rafts [342].

Whether the IR itself is localised to lipid rafts/caveolae is a matter of debate. The IR has been found in caveolin-enriched membrane fractions prepared without detergent [311, 343], and in DRMs by some investigators [344] but not by others [345]. Further, the IR was observed in caveolae by immuno-electron microscopy [311, 345] but not detected in immuno-isolated caveolae [346]. Studies on caveolin knock-out mouse models however, suggest a role for caveolins/caveolea in IR function: caveolin-1 knock-out mice are resistant to diet-induced obesity and this appears to be at least partly due to drastically-decreased IR levels in adipocytes [347]. Muscle cells from the caveolin-3 knock-out mouse exhibit impaired IR signalling without notable changes in the expression of IR, or its downstream targets [348]. The participation of non-caveolar lipid rafts in IR signalling has been reported in pancreatic beta-cells, in which insulin regulates its own secretion [349].

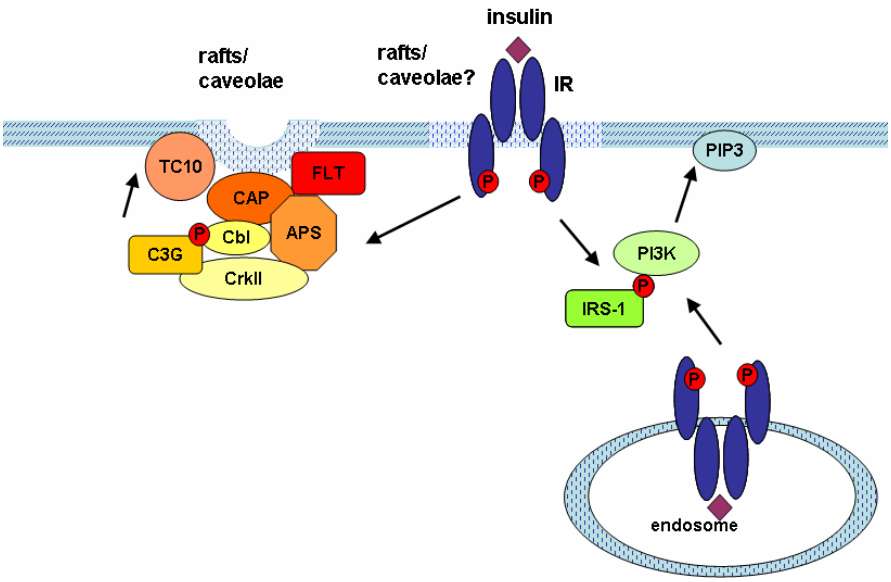

Figure 6. Lipid rafts/caveolae and endosomes in insulin signalling. The Cbl-CAP-TC10 pathway has been shown to assemble in caveolae, while IRS-1/PI3K signalling occurs in the cytoplasm and endosomes. PIP3 is created by the action of PI3K at the plasma membrane and probably other membrane compartments as well. Whether the IR itself localises to caveolae/rafts is unclear, although cell type specific differences might explain some of the reported discrepancies. Lighter blue colour and straight lines indicate lipid rafts/caveolae, darker blue and hatched lines show the rest of the membrane. CAP, cbl-associated protein; FLT, flotillin; PIP3, phosphatidyl inositol 3-phosphate; P, phosphotyrosine.

3.3 Insulin resistance

The outbreak of a world-wide epidemy of obesity, metabolic syndrome and insulin resistance is among today's biggest health problems [350]. Patients with these metabolic abnormalities have a highly increased risk for cardiovascular diseases, which significantly contributes to the related morbidity and mortality. This increased risk has several causes, and among them are the dyslipidemias invariably associated with these syndromes, especially increased concentrations of total and VLDL-associated triacylglycerols and decreased HDL-cholesterol [351]. The connections between disorders of lipid and glucose metabolism are being intensively investigated. As the major regulator of both lipid and glucose metabolism, the liver appears the be

the central organ in many aspects of the pathophysiology of insulin resistance and related disorders. Organ-specific knock-outs of the IR in mice have also shown that the deletion of the receptor from adipocytes or muscles does not impair whole-body glucose homeostasis, whereas the liver-specific knock-out induces a severe insulin resistance and glucose intolerance [352].

3.3.1 Mechanisms of insulin resistance

Insulin resistance is often determined as a state when a certain amount of insulin does not yield the expected biological outcomes [350]. At the cellular level, resistance to insulin action could stem from two principal causes: defective binding of insulin to the IR, or impaired activation of the IR signalling cascade in the presence of normal insulin binding. Although defective binding has sometimes been reported [353], in most cases insulin resistance is accounted for by post-binding effects. Compromised activity of the components of the insulin signalling cascade, such as IR, IRS1 and PI3K, has been reported in the tissues of type 2 DM patients [354-356]. Mutations of the IR underlie some rare hereditary cases of severe type 2 DM [357]. There is a strong familial component also in the common forms of insulin resistance, but the genetic background appears very heterogeneous. In contrast, the environmental factors predisposing to insulin resistance and frank type 2 DM are well-known, the most important of them being a high-fat (Western type) diet, physical inactivity and obesity [356, 358].

In recent years, it has been recognized that instead of being just an inert lipid storage organ, white adipose tissue is hormonally and metabolically highly active. Its amount as well as a variety of adipocyte-derived molecules is increased in obesity. It has thus been suggested that these molecules could play a causative role in insulin resistance.

Adipocytokines. Increased secretion of proinflammatory cytokines by adipose tissue (adipocytes and/or adipose tissue resident macrophages) has been observed in obesity and metabolic syndrome [350]. Tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) have been most extensively studied in this aspect [359, 360]. In rodent models of insulin resistance, neutralisation of circulating TNFα by monoclonal antibodies improves insulin sensitivity [361], but similar experiments in humans have been

unsuccessful [362]. However, TNFα in humans might also act in a paracrine fashion. Several possible mechanisms for the action of TNFα have been suggested, such as the down-regulation of genes needed for insulin action [363], increased serine/threonine phosphorylation of IRSs [364] and increased lipolysis leading to elevated FFAs (see below) [365, 366].

IL-6 levels have also been reported to be elevated in obese individuals, but the levels correlate better with the amount of adipose tissue than insulin sensitivity [359]. In cell culture experiments, IL-6 has been shown to reduce the insulin sensitivity of hepatocytes [367, 368], and *in vivo* studies support this notion [369]. The mechanisms of action of IL-6 seem to be partly similar to those of TNFα, but IL-6 also activates transcription of a family of proteins called the suppressors of cytokine signalling (SOCS). These can, in turn, reduce the activity of molecules in the IR signalling pathway, such as IRS-1 [368]. In other cell types, especially striated muscle, IL-6 however seems to rather increase insulin action, and thus the effect of this cytokine in the whole body insulin sensitivity is not evident [359].

Adipokines. Several small peptide hormone products of the adipose tissue – including leptin, resistin and adiponectin, collectively called adipokines – have emerged in the concept of insuling resistance [350]. Leptin was identified as the causative mutation in the ob/ob mouse model of obesity [370], and is known to play a central role in the regulation of apetite and energy expenditure. Leptin levels are often increased in obesity, and it has been shown to affect insulin action both *in vivo* and *in vitro* [371]. These studies are however rather controversial and their physiological significance remains obscure [372]. Resistin was originally discovered as a gene suppressed by peroxixome proliferator-activated receptor γ (PPARγ) agonists in mice, and in murine models of insulin resistance and obesity, resistin levels are increased and appear to significantly contribute to the observed phenotypes [373]. In humans, however, the data is far less convincing and the function of resistin – if any – poorly understood [373].

Adiponectin, in contrast to the other adipokines, is decreased in obesity [374]. Transcription of the adiponectin gene is induced during adipocyte differentiation and

by PPARγ agonists, but inhibited by both TNFα- and IL-6-mediated signalling [375]. Adiponectin-deficient mice develop insulin resistance on a high-fat diet [376], and trans-genic expression of adinectin in adipose tissue reverses the phenotype [377]. Also in lipodystrofy, which regularly leads to insulin resistance [378], adiponectin levels are low but transplantation of even a small amount of normal white adipose tissue can correct the metabolic abnormalities [379]. In humans, adiponectin decreases the accumulation of triacylglycerols in the liver [380], which appears to play a key role in the insulin sensitivity of the liver (see below).

Elevated FFAs have been known to associate with insulin resistance for years, but recently, new mechanisms to explain the connection have emerged. Elevated circulating FFAs lead to the increased flux and metabolism fatty acids in insulin target tissues, especially the liver, and studies have reported a strong correlation between the amount of fat in the liver and its insulin sensitivity [358, 381-383]. Accumulation of fat in the liver is often observed in not only obese but also lipodystrofic patients, who also have high circulating FFAs [383]. The white adipose tissue is the main organ for fatty acid uptake, storage and release, thus a dysfunction of this tissue – present in both obesity and lipodystrophy – seems to underlie the associated FFA increase. This, in turn, leads to "ectopic" depositon of FAs in other tissues and their reduced insulin sensitivity. The insulin sensitivity of striated muscle is also decreased by intracellular lipid accumulation [358]. The increased flux of FFAs into the liver, together with reduced hepatic insulin responsiveness, could also lead to increased secretion of VLDL and to the other lipoprotein abnormalities often observed in insuli resistant states [383].

Possible cellular mechanisms of FFA-induced insulin resistance have been intensively studied [358, 384, 385]. It is known that increased metabolism of FFAs by the liver and striated muscle increases the formation of lipid secondary messangers, e.g. diacylglycerol, fatty acyl-CoA and ceramide, which can interfere with several intracellular signalling cascades. A candidate pathway leading to the development of insulin resistance involves the atypical isoforms of the serine/threonine kinases PKC, θ and ε, which can phosphorylate IR and IRS-1 molecules in serine residues. This

modification reduces the activity of these molecules by making them less optimal substrates for the IR kinase domain [386-388].

3.3.2 Membrane hypothesis of insulin resistance

Even before identification of the IR, changes in membrane biophysical parameters, especially microviscosity and fluidity, were reported upon insulin binding [389, 390]. Moreover, it was found that altered membrane properties, decreased membrane fluidity in particular, compromised insulin-induced glucose transport [391]. When decreased membrane fluidity was then reported in erythrocytes from type II DM patients [392], a connection between the properties of cell membranes and insulin signalling efficiency was suggested [6]. Reconstitution studies on the IR in liposomes demonstrated that insulin binding induces extensive changes in membrane structure in the vicinity of the receptor, and that cholesterol strongly inhibited these changes [393, 394]. IR activity was also found to be sensitive to the phospholipid and GSL composition of the membranes [395], and cell biological studies confirmed that altering the composition of cell membranes affects insulin sensitivity at the level of receptor activation [396-398].

Subsequently, the lipid composition and fluidity of cell membranes (mostly erythrocytes) from diabetic and obese patients have been measured in several studies yielding controversial results [399-405]. Increases in membrane cholesterol/phospholipid ratio and fluidity have, however, been reported in some of them [400, 402, 405], and similar observations have been made in animal studies, especially in the livers of aging Wistard rats [406, 407]. Curiously, the antidiabetic agent Metformin has been suggested to act at the level of plasma membrane, possibly by correcting the supranormal fluidity of cell membranes in the diabetic state [408]. While membrane fluidity as such can affect the function of membrane proteins (see Section 2.2.4), the altered function of lipid microdomains associated with increased cholesterol levels could also be considered as an underlying mechanism. Interestingly, it was also recently reported that TNFα-induced insulin resistance may involve lipid microdomains via increased synthesis of the ganglioside GM3. It was found that in TNFα-treated adipocytes, the amount of GM3 in DRMs was increased but the amount

of IR decreased, thus the mechanism of augmented IR function might be its replacement from the lipid microdomains [344].

The membrane hypothesis might also provide an explanation for the deleterious effects of intrahepatic triacylglycerol accumulation for insulin signalling. In animal studies, changes in the hepatocyte plasma membrane lipid composition related to fatty liver and the quality of dietary lipids have been reported [409, 410]. Similar changes might happen in the liver as well. Recent reports have also described a close relationship between plasma membrane free cholesterol and lipid droplets. The free cholesterol surrounding the lipid droplets can represent ~30% of total cellular cholesterol, and upon plasma membrane cholesterol depletion, can readily redistribute to the plasma membrane [100, 411]. This work has been carried out in adipocytes, but we have observed layers of free cholesterol surrounding lipid droplets in hepatocytes as well (our unpublished results).

AIMS OF THE STUDY

The importance of lipid microdomains in insulin signalling has been revealed in recent years, but the research has concentrated on adipocytes. The current study aimed at characterising the role of lipid microdomains in hepatocyte insulin signalling, at the level of the IR in particular. In addition, the study sought insight into the effects of disturbed cholesterol homeostasis on the composition of cell membranes and into the special structural characteristics of cholesterol in membrane function. The specific aims of the individual research projects are listed below:

I) To study the association of IR with lipid microdomains and the effects of domain disruption on IR function in hepatic cells.

II) To investigate the function of the IR in hepatocytes with intracellular lipid accumulation (primary hepatocytes from the NPC-cholesterolosis mouse), and to characterise the plasma membrane lipid composition of these cells.

III) To study the effect of altered domain-forming capacity of cell membranes on IR function by exchanging cholesterol for desmosterol, and to compare the properties of desmosterol and cholesterol in model systems and cell membranes.

METHODS

All the experimental procedures used to create the data presented in this thesis are summarised in the table below. The number of the original publication in which the method has been described is indicated.

Table 1.

METHOD	*PUBLICATION*
Cell culture	I, III
Protein determination	I, II
Cholesterol determination	I, II
Insulin stimulation and IR activation	I, II
SDS-page and Western blotting	I, II
Quantification of Western blots	I, II
Cholesterol depletion	I, II, III
Cholesterol/desmosterol-cyclodextrin complexes	I, III
Fluorescent stainings and microscopy	I, II, III
Electron microscopy	I, II
GM2 clustering	I
GSL determination	I
DRM isolation	I, II, III
Primary hepatocyte isolation and culture	I, II
Plasma membrane isolation	II
IR *in vitro* kinase assay	II
Lipid extraction	II
Phosphorus determination	II
Electronspray ionization mass spectrometry	II
[^{125}I]-insulin binding	II, III
[^{14}C]-cholesterol and [^{3}H]-choline labelling	II
Thin-layer chromatography	II
DPH anisotropy measurement	II, III
Liposome preparation	III
Detergent turbidity	III
Fluorescence quenching assay	III
Subcellular fractionation	III
Ag+-high performance liquid chromatography	III
Albumin secretion	III
Atomic-scale simulations	III

RESULTS AND DISCUSSION

1. Active IR associates with non-caveolar lipid rafts in hepatocytes

1.1 The lack of caveolae at the hepatocyte plasma membrane

The presence of the IR in caveolae/lipid rafts in adipocytes is controversial and moreover, in hepatocytes, the existence of caveolae is a matter of debate [412-414]. Thus, we first aimed at determining the expression of caveolar proteins in the cell type used in the study, Huh7. Huh7 is a human hepatoma cell line, which expresses IR at high levels and the receptor is activated in response to insulin. We were unable to detect any expression of caveolin-1 or caveolin-2 in these cells by Western blotting (I, Figure A1). When inspected by electron microscopy, morphological caveolae were not observed at the plasma membrane of Huh7 cells (I, Figure 1B). As a positive control, we used mature 3T3-L1 adipocytes, which express caveolin-1 and -2 at high levels and contain abundant caveolae.

We also isolated primary hepatocytes from mice, and studied the expression of caveolin-1 and -2 in them. Again, no expression of either of the proteins could be detected by Western blotting (I, Supplementary Figure 1A). To confirm that this lack of detection was not because of weak or non-functional antibodies, we tested several anti-caveolin-1 antibodies (data not shown). Others have, however, detected expression of caveolins in liver lysates and even in isolated hepatocytes [412, 414]. In the former case, other liver resident cell types like endothelial cells and stellate cells could contribute to the observations. For hepatocytes, the state of differentiation might be involved, as hepatocytes have been reported to up-regulate the expression caveolins during liver regeneration [411]. In any case, the hepatic cells used in this study were negative for caveolin expression and could thus be used to study the role of non-caveolar lipid rafts in IR function.

1.2 Treatments affecting lipid raft components compromise IR activity

Cholesterol removal by methyl-β-cyclodextrin is commonly used to study lipid raft – or caveolar – dependence of cellular processes, and was reported to affect IRS1, but

not IR activation in adipocytes [345, 415]. We tested the effect of cholesterol removal on IR function in Huh7 cells. The cells were treated with 10 mM methyl-β-cyclodextrin for 15 min, stimulated with insulin and the phosphorylation status of IR then analysed by Western blotting with anti-phosphotyrosine antibodies. We found that IR autophosphorylation was significantly decreased in the cyclodextrin-treated cells, and phosphorylation of IRS1 was also affected (I, Figure 2A, B). A possible explanation for the discrepancy between our observations and the previous studies is that the lack of caveolae makes the IR in Huh7 cells more sensitive to cholesterol depletion than in adipocytes.

Cholesterol depletion can have other effects on cells than lipid raft disruption [202, 269] and see Section 2.2.4), thus we wanted to confirm our results by using other methods. Crosslinking of GSLs on live cells leads to a clustering of these components in large patches [209], and given the proposed dynamic nature of lipid rafts on cell membranes, such a treatment would be likely to disturb the normal function of these domains. Multivalent antibodies can be used to crosslink GSLs, and we chose to use an antibody against the ganglioside GM2, as Huh7 cells contained high amounts of this lipid (I, text, page 3). The cells were incubated in the presence of anti-GM2 or control antibodies (a mixture of mouse IgM antibodies), followed by secondary anti-IgM antibodies to induce clustering. The incubations were performed at 12˚C to prevent endocytosis of the bound antibodies. Micronscale clusters of GM2 were observed by fluorescent microscopy when both primary and secondary antibodies were added to live cells, while adding the secondary antibodies to fixed cells resulted in a uniform staining pattern (I, Figure 3A). IR phosphorylation was markedly reduced after GM2 patching, whereas treatment with control antibodies had no effect (I, Figure 3B, C). To confirm that the result was not restricted to the Huh7 cell line, which – like other cultured cells – may exhibit characteristics not found in primary cells, we tested the effect of GM2 patching also in mouse primary hepatocytes. Again, a defect in IR activation was observed (I, Supplementary Figure 1B).

1.3 Active IR associates with detergent-resistant membranes (DRMs)

We next studied the DRM association of the IR. In adipocytes, despite the immuno-electron microscopy findings, the IR was not recovered in DRMs [345]. In that study,

1% Triton X-100 was used, we therefore chose to use a lower detergent concentration (0.1%). It has been previously shown that lower concentrations of Triton X-100 or milder detergents can be useful when detecting possible but weak DRM association of molecules [2, 203]. Huh7 cells were extracted with the detergent at 4˚C and the DRMs separated from soluble membranes by density-gradient fractionation. In the absence of insulin, the IR was completely solubilised, but after insulin stimulation, it partitioned into DRMs to a significant extent (I, Figure 4A). Under the same conditions, another plasma membrane receptor, the transferrin receptor, was exclusively found in the soluble fractions (I, Figure 4A). We also tested 1% Triton X-100, and found that the ligand-occupied IR was recovered in DRMs also after the more stringent detergent extraction, albeit to a smaller degree (I, Supplementary Figure 2).

To confirm the connection the results of the DRM-association experiments, we depleted Huh7 cells of cholesterol and performed the DRM isolation thereafter. Even in the presence of insulin, the IR was found in the soluble fractions in cholesterol-depleted cells (I, Figure 4B). To further validate the specificity of the treatment, we added back cholesterol to the depleted cells and analysed the DRM association of the IR in the cholesterol-repleted cells. Cholesterol repletion rescued the detergent resistance of the IR, although not quite up to the original level (I, Figure 4B). Others have also reported only partial rescue of raft-dependent phenomena after cholesterol depletion-repletion [244], thus it may not be possible to fully restore the delicate structure of the plasma membrane by adding back cholesterol. The activation of the IR was, however, markedly improved by cholesterol repletion (data not shown).

In addition, we tested if the effect of GM2-patching on the DRM association of IR was analogous to that of cholesterol depletion. We isolated DRMs from GM2-crosslinked cells, and found that the ligand-bound IR was not recovered in the DRM fraction (I, Figure 5A). Morphologically, we observed that fluorescently-labelled insulin was excluded from the GM2 clusters, whereas in non-clustered cells, insulin and GM2 partially co-localised (I, Figure 5B).

Together, the data indicate that the function of the IR is sensitive to changes in its membrane environment, and a possible explanation for this sensitivity is the

partitioning of the active IR into cholesterol-sphingolipid microdomains. In adipocytes, the reported interaction of IR with caveolin-1 might mediate caveolar localisation of the receptor, but in cells lacking caveolins, other mechanisms must exist. DRM association could be triggered by such events as a conformational change in the IR upon insulin binding. The IR contains palmitoyl and myristoyl modifications that might become more exposed in the active conformation, affecting the receptor-membrane lipid interactions. Previous research has also shown that reversible palmitoylation of signalling proteins can regulate their association with microdomains [416]. Segregation into domains, in turn, could augment IR signalling by several mechanisms: Domain partitioning might drive the reported self-aggregation of the IR [298] or support the active conformation of the receptor. In addition, localisation into specific domains might protect the active receptor from its down-regulators, such as tyrosine phosphatases.

Our observations also bring together data from several previous reports on the effect of membrane lipids on IR function (see Section 3.3.2). The reported changes in membrane biophysical characteristics upon insulin binding and the partitioning of the active IR into DRMs might actually be reflections of the same phenomenon.

2. Function of IR and lipid rafts in NPC hepatocytes

2.1 IR localisation in NPC hepatocytes

To gain further insight into the role of cholesterol and lipid rafts in IR signalling, as well as to the effects of intracellular cholesterol accumulation on the composition of cell membranes, we studied primary hepatocytes from the NPC-cholesterolosis mouse. The NPC disease offers a model system for investigating the effects of disturbed cholesterol homeostasis on cellular processes. A naturally-occurring mouse model of the NPC1 disease closely resembles the human disease [151], and was used in this study together with wild type (WT) littermates of the same strain.

Hepatocytes were isolated from seven-week-old mice using the collagen perfusion method [417]. Before plating, the viability of the cells was controlled. As rapid dedifferentiation of primary hepatocytes in culture is a well-known phenomenon, the

cells were always used within 24 hours of isolation and plated on gelatine-coated dishes [418]. The NPC cells exhibited the characteristic perinuclear accumulation of free cholesterol as visualised by filipin staining (II, Figure 1A). Anti-IR immunofluorescent staining revealed a punctuate pattern at the plasma membrane of both WT and NPC cells, with no co-localisation with the late-endosomal/lysosomal marker Lamp-1 (II, Figure 1B). We also studied the distribution of IR in the presence of insulin and after insulin washout. In both cell types, the plasma membrane staining pattern was completely recovered after a 60 minute washout, indicating that the IR in NPC cells does not become sequestered to the storage organelles upon receptor endocytosis (Figure 7).

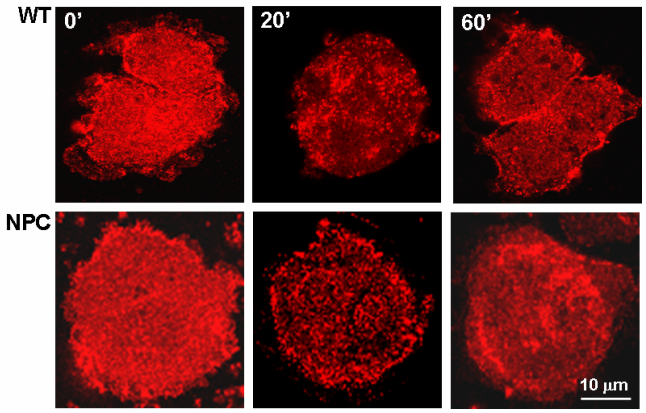

Figure 7. IR recycling in WT and NPC mouse primary hepatocytes. Insulin was bound to the cells for 15 min on ice, and the cells were then washed and moved to 37˚C for indicated timepoints (0, 20 or 60 min). Thereafter, the cells were fixed with acetone and stained with anti-IRβ antibodies. The plasma membrane staining present at the 0 min time point was recovered after 60 min of insulin washout in both WT and NPC cells.

2.2 IR levels and activation in NPC hepatocytes

Next, the amount of IR in WT and NPC hepatocytes was studied by Western blotting. Unexpectedly, we found that the receptor levels in NPC cells were about 50 per cent higher than in WT cells (II, Figure 2A, B). This finding was highly consistent and observed in all the mice analysed, as well as in whole liver and brain lysates from the

NPC mice (data not shown). Interestingly, the up-regulation of IR mRNA in several NPC patient fibroblast lines has also been observed (Dr. Suzanne Pfeffer, personal communication). To analyze the activation of the IR, WT and NPC cells were stimulated with insulin and IR phosphorylation was determined by immunoblotting with anti-phosphotyrosine antibodies. Despite the higher expression levels, the amount of phosphorylated IR in NPC cells was similar to that observed in WT cells, indicating that the activation efficiency of the NPC IR was compromised (II, Figure 2A). When activation of the IR was analysed as a function of time, the difference between WT and NPC was detected at several time points (II, Figure 2C).

A possible explanation for the increased number but decreased phosphorylation of the IR in NPC cells could be that the receptors are located in a compartment inaccessible to insulin. However, the results from IR immunofluorescent stainings suggested that the IR localised at the plasma membrane in NPC cells, and is thus available for insulin binding. Subsequent experiments with [^{125}I]-labelled insulin and isolated plasma membranes (II, Figures 6 and 7) also indicated that insulin binding to the IR was not affected in NPC cells. Thus, we concluded that the up-regulation of IR in NPC cells could be a compensatory mechanism to overcome the decreased activity of the receptor. In mice, this compensatory mechanism is apparently able to balance the system, as we did not observe differences in the fasting insulin levels of WT and NPC mice (data not shown). This also corroborates the fact that insulin resistance, or at least frank diabetes, is not commonly observed in NPC patients. However, other aspects of insulin signalling, such as the stimulation of cell survival, especially in the central nervous system, might still be affected and contribute to the pathogenesis of the NPC disease. Moreover, the mechanism of defective signalling could be shared by other growth factor receptors, and indeed, impaired function of the raft-associated neurokine receptor TrkB in NPC cells has been reported [275, 419].

2.3 Activation of IR in isolated plasma membranes

To further analyse the mechanisms of defective IR activation in NPC cells, we isolated plasma membrane fractions from WT and NPC mouse livers. The livers were homogenised and fractionated in a discontinuous sucrose-density gradient. This fractionation was fairly efficient in separating the plasma membranes of WT cells from other cellular membranes, like the ER and the late endosomes/lysosomes, as

judged by anti-calnexin and anti-Lamp1 immunoblotting, respectively, of the plasma membrane-enriched fraction (the interphase between 0.8 M/1.2 M sucrose - II, Figure 3A). The fraction representing the plasma membrane in NPC samples, however, contained considerable amounts of lysosomal membranes, as revealed by anti-Lamp-1 Western blotting (II, Figure 2A), and ultra-structural analysis of the fraction by electron microscopy (II, Figure 2A). This difference between WT and NPC most likely reflects the proliferation of the storage organelles in NPC cells, as well as their high cholesterol content, which affects the density of the membranes. Thus, the plasma membrane-enriched fractions recovered from the first gradient were subjected to a second gradient purification step. After this second gradient, WT and NPC cells appeared morphologically similar, and only trace amounts of Lamp-1 and calnexin were detected in the plasma membrane fractions (II, Figure 2B). In comparison to the starting material (II, Figure 3C), the final preparations of both the WT and NPC samples exhibited an eightfold enrichment of the plasma membrane markers IR and Na^+/K^+ ATPase.

We next set up an *in vitro*-assay for studying IR activation in the isolated plasma membranes. IR phosphorylation in the membrane preparations was observed in the presence of insulin and ATP, but not if either of these components was excluded (II, Figure 4A). The difference between IR phosphorylation in WT and NPC samples was also detected in the isolated membranes (II, Figure 4B, C). Indeed, when the samples were normalised for the amount of IR, the compromised activation of the receptor in NPC membranes was even more evident than in intact cells (II, Figure 4B). These observations indicated that the IR activation defect in NPC cells was caused by factors present at the plasma membrane. Increased tyrosine phosphatase activity could account for decreased IR phosphorylation, and has been shown to be one mechanism of defective IR activation in insulin-resistant Wistard rats [406]. Membrane-associated phosphatases could be present in the plasma membrane preparations; therefore, we performed the IR activation assay in the presence of phosphatase inhibitors. This treatment was accompanied by a slight increase in IR phosphorylation in both WT and NPC preparations, but the difference between the samples was maintained (data not shown).

2.4 Lipid composition of the NPC hepatocyte plasma membranes

We then studied the lipid composition of the isolated plasma membranes. The amounts of cholesterol and total phospholipids were determined by enzymatic assays and the detailed composition of the constituent phospholipids by electronspray ionization mass spectrometry. We detected a twofold excess of cholesterol in the NPC samples in comparison to WT, but no significant difference in the total amount of phospholipids, thus the cholesterol/phospholipid ratio in the NPC membranes was increased (II, Figure 5A). A previous study reported a tenfold increase in the cholesterol content of NPC mouse livers [420], suggesting that our plasma membrane preparation method was relatively efficient in removing the storage organelles. Minor contamination of the plasma membrane preparations by late-endosomal/lysosomal membranes can however not be ruled out, but we have several reasons to believe that the plasma membrane of NPC cells was indeed enriched in cholesterol.

Previous studies have reported controversial results concerning the cholesterol content of the NPC plasma membranes [139, 140, 421-423], but these studies have used variable techniques and cell types that are likely to explain at least part of the difference [5, 424]. To our knowledge, the current study is the first to investigate the plasma membrane of NPC hepatocytes. Hepatocytes differ from other cell types in several aspects of cholesterol metabolism, as they are constantly dealing with high amounts of both endogenously-synthesised and lipoprotein-derived cholesterol. Prior research has shown that in addition to LDL cholesterol, other cellular cholesterol pools (e.g. newly synthesised cholesterol) contribute to the cholesterol accumulation in NPC cells [425]. In addition, despite the late-endosomal transport block in the NPC cells some LDL-derived cholesterol still reaches the plasma membrane [140]. It can therefore be surmised that eventually, the “backwards” leakage of cholesterol from the late endosomes/lysosomes fills up other membrane compartments as well. This does indeed appear to be the case, as the whole endosomal system in NPC cells appears paralysed [132] and even the early endosomal compartment, which derives directly from the plasma membrane, has been shown to be cholesterol-enriched [138]. Finally, an additional mechanism in hepatocytes, i.e. cholesterol uptake from HDL particles via SR-B1, appears to deliver cholesterol to the plasma membrane without passing via the late endosomes [426, 427].

The mass spectrometric analysis of the major phospholipid classes (PC, SM, PE) revealed that in the NPC plasma membranes, the average degree of acyl chain unsaturation of PC and SM was decreased in comparison to WT, and especially in SM, the average chain length was decreased (II, Figure 5B). In PE, only minor differences were detected. The acyl chain composition of SM and PC indicated that at least the outer leaflet of the NPC plasma membrane would have a more rigid and ordered phase-favouring structure than the plasma membrane of WT cells. To assess the fluidity of our membrane preparations, we studied the fluorescence polarisation of 1,6-diphenyl-1,3,5-hexatriene (DPH) in WT and NPC membranes. The anisotropy of DPH polarisation can be used as a measure of membrane organisation, or fluidity (high values indicating high ordering and low values low ordering) [428]. We found that DPH anisotropy values in NPC membrane preparations were significantly increased in comparison to WT (II, text, page 11).

These results further supported our notion that the cholesterol content of NPC plasma membranes increased. Indeed, as cholesterol packs best next to saturated acyl chains and the metabolism of cholesterol and SM, and to some extent also PC, are interconnected [15], the detected acyl chain changes could reflect an unavoided consequence of the cholesterol overload. In a previous study, the changes in the phospholipids at the plasma membrane of NPC1 fibroblasts were in the opposite direction [421]. The discrepancy between the current data and that obtained in fibroblasts might be related to cell type differences, or to the fact that the material used in this study was from primary cells. Cell lines, even if they are of primary origin, can develop varying phenotypes when cultured for a long time. In favour of this explanation, decreased fluidity and fatty acyl unsaturation of cellular membranes in another NPC1 fibroblast cell line have been reported [422]

2.5 DRMs in NPC hepatocytes

The lipid composition of the NPC plasma membranes (i.e. high cholesterol content and saturated phospholipids) pointed towards increased formation of DRMs. We therefore studied the DRM association of the IR and other lipid raft components in NPC cells. Because of technical difficulties, it was not possible to detect the DRM association of the inactive IR in hepatocytes, so we performed detergent extraction and density gradient on the isolated plasma membranes. Both hepatocytes and the

isolated membranes were found to be less sensitive to the detergent treatment than Huh7 cells, so 1% Triton X-100 was used in these experiments. The inactive IR in WT plasma membranes exhibited no detergent resistance, whereas in NPC membranes, small amounts of IR were detected in DRMs even in the absence of insulin (II, Figure 6A). The DRM association of the active receptor was studied in hepatocytes by using [^{125}I]-labelled insulin. Cells were stimulated with the radio-labelled ligand and the amount of the label in each fraction was detected. In WT cells, the amount of [^{125}I]-insulin in DRMs was approximately 20 % of the total, whereas in NPC cells, the amount recovered in DRMs was around 40 % (II, Figure 6B). In this experiment, we also determined the total amount of [^{125}I]-insulin binding to WT and NPC cells, and found that NPC cells exhibited increased insulin binding, consistent with increased IR levels, plasma membrane localisation, and the normal ligand binding capacity of the receptor (data not shown).

The association of membrane lipids with DRMs was studied using [^{14}C]-cholesterol and [^{3}H]-choline labelled WT and NPC cells. Choline becomes incorporated into both PC and SM, thus to detect the amounts of these two species individually, lipids in the gradient fractions were extracted and separated by thin-layer chromatography. We found that the DRM association of all these lipids was increased in NPC cells, although in the case of SM, the difference was not statistically significant (II, Figure 6C). The clearest difference was in the DRM association of PC, as this lipid in the WT hepatocytes (and in other normal cells) is almost completely soluble. The increased DRM association of PC in NPC cells probably reflects the increased amount of saturated PC species, as well as the decreased fluidity of the NPC membranes in general. It can indeed be envisioned that only a minor fraction of NPC cell membranes would actually be solubilised by the detergent treatment.

2.6 Cholesterol depletion improves IR activation in NPC membranes

The decreased fluidity of NPC membranes and the increased DRM association of the IR prompted us to test the effect of cholesterol depletion on IR activation in NPC cells. Because of the harmful effects of the treatment on live cells, we tested the effects of cholesterol removal on isolated plasma membranes. Prior research has shown that methyl-β-cyclodextrin treatment of plasma membrane vesicles decreases

the ordering of the membrane lipids [429]. We incubated the membrane preparations in the presence of methyl-β-cyclodextrin and analysed IR activity thereafter. In WT samples, the treatment compromised IR activation, in accordance with our previous data (I and data not shown), but in NPC membranes, we detected a significant increase in IR phosphorylation (II, Figure 7A, B).

Together, the data describes increased levels, but compromised activity of the IR in NPC mouse primary hepatocytes. These cells also exhibited an altered lipid composition and a decreased fluidity of the plasma membrane and the DRM association of the IR and major membrane lipids were increased. The activity of the IR can be improved by removing cholesterol from the membranes. This improvement can result from an increased fluidity of the membrane as such, or be more specific for cholesterol removal, but either way, the finding is highly interesting. Decreased membrane fluidity, owing in many cases to increased cholesterol/phospholipid ratio, has been reported in cell membranes from insulin-resistant subjects, and evidence suggests that the antidiabetic agent Metformin acts, at least in part, by correcting these unfavorable cell membrane properties (see Section 3.3.2). The mechanism, by which decreased membrane fluidity impairs IR function, could be disrupted membrane dynamics and function of lipid microdomains. In addition to the NPC cells, we were able to demonstrate the inhibitory effect of increased plasma membrane cholesterol in Huh7 cells by loading them with cholesterol/cyclodextrin complexes (Figure 8).

Another important point in this work is the demonstration of altered lipid composition of the plasma membrane as a result of intracellular lipid accumulation. This holds at least in the case of hepatocytes, and as discussed in Section 3.3.3, the accretion of intrahepatic lipid strongly correlates with insulin resistance. The mechanisms of lipid storage in NPC and the more common forms of fatty liver are very different, but the composition of the plasma membrane could be affected also in the latter. In particular, the suggested interaction between cytoplasmic neutral lipid droplets and plasma membrane free cholesterol could be a mechanism modulating the properties of plasma membrane, and possibly IR function, in the common forms of fatty liver.

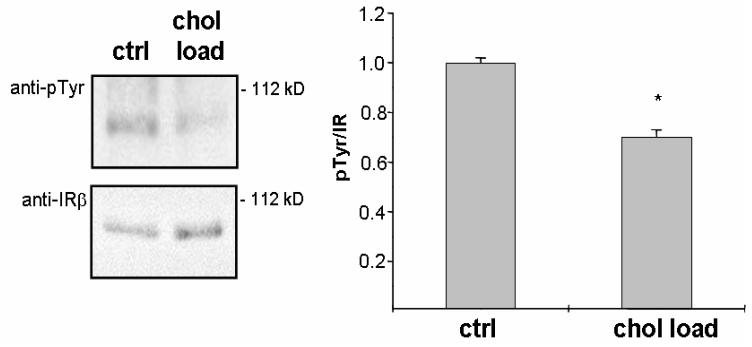

Figure 8. IR activation in cholesterol-loaded Huh7 cells. The plasma membrane of Huh7 cells was loaded with cholesterol by incubating the cells in the presence of cholesterol/methyl-β-cyclodextrin complexes for 1 h. IR activation in response to insulin stimulation (3 min) was analysed thereafter. A representative immunoblot together with quantitation of the data is shown. Anti-pTyr, anti-phosphotyrosine; pTyr/IR, the intensity of the anti-pTyr band divided by the intensity of the anti-IR band.Values are mean ± SEM, n = 10, *p< 0.01 between ctrl and chol load.

3. The effect of desmosterol on domain formation and IR function

3.1 Domain-forming properties of desmosterol in liposomes

Desmosterol is an abundant membrane component in certain cell types, and its structure differs from that of cholesterol only by the presence of an additional double bond at position C24 (III, Figure 1). The effects of this structural difference on the properties of desmosterol-containing membranes are not clear. In humans, mutations in the gene coding for 24-dehydrocholesterol reductase results in severe phenotypes [161, 162], whereas knock-out mice lacking the enzyme are viable, although small and infertile [430]. In dioleyl-PC model membranes, the acyl chain-ordering effect of desmosterol was found to be similar to that of cholesterol [239], while in a saturated PC matrix, the effect of desmosterol was weaker [240].

We studied the properties of cholesterol and desmosterol containing ternary liposomes (consisting of dipalmitoyl-PC, DPPC, dioleyl-PC, DOPC and the sterol, III, Table I). First, the liposomes were subjected to detergent (Triton X-100) solubilisation and the light scattering remaining after detergent addition was determined by measuring the

optical density (OD) values. This method has been widely used in detecting Lo domains in model membranes, and is based on the formation of small micelles in samples that are solubilised, and the maintenance of big, turbid vesicles in samples that are not solubilised, upon detergent addition [172]. We found that the presence of cholesterol significantly increased the OD values in comparison to non-sterol-containing liposomes (III, Figure 2A), in accordance with several previous reports [172]. The effect of desmosterol was considerably milder, although a clear difference between desmosterol and no sterol was detected (III, Figure 2A).

We obtained analogous differences between the sterols when the DPH fluorescent polarisation, as a measure of fluidity and lipid ordering in the vesicles was determined (III, Figure 2B). The vesicles in these experiments contained 15 mol% of sterol, which is less than the amount of sterol normally present at the plasma membrane. This concentration was chosen because it has been used in previous studies assessing the effects of different sterols in domain formation [238] and because the domains formed with this concentration are relatively stable and present in the majority of the vesicles [189]. However, we also tested liposomes with 30 mol% of sterol, and found that the results for OD measurements were essentially similar to those obtained with the lower amount of sterol (data not shown).

Fluorescent quenching assay is another commonly-used method to assess domain formation and stability in model membranes [234]. In this method, an ordered-phase-favouring quencher lipid and a reporter lipid distributing equally among different phases are included in the membranes (III, Table I). When Lo phase forms, the quencher is sequestered away from the reporter and consequently, more fluorescence is detected, whereas disruption of the Lo phase has the opposite effects. When the fluorescence quenching is measured as a function of temperature, the thermal stability of the Lo domains can be estimated [431]. We studied the fluorescent quenching in cholesterol and desmosterol-containing vesicles. In comparison to non-sterol-containing liposomes, both sterols increased the fluorescent intensity at 20˚C, but the effect of desmosterol was again milder (III, Figure 2C, D). When the relative stability of the domains was determined by calculating the apparent Tms, we found that cholesterol significantly increased the Tm while the Tms for desmosterol and non-sterol containing vesicles were essentially similar (III, Figure 2E). Together, data

from the liposome experiments showed that desmosterol exhibits some ordering potential in the DPPC-DOPC membranes but this effect is significantly weaker than that of cholesterol.

3.2 Distribution of desmosterol in cell membranes

We next studied the distribution of desmosterol in cell membranes. To obtain measurable amounts of desmosterol, we chose to use Chinese hamster ovary (CHO) cells that are known to efficiently synthesise cholesterol via desmosterol [432]. To up-regulate cholesterol biosynthesis, the cells were cultured in the absence of lipoproteins for 7 days. This resulted in a significant increment in the amount of desmosterol (up to 10% of total cellular sterol). Cellular membranes were then separated by sucrose-density gradient fractionation. Cholesterol and desmosterol exhibited essentially similar distribution within the gradient, and the majority of both sterols was detected in intermediate-buoyant density fractions (0.8 – 1.2 M sucrose) corresponding largely to the plasma membrane and endosomes (III, Figure 3A). These membranes also contained a major pool of caveolin-1 (data not shown). The ER marker calnexin (data not shown) was mostly detected in the low-density fractions that contained little cholesterol and desmosterol (III, Figure 3A), implicating that neither of the sterols was enriched in the ER membranes. Our results are in accordance with a previous study reporting that desmosterol in cells localises to the plasma membrane [159].

To investigate whether desmosterol associates with DRMs, we subjected the plasma membrane-enriched membrane fractions to detergent extraction, pelleted the insoluble membranes and analysed the amount of desmosterol and cholesterol in the pellet and in the supernatant. While the majority of cholesterol was detected in the DRMs, desmosterol was almost completely soluble (III, Figure 3B). Thus, even though desmosterol is present at the plasma membrane, it does not efficiently partition into DRMs. Similarly to model membranes, desmosterol may therefore have a weaker organising potential than cholesterol in cell membranes as well.

3.3 Acute exchange of cholesterol with desmosterol in cell membranes

To study if exchanging cholesterol with desmosterol would have functional effects in cells normally containing cholesterol, we set up a protocol for acutely changing the majority of the plasma membrane sterol. Changing the sterol could affect cholesterol

and/or lipid microdomain-dependent processes, such as IR signalling, and we also sought to find out how IR function in particular would react to the sterol exchange. Huh7 cells were used for these experiments. They normally contain cholesterol as their sole membrane sterol, and conversion of desmosterol as well as other precursors into cholesterol occurs rapidly [158]. We used methyl-β-cyclodextrin to remove about 70 per cent of plasma membrane cholesterol and then fed back either cholesterol or desmosterol from a cyclodextrin complex. We tested several repletion times, and found that the total amount of sterol returned to control levels within one hour (III, Figure 4A). The majority of the added desmosterol was not yet converted to cholesterol at this point (III, Figure 4A).

To ensure that the cells that had undergone the sterol exchange protocol were still viable, we studied their morphology by fluorescent microscopy. As expected, the filipin staining intensity dropped dramatically after methyl-β-cyclodextrin treatment, but returned back to normal after either cholesterol or desmosterol repletion (III, Figure 4B, upper panel). Filipin staining also revealed that the basic morphology of the repleted cells was similar to that of control cells. To visualise the Golgi apparatus, the cells were stained with fluorescently-labelled lectin. Cholesterol depletion caused a mild dispersion of the staining, but repletion with both of the sterols reversed the phenomenon (III, Figure 4B, lower panel).

As an indicator of the general well-being of the sterol-exchanged cells, and also as an example of a cellular process not thought to be lipid raft-dependent, we studied protein secretion in the sterol-exchanged cells. Huh7 cells secrete high amounts of albumin, with 50 % of newly-synthesised albumin being secreted within one hour [158]. Thus, we incubated the cells for one hour after the sterol exchange protocol in serum-free media, recovered secreted proteins from the media by tricholoroacetic acid precipitation, and analysed the amount of albumin by Western blotting. We found no difference in albumin secretion between control and cholesterol- or desmosterol-repleted cells (III, Figure 4C). Lipid analysis of the cells after the secretion period showed that the desmosterol-repleted cells still had desmosterol as their major sterol (~60% of total, III, text, page 7). From these results, we concluded that the sterol-

exchanged cells were in relatively good condition and gross functions like protein secretion were not affected by the treatment.

3.4 Desmosterol compromises IR activity and DRM-association

We then moved on to analyse the consequences of sterol exchange in IR function. The sterol-exchanged cells were stimulated with insulin and IR activation analysed by Western blotting, as in our previous studies. We found that while insulin robustly stimulated IR phosphorylation in cholesterol-repleted cells, receptor activation was almost completely abolished in desmosterol-containing cells (III, Figure 5A). This was not due to decreased insulin binding, as both cholesterol- and desmosterol-repleted cells showed similar [^{125}I]-insulin binding properties (III, Figure 5B). To study whether the impaired function of the IR was coupled to changes in the DRM association of the receptor, the sterol-exchanged and insulin-stimulated cells were subjected to detergent extraction. In cholesterol-repleted cells, the IR was detected in DRMs; although similar to the cholesterol depletion-repletion experiment described in study I, the amount of the active receptor in DRMs was lower than in untreated cells. However, in the desmosterol-repleted cells, IR localised exclusively to the soluble fractions (III, Figure 5C).

The results indicate that desmosterol is not equivalent to cholesterol in cell membranes, at least not in supporting a specialised signalling function. Currently, we do not know whether the inhibitory effect of desmosterol on IR function is specific to desmosterol itself or accounted for by the lack of cholesterol and/or altered membrane properties and domain formation. The latter possibility would however be well in line with our previous results (I and II) as well as with the liposome experiments in this study.

We also studied the DRM association of PC and SM in the sterol-exchanged cells. The lipids in the different gradient fractions were analysed by electronspray ionization mass spectrometry. PC was, as expected, almost totally soluble, but somewhat surprisingly, SM exhibited considerable detergent resistance also in desmosterol-repleted cells (our unpublished results, Figure 9). The low domain-forming capacity of desmosterol in model membranes would predict little DRM assembly in the desmosterol-repleted cells as well. However, the desmosterol-repleted cells do contain

some cholesterol, which – although not sufficient to support the DRM-association of IR – might partly fulfil the requirements for Lo phase creation. It was recently reported that tiny amounts of cholesterol are enough to support the viability of mammalian cells, if some other sterol is present in larger amounts [21]. Alternatively, as it is currently unclear whether DRMs from cell membranes actually represent Lo phase domains or lipid/lipid-protein complexes of some other kind, the DRMs obtained from desmosterol-repleted cells could be e.g. sterol-independent clusters of SM and GSLs [199, 200].

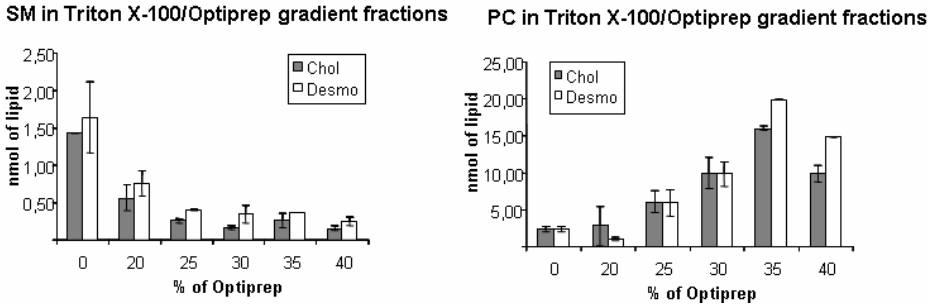

Figure 9. The amounts of SM and PC in Triton X-100/Optiprep gradient fractions. Sterol-exchanged cells were subjected to the detergent treatment (1% Triton X-100 for 10 min at 4˚C) and the Optiprep density gradient fractionation. Lipids in the fractions were extracted and the amounts of PC and SM analysed by electronspray ionization mass spectrometry. Values show means ± st dev, data from a representative experiment performed in duplicates.

3.5 Atomic-scale simulations of desmosterol-containing membranes

Recent atomic-scale stimulations have demonstrated the ordering effect of cholesterol on membrane lipids [174, 433], so we took this approach to gain further insight into the biophysical and functional differences between desmosterol and cholesterol. We studied the properties of dipalmitoyl PC-cholesterol (DPPC-chol) and DPPC-desmosterol (DPPC-desmo) bilayers at a sterol concentration of 20 mol% in the fluid phase and compared them to those of a pure DPPC bilayer. To our knowledge, previous atomic-scale simulation studies of lipid-desmosterol systems are not available.

To assess the sterol-dependent ordering of lipid hydrocarbon chains we determined the molecular order parameter, S_{CD}. Profiles of the order parameter for the *sn2* chain of DPPC in the PC-chol and PC-desmo systems revealed that both sterols increased the acyl chain order compared to the pure DPPC but cholesterol was significantly more effective in this respect (III, Figure 6A). The hydrocarbon chains of DPPC lie, on average, almost parallel to the bilayer normal in the presence of cholesterol, while the effect of desmosterol is less prominent (III, Figure 6B). An increase of membrane order is associated with changes in the number of gauche defects in the acyl chains and in the tilt angles of hydrocarbon chains with respect to the membrane normal. Cholesterol was found to be more effective than desmosterol in ordering the bilayer also when judged by these parameters (III, Table II). Moreover, the structural properties describing the condensing effect showed that cholesterol reduces the average surface area per DPPC and increases membrane thickness more effectively than desmosterol (III, Table II). We also determined the orientation of the sterols in the bilayer by considering the tilt angle between the sterol ring axis (vector between carbon atoms C3 and C17) and the bilayer normal. The average tilt angles of cholesterol and desmosterol were found to be clearly different, about 20º for cholesterol and 27º for desmosterol (III, Table II).

To study the molecular mechanisms responsible for the different actions of the two sterols, we focused on the conformations of the sterol tails and their interactions with the hydrocarbon chains of the DPPC molecules. The molecular order parameters of the tail segments showed that the cholesterol tail is strongly ordered, whereas in desmosterol the ordering of the tail is significantly lower and decreases strongly after the first segment of the chain (III, Figure 6C). This decrease is associated with the different conformations of the first two torsion angles in the chain (torsion around the bonds C17-C20 and C20-C22) (III, Figure 6D). Thus, it appears that the double bond in the tail of desmosterol changes the shape and flexibility of the end of the tail – the last four atoms lie on a surface and create a rigid structure. Consequently, the van der Waals interactions of the desmosterol tail with the hydrocarbon chains of the DPPCs should be different from those of cholesterol. To elucidate these differences, we conducted an analysis similar to that performed by Róg and Pasenkiewicz-Gierula [434]. We observed that van der Waals interactions of the last four atoms of the tail with DPPC hydrocarbon chains are stronger in the case of desmosterol than

cholesterol. At the beginning of the tail, close to the steroid ring structure, the effect was the opposite, as the hydrocarbon chains of DPPC packed better around the upper part of the tail of cholesterol than that of desmosterol.

Together, the simulations show that the double bond in the hydrocarbon tail of desmosterol gives rise to an additional stress in the tail, changing its conformation at the beginning of the tail compared to cholesterol. This seemingly minor difference has rather profound implications for various structural properties of the bilayer (III, Figure 7), in particular fluidity. These results are well in accordance with our findings in liposomes and in cell membranes. The finding that desmosterol impairs lipid microdomain-dependent signalling events pinpoints the importance of the special structural characteristics of cholesterol for cellular processes. Interestingly, the two pathways of cholesterol synthesis, Kandutsch-Russell and Bloch, produce precursor sterols with different domain-forming capacities (lathosterol and 7-dehydrocholesterol and desmosterol, respectively, see Section 2.1.3). Thus, as the precursors get incorporated into the cell membranes, the use of these alternative pathways might provide the cells with additional means to regulate their membrane properties.

Table 2. Biological processes, in which differences between cholesterol and desmosterol have been reported

Process	**Ref.**
Induction of conformational change in SCAP: desmosterol > cholesterol	[435]
ACAT substrate: desmosterol<cholesterol	[436]
Suppression of HMG-CoA activity: desmosterol>cholesterol	[437]
Precursor for 25-hydroxycholesterol: desmosterol<cholesterol	[438]
LCAT* substrate: desmosterol>cholesterol	[439]
Hedgehog morphogen signalling: desmosterol<cholesterol	[10, 440]

*LCAT, lecithin: cholesterol acyl transferase

PROSPECTIVES

The studies in this thesis describe the effects of membrane lipids, especially cholesterol, on IR signalling. It appears that the function of the receptor is highly sensitive to changes in its membrane environment, and we suggest that this sensitivity is mediated via cholesterol-dependent lipid microdomains. This view is reinforced by the finding that a sterol with a weak domain-forming capacity is unable to support IR activation. However, the exact nature and composition of these microdomains awaits characterisation. Especially the field of cell biology suffers from the lack of proper methods for studying them. New methods, microscopic in particular, are constantly being developed [441], but real breakthroughs are not yet in sight. In addition to the advances needed in cell biological studies, model membrane research should aim at characterising the behaviour of complex systems under physiological conditions (in terms of such measures as temperature, lipid species and the inclusion of proteins). Otherwise, the results from these studies will be difficult to apply to biological systems [266].

Despite these setbacks, it is interesting to speculate on the possible mechanisms how lipid microdomains might modulate IR function. Membrane domains could regulate or drive the microaggregation of IR, or receptor palmitoylation might govern the association with DRMs. We are currently characterising the function and DRM association of the IR in cells with deficient function of the enzyme palmitoyl protein thioesterase (an enzyme regulating protein palmitoylation).

Another concept addressed in the current study is the effect of intracellular lipid imbalance on the composition of plasma membranes. These findings relate to the pathophysiology of several diseases, including NPC and other lipid storage diseases, as well as more common conditions like non-alcoholic fatty liver. Alteration of membrane lipid composition could be a mechanism shared by these conditions, and in addition to IR, it could affect the function of several other membrane proteins. In an ongoing project, we are analysing the lipid composition and activation efficiency of IR in erythrocyte membranes from insulin-resistant subjects.

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