Analysis of lipid uptake and processing in cultured cells

(Lipid-Aufnahme und Stoffwechsel in kultivierten Zellen)

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Hiermit versichere ich, dass ich diese Arbeit selbstständig und ohne jede unerlaubte Hilfe angefertigt habe, dass diese oder eine ähnliche Arbeit noch keiner anderen Stelle zur Prüfung vorgelegen hat.

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Ort, Datum Radwan Hameed

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Content

1. Summary

To characterize lipid uptake pathways in cultured cells, uptake and metabolism of different isotope-labeled lipid probes were investigated in human skin fibroblasts, a mouse macrophage-like cell line (RAW264.7), human hepatocellular carcinoma cells (HepG2 cells) and a human squamous carcinoma cell line (A431 cells). As lipid probes, we used different fatty acids, cholesterol, a cholesteryl ester, a triacylglycerol, and the phospholipids phosphatidylcholine and sphingomyelin. The uptake and metabolism of exogenously added lipid probes differed with cell type, lipid structure, and mode of delivery. Cationic amphiphilic drugs (CADs) are widely used drugs that are known to interfere with lipid metabolism and to induce phospholipidosis in human patients. We investigated the influence of the desipramine, imipramine, chlorpromazine, chloroquine, and FTY720 as representative CADs on uptake and processing of the phospholipid probes. Desipramine was found to have drastic and cell-type specific effects on FA processing. Lipid processing was also impaired in a genetic phospholipidosis, Niemann-Pick disease, type A.

Fatty acids: to study the uptake and processing of C-18 fatty acids in cultured cells, uptake and incorporation of four FA probes in membrane phospholipids and in nonpolar lipids were monitored. We used FA probes that differed in the degree of unsaturation: stearic acid (18:0), oleic acid (18:1,ω-9), linoleic acid (18:2, ω-6), and linolenic acid (18:3, ω-3). These FA were applied in complex with bovine serum albumin (BSA) to the four different types of cultured cells. Significant differences were found between uptake and metabolism of these fatty acids, when fatty acid class and cell type were varied. FA uptake by fibroblasts and macrophages was highest with 18:1, and lowest with 18:3, and 18:0, respectively. Uptake by A431 cells and HepG2 cells was lowest with 18:0, and highest with 18:2 and 18:3, respectively. In macrophages, stearic acid and oleic acid are predominantly incorporated into nonpolar lipid droplet (LD) lipids, while linoleic and linolenic acid are predominantly incorporated into polar lipids. Also in HepG2 cells, the level of FAs incorporated into polar lipids was much greater for unsaturated FAs than for saturated FAs. In fibroblasts, only a minor incorporation of FAs into neutral lipids and a major incorporation into polar lipids were observed. In A431 cells, 18:2 was best incorporated into neutral lipids, followed by 18:3, 18:1, and 18:0. The impact of a cationic amphiphilic drug (CAD, FIASMA = functional inhibitor of acid sphingomyelinase), desipramine, on this process was also analyzed. Treatment with desipramine caused a tremendous reduction of FA-incorporation into triacylglycerols of

macrophages and A431 cells, but only a slight decrease in HepG2 cells. Fibroblasts showed an unexpected increase in the incorporation of FAs into triacylglycerol (TAG) and diacylglycerol (DAG). We also measured the uptake of [^{3}H]desipramine by different types of cells, which was lowest in fibroblasts.

Cholesterol, cholesteryloleate, and triolein: to characterize the uptake pathways for these lipids, we investigated the effect of different lipid delivery methods. The exogenous lipid probes were applied to the four different types of cultured cells either in complex with bovine serum albumin (BSA), or as components of low density lipoprotein (LDL) particles. Significant differences in uptake and metabolism after application of these two methods were found. When incorporated into LDL, uptake of cholesterol, cholesterol ester, and triacylglycerol was 2-4-fold higher than when delivered by BSA. Furthermore, the uptake of cholesterol presented as BSA-complexes was best for A431 cells, while uptake of the other lipids presented as LDL- and BSA-complexes were higher in fibroblasts than the other cell types. Also the metabolic incorporation of cholesterol and oleate derived from cholesterol ester and triacylglycerol was higher. These findings indicate that LDL-associated lipid is incorporated into cultured cells via a pathway that differs significantly from that of BSA-lipid.

Cholesterol and phosphatidylcholine processing in Niemann-Pick disease, type A: to investigate the role of Niemann-Pick disease, type A (NPA), one of the lysosomal storage diseases, on the processing of [^{14}C]cholesterol and [^{14}C]phosphatidylcholine, we applied the methods mentioned above to human fibroblasts and to fibroblasts from patients with NPA disease. Incubation with LDL-associated [^{14}C]phosphatidylcholine and LDL-associated [^{14}C]cholesterol show reduced processing of [^{14}C]phosphatidylcholine and [^{14}C]cholesterol by 25, and 21%, respectively. This study indicates that in NPA disease, also nutrient delivery via the endolysosomal system is impaired.

Phosphatidylcholine (PC) and sphingomyelin (SM) processing in drug-treated cells: to investigate the influence of cationic amphiphilic drugs on uptake and processing of exogenously added choline-containing phospholipids, the influence of five cationic amphiphilic drugs, desipramine (DMI), imipramine (IM), chlorpromazine (CPZ), chloroquine (CQ), and fingolimod (FTY720), were studied. The lipid probes were delivered as components of LDL-particles, and the metabolic fate of their isotope-labeled fatty acid moieties was monitored in the four different cell types mentioned before. Concentrations of

10µM had slightly to no apparent effect on [^{14}C]-SM and [^{14}C]-PC processing for all CADs tested. Profound changes were observed when CADs were administered in high concentration (20µM, and 40µM). In macrophages, all investigated drugs lead to an impaired processing of SM and PC. Incorporation of the fatty acids released from PC and SM into diacylglycerols, triacylglycerols, and glycerophospholipids was drastically reduced in the presence of 20µM of the drugs. Furthermore, each cell type showed a characteristic neutral lipid and phospholipid pattern. The effect of the investigated CADs on SM and PC processing in terms of *p*mol per µg cell protein depend on the concentration of the investigated CADs, the cell type, and the identity of the FA in lipid probes. For example, 20µM FTY720 caused a drastically reduced incorporation of sphingomyelin-derived stearic acid into triacylglycerols in macrophages, fibroblasts and A431 cells, but not in HepG2 cells. While 20µM FTY720 caused a slightly reduced incorporation of phosphatidylcholine-derived palmitic acid into triacylglycerols in macrophages. Therefore, our method is able to detect metabolic steps that are affected in the presence of CADs and to predict the potential of CADs to induce phospholipidosis.

2. Introduction

In this work, the uptake and metabolism of different isotope-labeled lipid probes in fibroblasts (which can be obtained also from the patients), macrophages (which can eat/consum, or engulf/digest cellular debris), HepG2 cells (that are derived from the liver tissue which play a major role in lipid metabolism) and A431 cells (which have a high content of lipid droplets) were compared, to characterize lipid uptake pathways by cultured cells.

2.1. Cell culture

The cell is the basic structural and functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing, and is often called the building block of life. Organisms can be classified as unicellular (consisting of a single cell; including most bacteria) or multicellular (including plants and animals). Humans contain about 10^{14} cells, for every 10 of those, about one is actually human and the remainder from bacteria and other microorganism. The cell is size ranging from 135µm in the anterior horn in the spinal cord to 4µm in granule cells in the cerebellum, but a typical cell size is 10µm and a typical cell mass is 1ng. Cell culture is a term referred to the growth and maintenance of prokaryotic or eukaryotic cells under sterile and appropriate conditions (typically, 37 °C, 5% CO_2 for mammalian cells) out of their natural environment. Some cells can naturally survive in suspension cultures without being attached to a surface, such as cells that exist in the bloodstream. Adherent cells require a surface to continue to divide and fill the available area, such as a standard culture plastic dish. Sterile techniques are generally performed to avoid contamination with bacteria, yeasts, or other cells. To exclude contaminating microorganisms, antibiotics (e.g. penicillin and streptomycin) are added to the growth media, and the cell culture is carried out in a biosafety hood. Cell culture conditions can vary widely from one cell type to another. The growth medium is the most commonly varied factor among the culture conditions. It can vary in pH, glucose concentration, growth factors such as calf serum, and the presence of other nutrients. Cultured cells of different types serve as model systems of reduced complexity for living cells in their physiological surroundings. Although lipids of different classes have been applied to different types of cultured cells, a systematic study is missing that shows how different cells types take up and utilize lipids of different classes. In the present work, I provide a systematic comparison of four different cell

types towards their capacity to incorporate and metabolize different classes of lipids from the culture medium. This is part of a larger program to determine the kinetics by which exogenously applied lipids of different classes are taken up by cultured cells and processed to lipid droplets (LDs) constituents. LDs are intracellular storage organelles for nonpolar lipids (Goodman, 2008; Thiele, et al. 2008). They play a crucial role for lipid metabolism and homeostasis (Fujimoto, et al. 2008) and for diseases like obesity, metabolic syndrome, diabetes, and others (LeLay, et al. 2009). However, to make confident comparisons among studies using cells from different sources, the following cells were compared.

2.1.1. A macrophage-like cell line

In 1893 Metchnikoff was the first who used the term “macrophage”. Human macrophages are about 21µm in diameter. Macrophages are derived from monocytes that circulate in the blood. The most convenient source of macrophages are CD34 positive bone marrow progenitors that shed their progeny after proliferation and differentiation to promonocytes in the bloodstream. They then further differentiate into monocytes and extravasate into tissues (Ross, et al., 2002). Macrophages develop from the division of monocytes that migrate from the blood into many tissues throughout the body, including connective tissues, liver, lung, lymph nodes, spleen, bone marrow, skin, and others. The type of macrophage that results from monocyte differentiation depends on the type(s) of cytokines that these cells encounter. Cytokines are peptides and proteins produced by immune cells that can influence cell behavior and affect interactions between cells. They act as antimicrobial mediators and play critical roles in immune regulation and wound-healing.

Macrophages have a wide range of cellular functions; they engulf and destroy cellular debris, ingest foreign invaders of the body, damaged macromolecules, bacteria, and infected or dead body cells by phagocytosis. After infection, damaged tissue is repaired and the remaining macrophages and other leukocytes move out of the tissue and reenter the circulation. In atherosclerosis, macrophages migrate to inflamed areas of the vessels of the cardiovascular system, where they can endocytose substantial amounts of cholesterol from lipoproteins, and accumulate within the artery wall under some circumstances (Lucas, et al. 2001). As the imported cholesterol is converted into the esterform, they accumulate cholesteryl esters in lipid droplets. These lipid-filled macrophages are called foam cells because the lipid droplets have a foamy appearance. As macrophage foam cells accumulate in an artery wall, they initially form an early fatty streak, the first unique step in atherosclerosis. RAW 264.7 (Fig.

2.1B), a mouse leukaemic monocyte macrophage cell line, is the most commonly used monocyte-derived line. These cell lines show fundamental differences to the primary cells in that they grow continuously in culture due to permanent alterations in their genes that may have an effect on the signaling cascades that are activated by microbial ligands (Hartley, et al. 2008).

2.1.2. Human skin fibroblasts

The skin is the largest organ system in the body, acting as the protective barrier against the damaging effects of the environment. The skin consists of two basic layers, the epidermis, which is largely composed of keratinocytes, and the dermis. Fibroblasts have a branched cytoplasm surrounding an elliptical, speckled nucleus having one or two nuclei that often locally align in parallel cluster when crowded. Fibroblasts are large and flat with elongated cells possessing processes extending out from the ends of the cell body (Fig. 2.1A). Fibroblasts produce collagen proteins in order to maintain structural framework integrity for many tissues. They also appear to play an important role in the pathophysiology of fibrotic diseases as well as in cutaneous wound-healing tissues (Cevikbas, et al. 2011; Diegelmann, et al. 2004) by migrating to the site of damage, where they deposit new collagen and facilitate the healing process. Fibroblasts are morphologically heterogeneous with diverse appearances depending on their location and activity. Fibroblasts show distinct phenotypes in different anatomical locations, such as fibroblast-like cells that are found between the cartilaginous fibers in the synovial membrane of joints and are called synoviocytes. Fibroblasts produce one type of the extracellular protein fibronectin. This fibronectin is able to adhere fibroblasts to the extracellular matrix. Fibronectins are essential for the migration and differentiation of many cell types in embryogenesis. These proteins are also important for wound-healing because they promote blood clotting and facilitate the migration of macrophages and other immune cells into the affected area.

2.1.3. A human epidermoid carcinoma cell line (A431 cells)

A431 cells are a model cell line derived from a human epidermoid carcinoma from an 85 year old female patient (Giard, et al. 1973), which has been used for a variety of studies in cell biology. A431 cells (Fig. 2.1C) were found to have high levels of the epidermal growth factor receptor (EGFR) on its cell surface, nearly 3 million receptor sites per cell, and amplification of the EGF receptor gene (Merlino, et al. 1984). Therefore, they are used as a positive control

for EGFR expression, furthermore, they are used in studies of the cell cycle and cancer-associated cell signaling pathways. The growth of A431 cells has been shown to be inhibited by high concentrations of EGF (Kamata, et al. 1986), and paradoxically to stimulate A431 cell proliferation at low concentrations of EGF (Kawamoto, et al. 1983). This is in marked contrast to normal human fibroblasts, which have a much more reduced EGF receptor density.

2.1.4. Human liver hepatocellular carcinoma cells (HepG2 cells)

The HepG2 cell line was established by D.P. Aden, (Aden, et al. 1979) and is the most commonly used cell line for a variety of biochemical and cell biological studies of hepatocyte functions (Fig. 2.1D). This cell line is derived from a 15 year old male patient with primary liver cancer with differentiated hepatocellular carcinoma. In morphology, these cells are epithelial as monolayers and in small aggregates, have a model chromosome number of 55, and are not tumorigenic in nude mice. HepG2 cells exhibit numerous functions, including the synthesis and secretion a variety of major plasma proteins (Knowles, et al. 1980) such as albumin, transferrin and the acute phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, and plasminogen, and enzymes of carbohydrate metabolism (Verspohl, et al. 1984). HepG2 cells appear to be a useful model of the human hepatocyte and are widely used as an *in vitro* model of human hepatic lipid metabolism (Javitt, et al. 1990).

2.1.5. Niemann-Pick diseases, type A (NPA)

Niemann-Pick diseases, type A (NPA) is one of a group of metabolic diseases classified as lysosomal storage disorders (LSD's) in which lipids accumulate in certain tissues, caused by mutations in the sphingomyelin phosphodiesterase 1 gene (SMPD1) encoding for acid sphingomyelinase (Ferlinz, et al. 1991). NPA is a severe neurodegenerative disorder of infancy. It usually begins in the first few months of life; symptoms are an enlarged liver and spleen, enlarged lymph glands, swelling of the skin of the face, and brain and nervous system impairment. Acid sphingomyelinase (ASM) is the lysosomal enzyme affected by the gene defects. Its function is to degrade sphingomyelin (SM) into ceramide and phosphorylcholine, and is found in every cell of the body. The enzyme defect leads to sphingomyelin and cholesterol accumulation within the lysosomal compartment inside cells, causing cell death in early childhood. NPA occurs in all races and ethnicities, but higher rates are seen in the Ashkenazi (Eastern European) Jewish population. In this study, the uptake and subsequent

metabolism of both LDL-associated [^{14}C]cholesterol and LDL-associated [^{14}C]phosphatidylcholine into cultured skin fibroblasts from controls (healthy probands), and from a patient with Niemann-Pick diseases, type A was also examined.

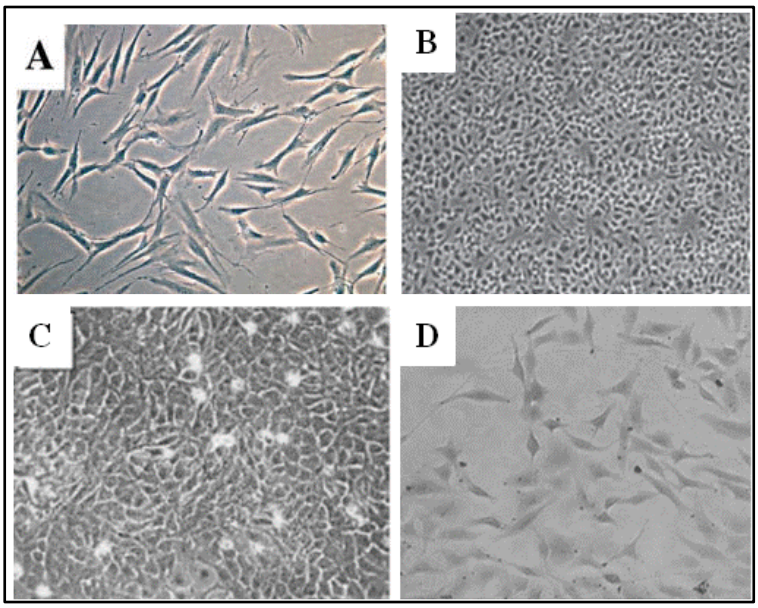

Figure 2.1: Morphological aspects of A: fibroblasts (Lysy, et al. 2007); B: RAW 264.7 macrophage (Cox, et al. 2009); C: A431 cells (Veldman, et al. 2005); and D: HepG2 cells (Reynaert, et al. 2004) that were used in the present study.

2.2. The biological membrane

Membranes in cells typically define enclosed spaces or compartments in which cells may maintain a chemical or biochemical environment that differs from the outside. Biological membranes are asymmetric structures. Both the lipids and the proteins of membranes exhibit lateral (when lipids or proteins of particular types cluster in the plane of the membrane) and transversal asymmetries. In eukaryotic cells, also the lipid composition of the membranes of different organelles is heterogeneous. For example, the plasma membrane is highly enriched in cholesterol and glycosphingolipids, which are nearly absent from the endoplasmic reticulum (ER) (Prinz, 2002; Munro, 2003). In addition, there is a transbilayer lipid compositional asymmetry within the same membrane (Pomorski, et al. 2001), and in polarized cells, apical and basolateral membrane have different lipid and protein composition. Biological membranes and their components serve a number of essential cellular functions: They act as a selective barrier within or around a cell, where many reactions and processes occur; function as a platform for signal transduction; allow cell recognition; provide anchoring sites for cytoskeletal filaments or components of the extracellular matrix; compartmentalize cells; regulate the fusion of the membrane with other membranes in the cell and provide a passage way across the membrane for certain molecules.

The major components of all biological membranes are lipids, proteins and small amount of carbohydrates (as part of glycolipids and glycoproteins) of less than 10% of the mass of most membranes in variable proportion. Membranes proteins mediate and regulate transport of metabolites, macromolecules, and ions in and out of cells or subcellular organells (Shkulipa, 2006). The lipids of cell membranes play a crucial role in the function and properties of cell membranes. The membrane lipids consist of many different lipid species, classified according to head-group and backbone structures. These include glycerophospholipids, sphingolipids and cholesterol (Pomorski, et al. 2001; Edidin, et al. 2003; Holthuis, 2001; Fahy, et al. 2005). The carbohydrate moieties attached to some proteins and lipids are particularly abundant on the extracellular surface of the plasma membrane where they form the glycocalyx. The function of this layer is to prevent uncontrolled membrane fusion and to participate in recognition phenomena. In endothelial tissues, the glycocalyx serves to shield the vascular wall from the shear stresses of blood flow, impede leakage of blood constituents across the endothelial lining, and prevent adhesion of leucocytes and platelets to the endothelium (Rehm, et al. 2004).

Many subcellular organelles in eukaryotes are surrounded by membranes (Voet, et al. 2011) such as nuclei, mitochondria, chloroplasts, endoplasmic reticulum, and Golgi apparatus. These organelles continually exchange biomolecules by a variety of membrane trafficking mechanisms (Sprong, et al. 2001; Mellman, 1996).

In 1972, S.J. Singer, and G.L. Nicolson proposed the fluid mosaic model for membrane structure (Fig. 2.2), a widely accepted model of biological membranes. They suggested that membranes are dynamic structures composed of proteins and phospholipids. In this model, the phospholipids form a fluid bilayer (Singer, et al. 1972) in which the nonpolar regions of the lipid molecules in each layer face the core of the bilayer and their polar head groups face outward, interacting with the aqueous phase on either side. The hydrophobic nature of lipid molecules allows membranes to form spontaneously, and to act as effective barriers to polar molecules. The fluidity of the hydrocarbon core of the bilayer increases with increasing content of unsaturated or branched alkyl chains or with decreasing alkyl chain length.

Membrane proteins can be embedded in the bilayer by hydrophobic interactions between the membrane lipids and hydrophobic domains of the proteins. Some proteins protrude from only one side of the membrane; others have domains exposed on both sides. The orientation of proteins in the bilayer is asymmetric, giving the membrane “sidedness”: the protein domains exposed on one side of the bilayer are different from those exposed on the other side, reflecting functional asymmetry. The individual lipid and protein units in a membrane form a

fluid mosaic because most of the interactions among its components are noncovalent, leaving individual lipid and protein molecules capable of rotational degrees of freedom and to move laterally in the plane of the membrane, but movement of either from one face of the bilayer to the other is restricted.

Singer and Nicolson defined two classes of membrane proteins: peripheral (or extrinsic proteins) and integral proteins (or intrinsic proteins). The first includes those proteins that do not penetrate the bilayer to any significant degree and are associated with the membrane by virtue of electrostatic interactions and hydrogen bonds between the polar groups on the membrane surface and the surface of the protein. Furthermore, peripheral proteins can easily be dissociated from the membrane by treatment with salt solutions or by changes in pH. Integral proteins, in contrast, possess hydrophobic surfaces that can readily penetrate the matrix of the phospholipid bilayer itself as well as surfaces that prefer contact with the aqueous medium. In eukaryotic cells, almost all integral membrane proteins are synthesized on the surface of the rough ER (Gilmore, et al. 2012). Integral membrane proteins are strongly associated with bilayer lipids that influence specific function of certain membrane proteins. Because of these interactions, integral proteins can only be removed from the membrane by agents capable of breaking up the hydrophobic interactions within the lipid bilayer itself such as detergents and organic solvents.

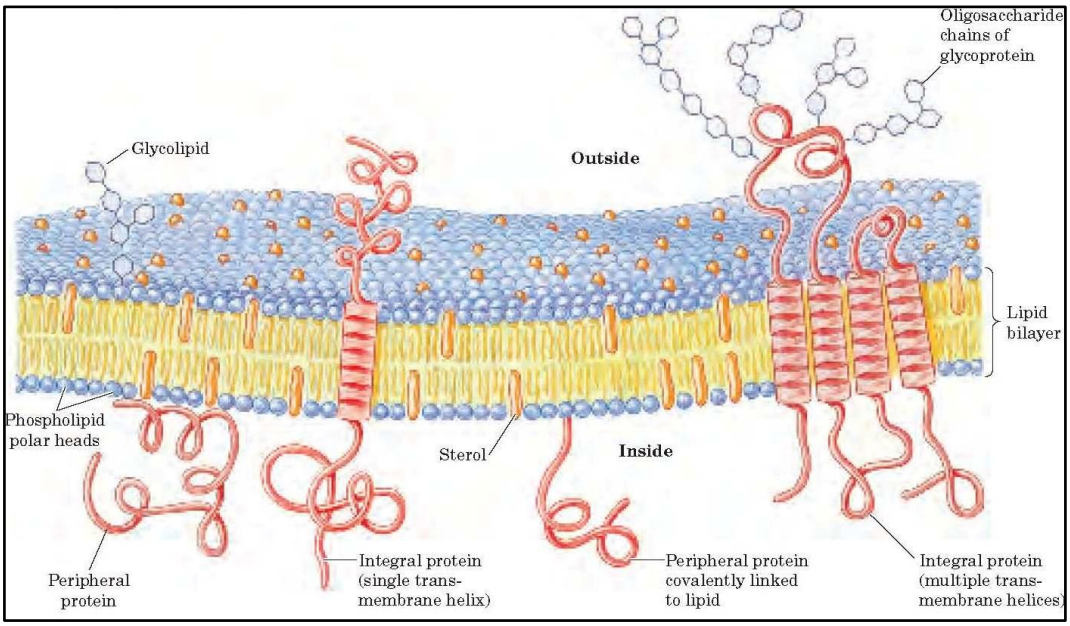

Figure 2.2: The fluid-mosaic model of membrane structure proposed by (Singer, S.J. and Nicolson, G.L. 1972). In this model, a lipid bilayer is composed of phospholipids, cholesterol, glycolipids, and proteins. Peripheral proteins are embedded in either the outer or inner leaflet of the lipid bilayer, while integral proteins are firmly embedded in the lipid layers. Many of the proteins and lipids have externally exposed oligosaccharide side chains (Nelson, et al. 2005).

2.2.1. Lipid bilayers

Amphipathic lipids spontaneously form a variety of supramolecular structures when added to an aqueous medium. They can aggregate into one of three forms: spherical micelles, liposomes, and sheetlike, two-molecule-thick bilayers (Fig. 2.3). All these structures form in ways that minimize the surface area in contact between the hydrophobic lipid chains and the aqueous milieu. For example, when small amounts of a fatty acid are mixed with an aqueous solution, a monolayer is formed at the air–water interface, with the polar head groups in contact with the water surface, shielding their hydrophobic tails from the water in contact with the air (Shkulipa, 2006). Micelles formed from an amphipathic lipid in water position the hydrophobic tails in the center of the lipid aggregate with the polar head groups facing outward. Micelles are the preferred form of aggregation in water for detergents and soaps. Phospholipids prefer to form bilayer structures in aqueous solution because their pairs of fatty acyl chains do not pack well in the interior of a micelle. Phospholipid bilayers form rapidly and spontaneously when phospholipids are added to water, and they are stable structures in aqueous solution. Extensive bilayers normally wrap around themselves and form closed vesicles. The nature and integrity of these vesicle structures are very much dependent on the lipid composition. Phospholipids can form either unilamellar vesicles (with a single lipid bilayer) known as liposomes, or multilamellar vesicles which are reminiscent of the layered structure of onions.

The lipid bilayer thickness is about 3nm, 30Å thick (Heimburg, 2009), and is defined by the length, degree of saturation, and packing of the fatty acid chains. The thickness of bilayers is not a static number since thickness can vary over the surface of a membrane if microdomains of lipids are formed with different alkyl chain lengths. For example, addition of cholesterol or increasing chain length causes membrane thickening, whereas increased chain unsaturation or the strength of head group repulsions causes the bilayer to thin (Cantor, 1999).

An important property of the lipid bilayer heterogeneity is that it contributes to membrane fluidity. The bilayer's fluidity allows lateral mobility within the lipid bilayer. It depends on the membrane phase and changes with the temperature. This fluidity is biologically important, influencing membrane transport. For example, the membranes of mammalian spermatozoa are composed of a complex mixture of lipids that provide the correct infrastructure and fluidity for the membrane mediated events that lead to fertilization (Ladha, 1998). Other roles for lipid diversity are the storage of precursors that can be metabolized to potent second messengers, e.g., diacylglycerol, ceramide, sphingosine, inositol trisphosphates

and eicosanoids. In addition, several phosphoinositides (phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol-4,5-biphosphate (PI45P2), phosphatidylinositol-3,4,5-triphosphate (PI345P3)) act as membrane recognition and attachment sites for protein complexes involved in protein traffic and membrane fusion events (Simonsen, et al. 2001; Barlow, et al. 2010).

2.2.2. Transmembrane lipid asymmetry

The fluidity of the lipid bilayer of biological membranes has been established by biophysical studies. The lipids can rotate freely about their axis perpendicular to the plane of the membrane and diffuse readily within the lateral plane. Movement of polar lipids from one leaflet of the bilayer to the other is severely constrained and is measured in half times of hours or days. This constraint results from the requirement of free energy to move a hydrated polar moiety from the aqueous interface through the hydrocarbon interior of the structure. As a consequence of this restricted motion, an asymmetric distribution of lipids can be created and maintained across biological membranes. In many naturally occurring bilayers, the external monolayer of the mammalian cell membrane is made up almost exclusively of the neutral zwitterionic phospholipids phosphatidylcholine (PC), and sphingomyelin (SM), together with some phosphatidylethanolamine (PE). Phosphorylcholine is the most common head group accounting for about half of the phospholipids in most mammalian cells. In contrast, the internal monolayer contains anionic phospholipids as a major component which account for about 30% of cell phospholipids (Verkleij, et al. 1973; Buckland, et al. 2000; Chaurio, et al. 2009), mainly phosphatidylserine, phosphatidylethanolamine, and the phosphatidylinositols are much more abundant in the inner (cytoplasmic) leaflet (Chaurio, et al. 2009; Quinn, 2002). This is illustrated in figure 2.4, which shows the percentage distribution of the major lipid classes between the cytoplasmic and outer leaflet of the human erythrocyte membrane.

Lipid asymmetry is maintained by translocases (Flippases) (Van Meer, et al. 2008). The aminophospholipid translocase is an ATPase II type enzyme that requires Mg^{2+} and specifically transports phosphatidylserine and phosphatidylethanolamine from the outer to the cytoplasmic leaflet of the membrane, while choline phosphatides are transported from the cytoplasmic to the outer leaflet.

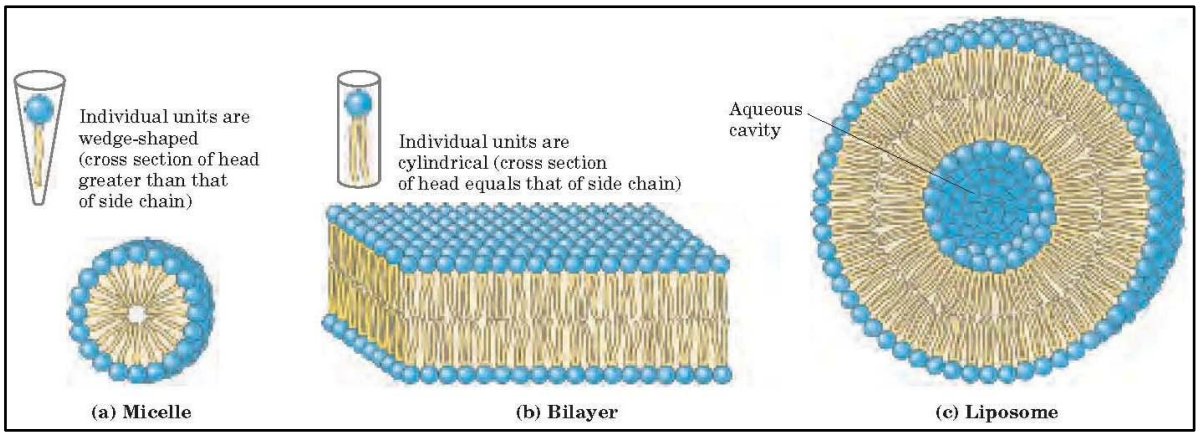

Figure 2.3: Variety of structures of amphipathic lipid that aggregates in water. (a) In micelles, the hydrophobic fatty acid chains are sequestered at the core of the sphere with only small amounts of water in the hydrophobic interior. (b) In an open bilayer, all fatty acids acyl side chains are protected from interaction with water except those at the edges of the sheet. (c) When a bilayer folds on itself, it forms a closed bilayer (liposome) enclosing an aqueous cavity (Nelson, et al. 2005).

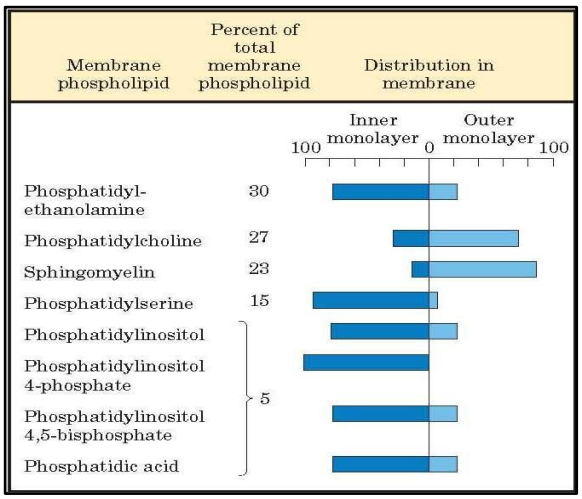

Figure 2.4: In erythrocyte plasma membrane, percentage asymmetric distribution of the major phospholipids between the cytoplasmic and outer monolayers leaflets. This distribution is determined by treating the intact cell with phospholipase C, which removes the head groups of lipids in the outer monolayer, but cannot reach lipids in the inner monolayer (leaflet). In the outer monolayer, the proportion of each head group released provides an estimate of the fraction of each lipid (Nelson, et al. 2005).

2.2.3. Membrane phospholipids

Lipids are a class of biological molecules defined by low solubility in water and high solubility in nonpolar solvents (Fahy, et al. 2005). The lipids found in biological systems are either hydrophobic or amphipathic. Phospholipids are the primary building blocks of most biological membranes. In eukaryotic cells, phospholipids are synthesized by enzymes located on the surface of the endoplasmic reticulum (ER). The membranes of mammalian cells contain more than 1,000 different phospholipid species (Vance, 2008). Phospholipids are abundant in all biological membranes and are derived from either glycerol or sphingosine, a long-chain unsaturated amino alcohol (Berg, et al. 2003). Phosphoglycerides (Fig. 2.6) consist of a glycerol backbone to which two residues (Fahy, et al. 2005), mostly fatty acids and a phosphorylated alcohol are attached in ester linkage. The fatty acid constituents are

usually even-numbered, most commonly of 16 or 18 carbons. Naturally occurring phospholipids contain a saturated fatty acid (such as stearic acid or palmitic acid) in position sn-1, whereas an unsaturated one (such as oleic acid, linoleic acid or arachidonic acid) in sn-2 position. The length and the degree of unsaturation of fatty acids in the membrane have an important effect on the fluidity (Chaurio, et al. 2009).

The major phosphoglycerides arise from phosphatidate through the formation of an ester bond between the phosphate group of phosphatidate and the hydroxyl group of one of several alcohols. The simplest phosphoglyceride is phosphatidic acid, which is sn-1,2-diacylglycerol 3-phosphate, a key intermediate in the formation of all other phosphoglycerides. In other phosphoglycerides, the 3-phosphate is esterified to an alcohol such as ethanolamine, choline, serine, glycerol, or inositol (Fig. 2.5). The second major class of phospholipids is sphingomyelin, which contains a sphingosine backbone rather than glycerol. A fatty acid is attached by an amide linkage to the amino group of sphingosine, forming ceramide.

The content of phospholipids also varies among organelles. For example, cardiolipin is a major constituent of the inner membrane of mitochondria, but is absent from other organelles (Van Meer, et al. 2008), whereas bis(monoacylglycero)phosphate is largely confined to late endosomes and lysosomes (Kolter, et al. 2010; Matsuo, et al. 2004; Kobayashi, et al. 2002). In mammalian cells, glycerophospholipids account for approximately 70% of the total membrane lipid content and thus play key roles in the structure and function of mammalian membranes; the other 30% consists of cholesterol, sphingomyelin, and glycosphingolipids (Leventis, et al. 2010). Among the phospholipids derived from glycerol, phosphatidylcholine (PC) is the most prevalent and accounts for 40-50% of the total phospholipids (Vance, 2008; Matsuo, et al. 2004). Of this amount, 76% is found in the outer monolayer, and 24% is found in the inner monolayer. Phosphatidylethanolamine (PE) is the next most abundant, which ranges from 20-45% of the total phospholipids, depending on the tissue (Vance, 2008; Murphy, et al. 2000), and is the major phospholipid in bacteria. Phosphatidylinositol (PI) (2-8% of the total PL), phosphatidylserine (PS) (2-10% of the total PL), phosphatidic acid (PA) (1% of the total PL), phosphatidylglycerol (PG) (<1% of the total PL), and bis(monoacylglycero)phosphate (BMP) (<1% of the total PL) (Kolter, et al. 2005) are present, but in lesser amounts (Leventis et al. 2010).

Most cells continually degrade and replace their membrane lipids. For each hydrolyzable bond in a glycerophospholipid, there is a specific hydrolytic enzyme in the lysosome and other subcellular compartments (Fig. 2.7). Phospholipases of the A type remove one of the two fatty acids at the sn-1 position, producing a lysophospholipid. Phospholipases A2

hydrolyze the ester bonds of intact glycerophospholipids at sn-2 of glycerol. Phospholipases C and D each split one of the phosphodiester bonds in the head group.

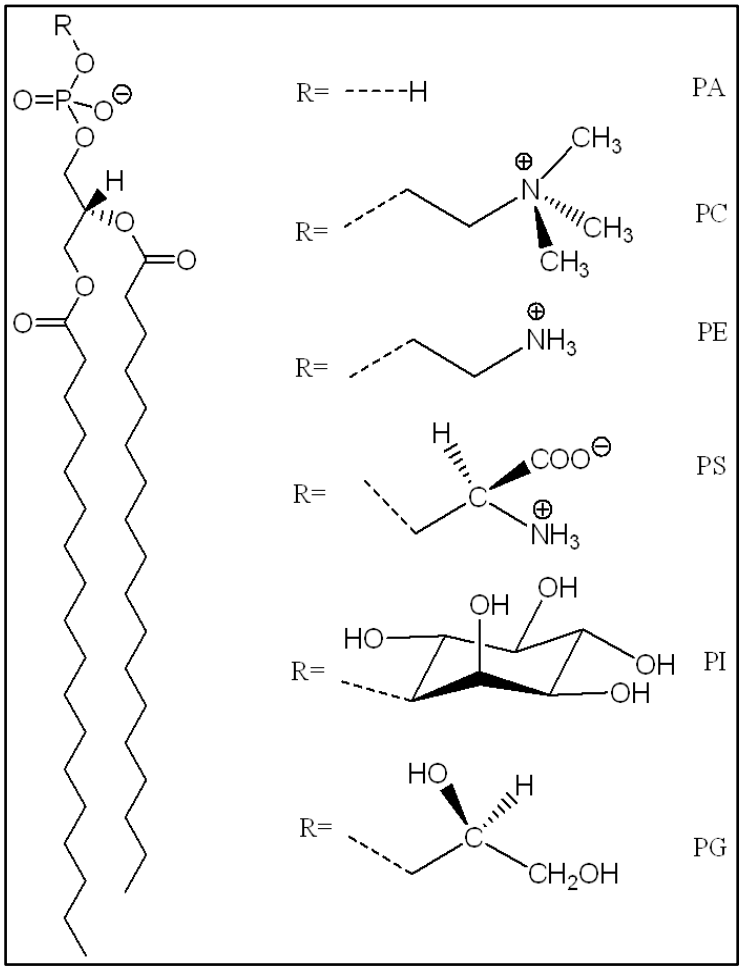

Figure 2.5: Structure of glycerol phosphate-based lipids. The lipid structures are shown with head group from top to bottom, phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG).

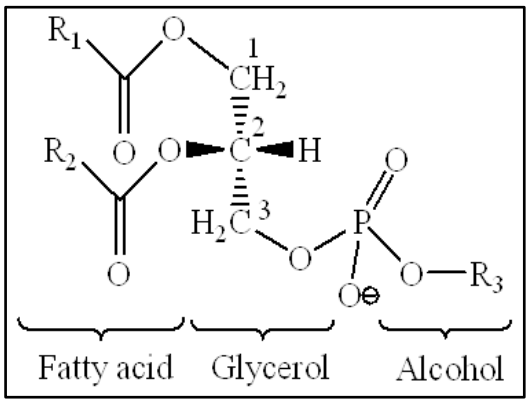

Figure 2.6: A phosphoglyceride showing the fatty acids (R_1 and R_2), glycerol, and phosphorylated alcohol components. In phosphatidic acid, R_3 is hydrogen

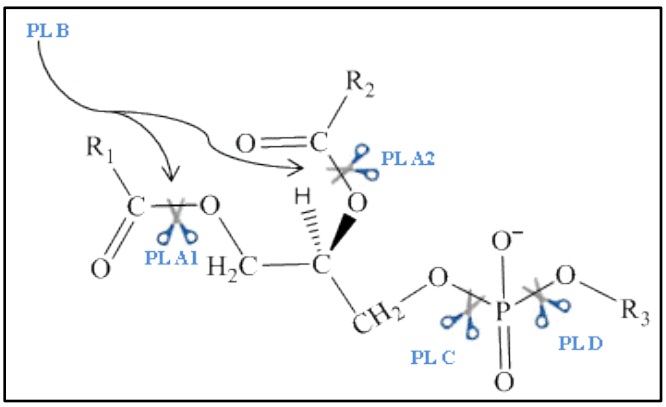

Figure 2.7: The specificities of phospholipases. The ester bonds of intact glycerophospholipids at sn-1 and sn-2 of glycerol are hydrolyzed by phospholipases A1, and A2, respectively. PLB cleaves both the sn-1 and sn-2 ester bonds. Phospholipases C and D each split one of the phosphodiester bonds in the head group. R_1 and R_2, $(CH_2)nCH_3$; R_3, various head groups. (Richmond, et al. 2011).

2.3. Endocytosis

2.3.1. Entry pathways into cells

In eukaryotic cells, endocytosis is a process by which cells take up large molecules, such as proteins, from the environment to the inside of the cell by engulfing them. In this process, plasma membrane lipids, integral proteins, and extracellular fluid become fully internalized into the cell (Doherty, et al. 2009). It is used by all cells of the body since many substances in the environment are large polar molecules that cannot pass through the hydrophobic plasma membrane. The endocytic pathway consists of distinct compartments that receive internalized molecules from the plasma membrane and recycle them back to the surface in early endosomes, or sort them to degradation in late endosomes and lysosomes.

Many endocytic entry pathways into cells have been identified, which differ in the cargoes they take up and in the protein machinery that mediates the endocytic process (Mayor, et al. 2007). Extracellular materials can enter the cell depending on the nature and size of the molecules either by endocytosis, phagocytosis, or macropinocytosis (Aderem, et al. 1999). The basic steps of the endocytic pathway are summarized in figure 2.8.

A few specialized cell types (e.g., macrophages and granulocytes) can ingest whole bacteria and other large particles whose size exceeds about 0.5μm such as viruses, cells debris, micro-organisms, and even apoptotic cells by phagocytosis. Phagocytosis is a nonselective, actin-mediated process and is usually independent of clathrin in which extensions of the plasma membrane envelop the ingested material, forming large vesicles called phagosomes. Once inside the phagocyte, the phagosome contains the pathogenic microorganism such as *mycobacterium avium* or *mycobacterium tuberculosis*. It appears to fuse well with early and poorly with late endocytic organelles, since the pH of phagosomes was precisely that of the early endosomes (≈ 6.3) (Kuehnel, et al. 2001). Macrophages are extremely active in this regard and may ingest 25% of their volume/hour. Thus, phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells, and it participates in tissue remodeling and inflammation.

Macropinocytosis is another form of endocytosis, a mainly actin-dependent endocytic process that usually starts from highly ruffled regions of the plasma membrane and is used to internalize nonspecifically large amounts of fluid, growth factors and small droplets of extracellular fluid to form an external macropinocytic structure that is then enclosed and internalized, forming discrete vacuoles, the macropinosomes, which are accumulated within

the cell (Lim, et al. 2011). Vesicles mediating this form are filled with a large volume of extracellular fluid and molecules. The formation of these vesicles is an extremely active process. It is highly active in macrophages and dendritic cells; for example, fibroblasts internalize their plasma membrane at about one-third of the rate of macrophages. The vesicle then travels into the cytosol and fuses with other vesicles such as endosomes and lysosomes (Falcone, et al. 2006). Internalized macropinosomes share many features with phagosomes and both are distinguished from other forms of endocytic vesicles by their large size, morphological heterogeneity, and the lack of coat structures (Jones, et al. 2007).

In clathrin-mediated endocytosis, clathrin subunits form a three limbed structure called a triskelion, with each limb being made up of one light and one heavy chain of clathrin. The clathrin is polymerized into a basket by assembly particles, composed of four adapter proteins. These interact with certain amino acid sequences in the cytoplasmic domain of membrane-bound receptors that become endocytosed, ensuring selectivity of uptake. The clathrin lattice grows as more receptors are occupied by target proteins, until a complete membrane-bound endocytic vesicle buds off the plasma membrane mediated by the protein dynamin and enters the cytoplasm. Clathrin coated pits make up about 2% of the surface of cells such as hepatocytes and fibroblasts. The clathrin is quickly removed by uncoating enzymes, and the individual vesicles fuse with each other to form early endosomes. Coated pits can concentrate large extracellular molecules that have different receptors responsible for the receptor-mediated endocytosis of ligands, such as low-density lipoprotein (LDL); the iron-carring protein transferrin; many protein hormones (e.g., insulin); and certain glycoproteins. Coated pits also involved in desensitizing of G-proteins coupled receptors (Kobilka and Lefkowitz, 2012).

Caveolae are dynamin-dependent and non-clathrin-coated plasma membrane buds. Caveolae are 50-80nm flask shape pits in the plasma membrane that resemble the shape of a cave (hence the name caveolae). They exist on the surface of many cell types, especially in endothelial cells and adipocytes. They consist of the cholesterol-binding protein caveolin, eventually in a putative domain enriched in cholesterol and sphingolipids (Simons, et al. 1997), signaling proteins and clustered glycosyl phosphatidylinositol-anchored proteins (GPI-Aps) (Aboulaich, et al. 2004; Lemaitre, et al. 2005; Sprenger, et al. 2004) and have several functions in signal transduction (Anderson, R.G. 1998). They also play a role in endocytosis, oncogenesis, and the uptake of pathogenic bacteria and certain viruses (Frank, et al. 2004; Li, et al. 2005; Pelkmans, 2005). Caveolar cargoes are diverse, ranging from lipids, proteins and lipid-anchored proteins to pathogens.

ATPases in the endosomal membranes reduce the pH in the endosomal lumen, facilitating dissociation of receptors from their target ligands. The imported ligands and receptors dissociate in the late endosome, the ligand is released, and the receptors recycle back to the cell surface. Ligands are then transported from late endosomes and are delivered to the lysosomes for degradation.

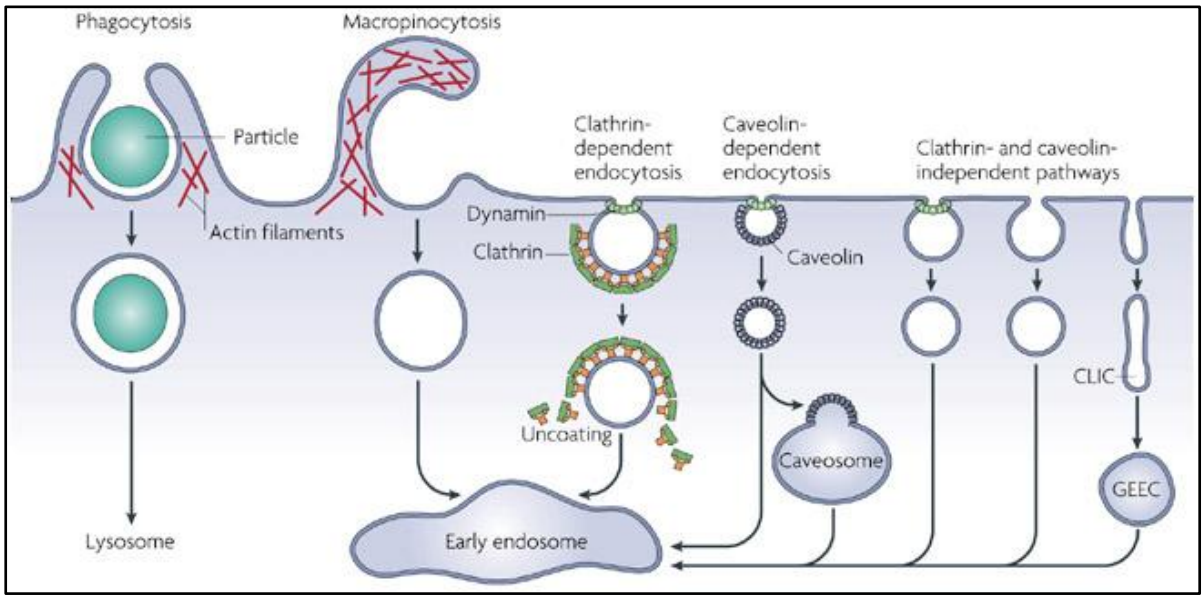

Figure 2.8: Endocytic entry pathways into cells. Phagocytosis can take up large particles, whereas fluid uptake occurs by macropinocytosis. The size of the vesicles formed by phagocytosis and macropinocytosis is much larger, compared with the other endocytic pathways. Some vesicles derived from the plasma membrane such as clathrin- or caveolin-coated vesicles and tubular intermediates internalize cargoes (known as clathrin-and dynamin-independent carriers (CLICs)) and deliver it to the early endosome. Some cargo may traffic first to intermediate compartments, such as the caveosome or glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC), before it moves to the early endosome (Mayor, et al. 2007).

2.3.2. Lysosomes

Lysosomes are acidic organelles enriched with catabolic enzymes for the degradation of various products of cellular turnover (Pastores, 2006) that have been taken up from the outside or have become obsolete for the cell. Lysosomes vary in size (0.1–1.2μm; Kuehnel, 2003) and shape. Lysosomes are found exclusively in animal cells, while in yeast and plants the same roles are performed by lytic vacuoles (Samaj, et al. 2005). The process by which an aged organelle is degraded in a lysosome is called autophagy. Lysosomes contain a group of enzymes that degrade polymers into their monomeric subunits. For example, nucleases degrade RNA and DNA into their mononucleotide building blocks; proteases degrade a variety of proteins and peptides; phosphatases remove phosphate groups from

mononucleotides, phospholipids, and other compounds; still other enzymes degrade complex polysaccharides and glycolipids into smaller units. All the lysosomal enzymes work most efficiently at acidic pH values (pH 4.8) and collectively are termed acid hydrolases, compared to the slightly alkaline cytosol (pH 7.0 - 7.3). The lysosome maintains this pH by vacuolar proton pumps. The acid pH helps to denature proteins, making them accessible to the action of the lysosomal hydrolases, which themselves are resistant to acid denaturation. Lysosomal enzymes are poorly active at the neutral pH of cells and most extracellular fluids.

Mutations within the genes that encode distinct acid hydrolases lead to the progressive accumulation of incompletely metabolized substrates within various tissues, and ultimately a disruption of organ function (Beck, 2010; Pastores, 2006). Characteristic disease manifestations may include distinctive facial features, organomegaly, skeletal problems and central nervous system (CNS) dysfunction. As a group, these disorders are commonly referred to as the lysosomal storage diseases (LSDs); inborn errors of metabolism that have traditionally been classified according to the biochemical nature of the incompletely degraded tissue deposits (Futerman, et al. 2004). Furthermore, phospholipidosis is another lysosomal storage disorder that is characterized by the excess accumulation of phospholipids in tissues.

A large number of approved catonic amphiphilic drugs (CADs) can induce phospholipidosis (drug-induced phospholipidoses) that may interfere with cellular functions, such as increased autophagy and loss of nuclei leading to chronic disease or even death. CADs might also bind to phospholipids, creating a complex which is trapped in the form of lysosomal lamellar bodies (Nioi, et al. 2008) that are resistant to degradation by phospholipases (Reasor, et al. 2006). The identification of the potential to induce phospholipidoses at early stages of drug discovery can be advantageous for selecting improved drug development candidates (Kasahara, et al. 2006; Nonoyama, et al. 2008; Nioi, et al. 2008), since drug-induced phospholipidosis is one of the major reasons for the retraction of drug candidates (Reasor, et al. 2006). In cultured cells treated with CADs, phospholipids can accumulate intracellularly with the formation of lysosomal multi-lamellar bodies within only a few hours of exposure (Nonoyama, et al. 2008). In addition, the chemical modification of lipoproteins, enzymatically degraded LDL (eLDL) and oxidized LDL (oxLDL), induce lipid storage in different compartments. While eLDL preferentially induces rapid formation of large cytoplasmic lipid droplets, oxLDL provokes endolysosomal phospholipidosis and impaired cholesterolester hydrolysis (Orso, et al. 2011).

2.4. Lipoproteins

Lipoproteins are soluble complexes of proteins (apolipoproteins) and lipids that transport hydrophobic, water-insoluble lipids and other fat soluble nutrients like vitamins A, K, D and E to different organs (liver) and tissues (adipose and other cells) in the circulation of all vertebrates. In vertebrates, dietary triacylglycerols are solubilized by amphipathic bile salts such as taurocholic acid, which are synthesized from cholesterol in the liver. They convert dietary fats into mixed micelles of bile salts and triacylglycerols. Micelle formation enormously increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine, and lipase action converts triacylglycerols to monoacylglycerols, free fatty acids, and glycerol. These products of lipase action diffuse into the epithelial cells lining the intestinal surface, the intestinal mucosa. There, they are reconverted to triacylglycerols and packaged with dietary cholesterol and specific proteins into lipoprotein aggregates called chylomicrons and enter the blood stream, which carries them to muscle and adipose tissue.
Chylomicrons are the largest of the lipoproteins and the least dense, containing a high proportion of triacylglycerols and other lipid soluble nutrients, e.g, vitamins. Chylomicrons are synthesized in the ER of epithelial cells that line the small intestine, then move through the lymphatic system and enter the bloodstream via the left subclavian vein. The apolipoproteins of chylomicrons include apolipoprotein B-48 (apoB-48) unique to this class of lipoproteins, apoE, and apoC-II. ApoB-48 is the major protein which has a molecular weight of 240kDa and forms an amphipathic shell around the spherical fat globule. ApoB-48 is formed from the first 48% of apoB-100 and arises from the posttranscriptional editing of apoB-100 mRNA in the intestine. Chylomicrons carry dietary fats to the target tissues where they are consumed (muscle) or stored (adipose tissue) after cleavage by the extracellular enzyme lipoprotein lipase. The remnants of chylomicrons, which are depleted of most of their triacylglycerols, but still contain cholesterol, apoE, and apoB-48, then move through the blood stream to the liver. Receptors in the liver bind to the apoE in the chylomicron remnants and mediate their uptake by endocytosis. In the liver, the remnants release their cholesterol and are degraded in lysosomes. Triacylglycerols that enter the liver by this route are cleaved into glycerol and fatty acids, which may be oxidized to provide energy, or to provide precursors for the synthesis of ketone bodies. When the diet contains excess carbohydrate and more fatty acids than are needed as fuel, they are converted to triacylglycerols in the liver and packaged with specific apolipoproteins into very-low-density lipoprotein (VLDL, or pre-β-lipoproteins). In addition to triacylglycerols, VLDLs contain some cholesterol and cholesteryl

esters, as well as apoB-100, apoC-I, apoC-II, apoC-III, and apo-E. These lipoproteins are transported in the blood from the liver to muscle and adipose tissue, where the free fatty acids are released from the VLDL triacylglycerols through lipoprotein lipase activated by apoC-II. Adipocytes take up these fatty acids, reconvert them to triacylglycerols, and store them in intracellular lipid droplets. The size and lipid composition of chylomicrons and VLDLs vary according to the nutritional status of the animal. Most VLDL remnants are removed from the circulation by hepatocytes. The uptake, like that for chylomicrons, is receptor-mediated and depends on the presence of apoE in the VLDL remnants. The loss of triacylglycerol converts some VLDL to VLDL remnants (also called intermediate density lipoprotein, IDL), by the action of lipases associated with capillary surfaces and their consequent enrichment in cholesterol esters. These intermediate-density lipoproteins may be taken up by the liver and further processed or converted into low-density lipoproteins (LDLs) by hydrolysis of more triacylglycerol, representing a final stage in the catabolism of VLDL. LDLs are very rich in cholesterol and cholesteryl esters and contain apoB-100 as their major apolipoprotein. LDLs carry cholesterol to extrahepatic tissues that have specific plasma membrane receptors that recognize apoB-100. These receptors mediate the uptake of cholesterol and cholesteryl esters. The fourth major lipoprotein type, high-density lipoprotein (HDL), originates in the liver and small intestine as small, protein-rich particles. HDLs contain apoA-I, apoC-I, apoC-II, and other apolipoproteins, as well as the enzyme lecithin-cholesterol acyl transferase (LCAT). LCAT is a glycoprotein of 67kDa on the surface of nascent HDL particles, converts the cholesterol and phosphatidylcholine of chylomicrons and VLDL remnants to cholesteryl esters, which begin to form a core, transforming the disk-shaped nascent HDL to a mature, spherical HDL particle. This cholesterol-rich lipoprotein then returns to the liver for metabolism and excretion. Some of this cholesterol is converted to bile salts in a process referred to as reverse cholesterol transport. LCAT uses preferentially PC species with 18:2 or 18:1 fatty acids in the sn-2 position (by the action of PLA2), thus enriching cholesteryl esters in these fatty acids. In contrast, PC containing 18:0 or 20:4 fatty acids is a poor substrate for LCAT, explaining the decreased contents of these fatty acids in the cholesteryl esters.

ApoA, apoC and apoE are referred to as exchangeable apolipoproteins (Saito, et al. 2004) and they are responsible for regulating the traffic of lipids into and out of a cell by acting as cofactors for plasma enzymes and ligands for cell-surface receptors. The classification into chylomicrons (CM), very low-density (VLDL), low-density (LDL), and high-density (HDL) lipoproteins is based on their relative contents of protein and lipid that determine the densities of these lipoprotein classes. Chylomicrons have only 1-2% protein while HDL has about 50%

protein by weight. The diameters of lipoproteins are inversely correlated with their densities and range from about 1200nm for CM down to 8nm for the smallest HDL.

2.4.1. Low density lipoprotein (LDL)

Low density lipoprotein (LDL), the most abundant cholesterol-carrying lipoprotein in human plasma, is one of the five major groups of lipoproteins which are synthesized in the intestine and the liver. The particles are usually spherical in shape and are classified according to their buoyant density. Subclasses of LDL in the density range from 1.027 - 1.060 g/mL and size range from 270 - 210 Å (27 – 21nm in diameter) have been obtained and shown to have different metabolic properties (Shen, et al. 1981). LDL contains an amphipathic monolayer shell surrounding a hydrophobic lipid core (Fig. 2.9), which is typically composed of about 170 TAG molecules, and 1600 CE molecules per LDL esterified mainly with linoleate. The surface monolayer comprises about 700 phospholipids, the majority of which is PC, about 450 molecules per LDL, 600 molecules of unesterified cholesterol, and also a single copy of apolipoprotein B-100 (apoB-100). ApoB-I00 is an amphipathic protein, which has 4536 amino acids residues and a mass of 514kDa (Hevonoja, et al. 2000). Human apoB-100 contains 25 cysteine residues of which 16 exist as intramolecular disulfide bonds: 14 of these are clustered in the N-terminal region, disulfide bonds in this region are critical for the correct folding and secretion of apoB. The type of fatty acid supplied to hepatocytes influences the secretion of apoB-containing lipoproteins. For example, when oleate is added to HepG2 cells, the synthesis of TAG and phospholipids is stimulated and the amounts of apoB and TAG secreted are increased (Dixon, et al. 1991). Thus, an increased lipid supply enables a larger proportion of newly synthesized apoB to be translocated across the ER membrane and enter the secretory pathway. In contrast, compared to oleic acid, the (n-3) fatty acids eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6), found in fish oils, decrease plasma TAG levels in humans and decrease the secretion of apoB-containing lipoproteins from rat hepatocytes and hepatoma cells.

Once in the circulation, LDL may either take up free cholesterol from cells or deliver free or esterified cholesterol to cells. The cholesterol bound to LDL is referred to as bad cholesterol because it is thought to have deleterious health impacts (Brunzell, et al. 2008). For example, increasing levels of LDL-cholesteryl esters (LDL-CE) were associated with elevated risk of atherosclerosis, leading to cardiovascular diseases, including heart attack and stroke (Scott, 2004; Rosenson, 2004).

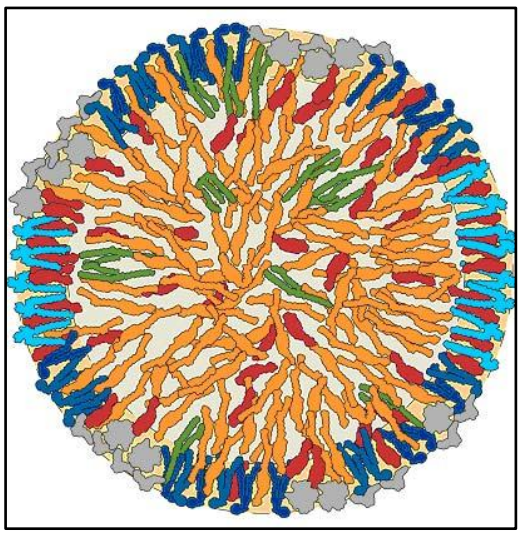

Figure 2.9: A schematic molecular model of an LDL particle. The colour coding for the molecules is: dark blue – phosphatidylcholine, light blue – sphingomyelin, dark yellow – cholesterol ester, red – cholesterol, green – triglyceride, and grey – apolipoprotein B-100. The molecular shapes and scales are derived from molecular dynamics simulations (Hevonoja, et al. 2000). The showed particle has a diameter of 20nm, including a surface monolayer of 2nm (yellowish background).

2.4.2. Lipoprotein uptake and trafficking

Lipoprotein receptors play an important role in lipoprotein metabolism and in cellular cholesterol homeostasis. The affinity and specificity of these receptors for the different lipoproteins facilitate the uptake and metabolism of lipoproteins in various tissues and cells. Each receptor can handle only one particle of LDL at a time. Brown and Goldstein in the 1970s identified the details of the uptake and metabolism of LDL following receptor-mediated endocytosis. When radiolabeled LDL was investigated to compare its uptake from the circulation of experimental animals into various tissues, high levels of hepatic LDL receptors were observed. Steinberg and co-workers and Dietschy and co-workers (Pittman, et al. 1982; Spady, et al. 1983) demonstrated that about 70% of the total-body uptake of radiolabeled LDL took place in the liver by LDL receptor-dependent pathways.

Most mammalian cells produce cell-surface receptors that specifically bind to the apoB-100 protein embedded in the phospholipid outer layer of LDL particles. In 1985, Brown and Goldstein were awarded with the Nobel Prize for their identification of the low density lipoprotein receptor (LDLR). LDLR complexes are present in clathrin-coated pits (or buds) on the cell surface, which when bound to LDL via adaptin, are pinched off to form clathrin-coated vesicles inside the cell. This allows LDL to be bound and internalized in a endocytosis process and prevent the LDL just diffusing around the membrane surface. Once the vesicle coat is shed, the uncoated endocytic vesicle (early endosome) fuses with an acidic late endosome. The acidic pH in this compartment causes a conformational change in the LDL receptor that leads to release of the bound LDL particle. The late endosome eventually fuses with the lysosome, and then the proteins and lipids of the free LDL particle are broken down to their constituent parts by lysosomal hydrolases that could easily digest all of the

components of LDL releasing cholesterol and fatty acids into the cytosol. Cholesterol that enters cells by this path may be incorporated into membranes or re-esterified by Sterol O-acyltransferase (SOAT) for storage within cytosolic lipid droplets. The apoB-100 of LDL is also degraded to amino acids that are released to the cytosol. The LDL receptor, which dissociates from its ligands in the late endosome, is either destroyed or can be recycled via an endocytic cycle back to the cell surface. The neutral pH of the exterior medium enables the receptor to undergo a conformational change so that it can function again in LDL uptake (Brown, et al. 1986; Rudenko, et al. 2002). ApoB-100 is also present in VLDL, but its receptor-binding domain is not available for binding to the LDL receptor. Conversion of VLDL to LDL exposes the receptor-binding domain of apoB-100.

Furthermore, various ligands including not only native lipoproteins (LDL, HDL, VLDL, and chylomicrons) but also modified lipoproteins (acetylated LDL, oxidized LDL, and oxidized HDL), anionic phospholipids, and maleyated BSA (Haberland, et al. 1985) can bind to specific amino acid residues of a cell surface transmembrane protein, the scavenger receptor glass B type I (SR-BI) (Trigatti, et al. 2003; McNutt, et al. 2007). Moreover, Ueda and colleagues have shown a reduction in plasma apoB in mice overexpressing SR-BI (Ueda, et al. 1999). In addition, CD36 is an integral membrane protein found on the surface in a variety of cells. In macrophages, CD36 binds oxidized LDL, acetylated LDL, native lipoproteins, oxidized phospholipids (Wang, et al. 2007) and long chain FA (Baillie, et al. 1996).

2.4.3. Regulation of LDL receptors

When cellular free cholesterol increases, the production of LDL receptors is reduced (Brown, et al. 1975). Together with the reduction in 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), this regulatory response decreases cholesterol input from plasma as well as from endogenous synthesis. The mechanism that mediated regulation of LDL receptors for this dual regulation was clarified by the discovery of sterol regulatory element-binding proteins (SREBPs) (Brown, et al. 1999). SREBPs are synthesized as membrane-bound proteins attached to the endoplasmic reticulum. In the presence of cholesterol, SREBP is bound to the protein SCAP (SREBP-cleavage-activating protein). In cholesterol-depleted cells, the complex migrates to the Golgi apparatus, where SREBP is cleaved by two proteases, S1P and S2P (site-1 and -2 protease). These two enzymes are activated by SCAP, to release a soluble fragment that enters the nucleus and acts as a transcription factor. It binds to the sterol response element (SRE) and it stimulates the transcription of many genes.

Among these are HMG CoA reductase and all the other enzymes of cholesterol biosynthesis as well as the LDL receptor (Horton, et al. 2002). LDLR mRNA is translated by ribosomes on the endoplasmic reticulum and the protein is modified in the Golgi apparatus before traveling in vesicles to the cell surface. The newly produced LDL receptors remove LDL from the blood, and deliver it to the interior of the cell where the LDL is digested and its released cholesterol becomes available for metabolic purposes. When LDL-derived cholesterol enters cells, it blocks the transport of SREBPs to the Golgi complex. By inhibiting the SREBP pathway, LDL also suppresses transcription of the LDL receptor gene (Brown, et al. 1999) which allows cells to adjust the number of LDL receptors to provide sufficient cholesterol for metabolic needs without producing cholesterol over accumulation (Brown, et al. 1975). The net effect is that the amount of cholesterol in the liver is maintained at a normal level while at the same time the level of LDL-cholesterol in blood is kept low (Brown, et al. 2004).

2.4.4. Bovine serum albumin (BSA)

In mammals, the blood plasma protein serum albumin is the most abundant plasma protein. In addition to its lipid transport properties, it plays a role in the regulation of lipid metabolism and it maintains the "osmotic pressure" needed for proper distribution of body fluids between intravascular compartments and body tissues, which causes fluid to remain within the blood stream instead of leaking out into body tissues. Its binding function in blood has been associated mostly with the transport of a wide varity of endogenous and exogenous compounds including fatty acids (Simard, et al. 2005), metal ions, amino acids, steroids and drugs (Carter, et al. 1989).

Bovine serum albumin (BSA) is a large globular protein (66kDa) with a single chain of 583 amino acid residues, and contains 17 pairs of disulfide bonds (Peters, 1985). BSA is a lipid-free soluble protein of the circulatory system and has many physiological functions (Carter, et al. 1994). It contains a limited number of saturable binding sites for lipids (Bhattacharya, et al. 2000) that are able to bind anionic and cationic ligands via both hydrophilic and hydrophobic interactions (Charbonneau, et al. 2010). In contrast to the many other proteins, bovine serum albumin has high affinity binding sites for anions with hydrophobic side chains (Choi, et al. 2002). However, the fatty acid binding affinity of albumin increases with increasing chain length, because of an increase in hydrophobic interactions (Choi, et al. 2002). BSA at pH 7.3 and molar ratio ≤ 1.5 has three equivalent binding sites for fatty acids

at temperatures from 0 ^{0}C to 38 ^{0}C (Bojesen, et al. 1994). It has long been known to serve as an extremely versatile transporter protein for a variety of compounds (Shcharbin, et al. 2007; Shcharbin, et al. 2005). BSA is considered as a 'soft' protein (Peters, 1985) because it does not have a high degree of structural stability. Therefore, conformational changes occur upon adsorption to a surface (Norde, et al. 1992; Kondo, et al. 1991; Giacomelli, et al. 2001).

There are marked similarities between BSA and human serum albumin (HSA) in their conformation due to 76% of amino acid sequence homology. BSA contains three homologous α-helical domains I, II and III, each domain contains two subdomains (A, and B) that share common structural motifs. HSA has only one tryptophan residue Trp-214, while BSA contains two tryptophans (Fig. 2.10), Trp-134 in the first domain located on the surface of the molecule and Trp-212 in the second domain located within a hydrophobic binding pocket of the protein (Tayeh, et al. 2009). Trp-212 in BSA and Trp-214 in HSA are located in a similar hydrophobic microenvironment in the sub-domain IIA (Peters, 1985).

The choosing of BSA in our experiments is based on its structural homology with HAS and due to the wide information available on BSA properties in solution (Doherty, et al. 1974; Bendedouch, et al. 1983). In addition, it is produced at industrial scale, therefore, it has been one of the most extensively studied member of this group of proteins (Charbonneau, et al. 2010).

Stremmel et al. 1983 showed that in rat liver, plasma membranes do not contain a specific albumin receptor of sufficient binding affinity to be detected or isolated by techniques that have been used for other kinds of cell surface receptors. It has been shown that lipid-BSA probes behave as reliable markers for fluid phase endocytosis in hepatocytes (Stromhaug, et al. 1997; Synnes, et al. 1999).

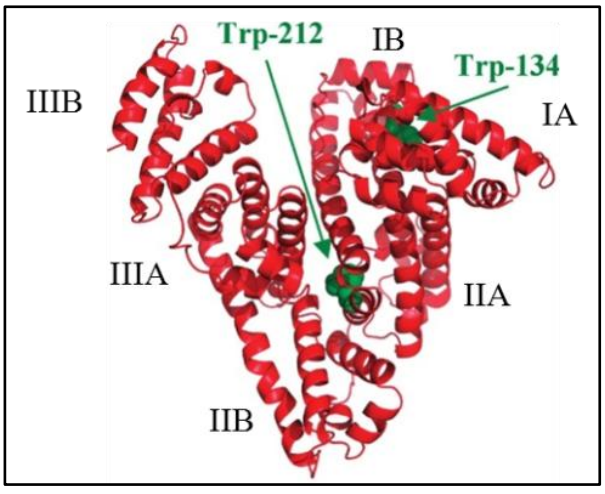

Figure 2.10: X-ray structure of bovine serum albumin with tryptophan residues in green color (Charbonneau, et al. 2010).

2.4.5. Interaction between BSA or LDL and lipids

The basis for the interaction between lipids and proteins is related to their amphiphilic nature and is due to their influence on the water structure, the so-called hydrophobic effect (Tanford, 1980).

Within living organisms, some proteins are adapted to form stable water-soluble complexes with lipids and to function in the mobilization of complex lipids. There are two main types of complexes; complexes formed between monomeric proteins and lipids and, secondly, large lipoprotein complexes. One of the most ubiquitous monomeric proteins that bind lipids is albumin. Serum albumin is a flexible protein that can adopt multiple conformations of approximately equal energy to accommodate the binding of ligands. One of the primary functions of albumin is to bind free fatty acids during mobilization of lipids in the body and transport them in the blood stream as a stable water-soluble complex. The protein has at least three fatty acid binding sites. The mechanism of binding of the fatty acids to the protein has been investigated by NMR methods (Lucas, et al. 2004). The dissociation of fatty acids from serum albumin takes place rapidly $0.04 – 0.14 s^{-1}$ (Weisiger, et al. 1987) with a dissociation constant of 1.4 ± 0.2 mM (Dubois, et al. 1993). Their dissociation from the complex at the site of entry into cells is assisted by the presence of proteins in the plasma membrane with a high affinity for fatty acids (McArthur, et al. 1999).

On the other hand, serum lipoproteins are specifically adapted to form structures designed to transport lipids throughout the body. These lipoproteins are circulating in the mammalian blood stream to distribute a cargo of lipids from their site of synthesis or uptake to the peripheral tissues. The lipids, mainly triacylglycerols, cholesterol, and cholesterol esters, occupy a central core surrounded by a shell of polar lipids and proteins. The proteins act to stabilize the lipid core and provide recognition sites for targeting the complex to the appropriate site of delivery. Moreover, the phospholipids that are associated with LDL are known to affect the physicochemical properties of the lipoprotein (Kleinman, et al. 1988) and may play a role in LDL metabolism by affecting the interaction between LDL and its receptor in the cell membrane (Aviram, et al. 1988). According to the LDL pathway (Goldstein, et al. 2009), LDL is endocytosed after binding to LDL receptors and transferred to lysosomes as mentioned before. Remarkably, LDL-associated PC is not only hydrolyzed in lysosomes, but also at other subcellular sites (Ishikawa, et al. 1989). LDL-phospholipids are known to affect epitope expression of apoB-100 on the surface of LDL particles (Aviram, et al. 1988), and consequently may alter the interaction between LDL and its receptor. It has been suggested

that hydrolysis of LDL-derived glycerophospholipids by hepatic lipase enhances the interaction between lipolysed LDL and LDL-receptor, might affect the apoB-100 epitope, and the cellular uptake of LDL via the receptor (Aviram, et al. 1988).

2.5. Lipid transfer proteins

Both cholesterol ester transfer protein and phospholipid-transfer protein act as specific mediators of lipid transfer between lipoproteins and lipoproteins and cells.

2.5.1. Cholesteryl ester transfer protein (CETP)

CETP is a hydrophobic glycoprotein of mass 74kDa, and plays an important role in reverse cholesterol transport. It promotes the efflux of cholesterol from peripheral tissues to the liver for excretion and degradation to bile acids, so LCAT is of great importance for cholesterol homeostasis. It is a suggested target for therapeutic intervention against atherosclerosis. The action of CETP is to transfer cholesteryl ester from HDL to the other triacylglyerol-rich lipoproteins such as LDL and VLDL, and reciprocal transfer of triacylglycerol from triacylglycerol-rich lipoproteins to HDL, and vice versa. Physiologically, the main effect of CETP may be to promote the transfer of LCAT derived cholesteryl esters out of HDL (where they were formed) into VLDL and LDL, in exchange for triacylglycerol (Fielding, et al. 2002). Inhibition of CETP offers a new approach to coronary artery disease therapy (Brousseau et al. 2004), since high CETP activity lowers the HDL/total cholesterol ratio, potentially increasing risk for atherosclerosis (Papp, et al. 2012).

2.5.2. Phospholipid-transfer protein (PLTP)

PLTP is a glycoprotein of mass 55kDa. PLTP is the second lipid transfer protein found in human plasma that enhances the transport of phospholipids and cholesterol from triacylglyerol-rich lipoproteins such as VLDL to HDL particles and promotes the generation of small pre-β-HDL particles that are initial acceptors of cell-derived cholesterol and the preferred substrate for LCAT (Jauhiainen, et al. 1993). PLTP has been shown to bind to both ApoA-I and ApoA-II (Pussinen, et al. 1998) and enhance CETP (Tollefson, et al. 1988). Three major classes of mammalian phospholipid transfer protein were identified, phosphatidylcholine transfer protein, phosphatidylinositol transfer protein, and the non-

specific lipid transfer protein denoted as sterol carrier protein 2 (SCP-2) that can transfer a wide range of phospholipids including sterols (Wirtz, 2006).

2.6. Fatty acid functions

Fatty acids have an amphipathic nature and constitute one of the most fundamental classes of biological lipids (Fahy, et al. 2005). They serve as the basic building blocks of complex lipids. In form of triacylglycerol, they represent a major form of storage energy. As part of glycerophospholipids, they provide an essential structural component of membranes, and through direct covalent linkage they are used to modify and regulate the properties of many proteins. They can also be metabolized to lipid signalling molecules that perform important roles as metabolic intermediates used for lipid synthesis and protein modification as well as can be broken down through mitochondrial beta-oxidation to generate ATP via oxidative phosphorylation (Puzio-Kuter, 2011). FAs directly regulate cellular processes via regulation of gene transcription (Jump, 2004), or via binding to membrane-bound receptors (GPR40, GPR41, GPR43, GPR84, and GPR120). For example oleic acid binds to GPR43 (Briscoe, et al. 2003), and ω3-unsaturated FAs to GPR120 (Oh, et al. 2010; Ichimura, et al. 2012). In addition, they regulate cell behaviour also indirectly through their bioactive metabolites (Fritsche, 2006; Calder, 2011). It is worth noting that fatty acids can regulate the expression of a range of genes involved in lipid metabolism. Recent studies have shown that poly unsaturated FA interacts directly with nuclear receptors such as peroxisome proliferator activated receptors (PPARs). PPARs are members of a sub-family of the nuclear hormone receptors (Montagner, et al. 2011) that act as sensors of fatty acids and fatty acid analogues. The PPAR family consists of three isoforms, PPARα, PPARβ (also known as δ) and PPARγ. These PPARs play essential roles in the regulation of fatty acid metabolism (Bishop-Bailey, 2011) and were found to be key regulators of lipid and carbohydrate metabolism (Varga, et al. 2011). The affinity of PPAR sub-types to FAs appears to be greatest for PPARα followed by PPARγ and PPARβ (Kliewer, et al. 1997). PPARα, the predominant isoform in liver, play a major role in the fatty acid-induced regulation of hepatic gene transcription, while PPARβ is abundant not only in skeletal muscle but also in adipose tissue, where it is involved in general and fundamental cellular processes (Montagner, et al. 2011). Both PPARα and PPARβ are activated by saturated and unsaturated fatty acids. PPARγ plays an important role in the storage of lipids in adipose tissue and is only activated by long-chain unsaturated fatty acid.

In a physiological context, the uptake of FAs is a highly regulated process, e.g., in endothelial cells, vascular endothelial growth factor B coordinates FA uptake with the energy demands of the surrounding tissue (Hagberg, et al. 2010). FA uptake and processing is also required for cancer cell survival (Zhan, et al. 2008; Hess, et al. 2010). A diet that is relatively high in unsaturated fatty acids, especially polyunsaturated fatty acids, may reduce the risk of heart attacks and strokes (Kwak, et al. 2012). By contrast, the incidence of cardiovascular disease is correlated with diets high in saturated fatty acids. In the present study, uptake and processing of exogenously added FAs by four different cell types were investigated. To avoid artefacts caused by the use of fluorescent or chemical reporter groups, labelling by radioisotopes was used to distinguish endogenous lipids from those that arose by incorporation of FAs of exogenous origin. The four investigated C_{18}-FAs (Fig. 2.11) differ in the degree of unsaturation (18:0, 18:1, 18:2, and 18:3) were applied in concentrations of 10µM. They were added to the culture medium of the cells in complex with bovine serum albumin (BSA). After different incubation time ranging from 15–120min, their cellular uptake and processing into different lipid classes in the different cell types were monitored.

2.6.1. Fatty acid biosynthesis

The liver and adipose tissue in most animals are the major sites of fatty acid biosynthesis. The two pancreatic hormones, insulin and glucagon exert essentially opposite effects on energy metabolism. Thus, during feeding, when the ratio of insulin to glucagon increases in the blood, the activities of several enzymes in the glycolytic and lipogenic pathways are elevated, while the activities of key gluconeogenic enzymes are decreased. As the ratio of insulin to glucagon decreases in the blood during prolonged periods of fasting, the activities of key enzymes in the glycolytic and lipogenic pathways are decreased, while the activities of key gluconeogenic enzymes are increased resulting in a reversal of the process. The fatty acid biosynthetic pathway is carried out by the multifunctional fatty acid synthase enzyme that catalyzes multiple condensations of malonyl-CoA with acetyl-CoA or the elongating lipid, eventually generating palmitate. A first step includes the formation of malonyl-CoA from acetyl CoA by the activity of the enzyme acetyl-CoA carboxylase. Formation of malonyl-CoA is a rate-limiting step of FA biosynthesis, because malonyl-CoA has no metabolic role other than serving as a precursor to FAs. Acetyl transacylase (acetyl transferase) and malonyl transacylase (malonyl transferase) catalyse the formation of acetyl ACP and malonyl ACP from acetyl-CoA and malonyl-CoA, respectively. Acetyl ACP (ACP= acyl carrier protein) is

condensed with malonyl ACP catalysed by acyl-malonyl ACP to form acetoacetyl ACP. A sequence of reactions follows in which the chain is extended to form butanoate. First, the reaction is catalysed by β-ketoacyl-ACP synthetase to produce 3-oxobutanoate, this is reduced to 3-hydroxy-butanoate by β-ketoacyl-ACP reductase, which is in turn dehydrated to E-2-butenoate by β-hydroxyacyl-ACP hydratase before it is reduced to butanoate by enoyl-ACP reductase. The addition of further six units of malonyl-ACP then continues until palmitoyl-ACP is formed. At this step, a thioesterase removes the fatty acyl product as the free acid (with the mammalian enzyme), which has to be converted to the CoA-ester before it can enter the various biosynthetic pathways for the production of specific lipids. Further elongation of palmitoyl-CoA by C_2 units occurs to form long- or very-long-chain fatty acids by Type III fatty acid synthetases (elongases).

A great diversity of fatty acid structures is produced by variations of the basic biosynthetic process. Fatty acids are either saturated such as stearic acid (18:0) or unsaturated such as oleic acid (18:1). Saturated fatty acids are built from two carbon units, initially derived from acetate, added to the carboxyl end of the molecule. The number of double bonds in an unsaturated fatty acid varies typically from one to six. They are introduced by desaturase enzymes at specific positions relative to the carboxyl group (Hunter, 2006). Elongases further extend the chain in two carbon units from the carboxyl end. All the possible odd- and even-numbered homologues with 2 to 36 carbon atoms have been found in nature in esterified form, but the most abundant saturated fatty acids in animal and plant tissues are straight-chain compounds with an even number of 14, 16 and 18 carbon atoms.

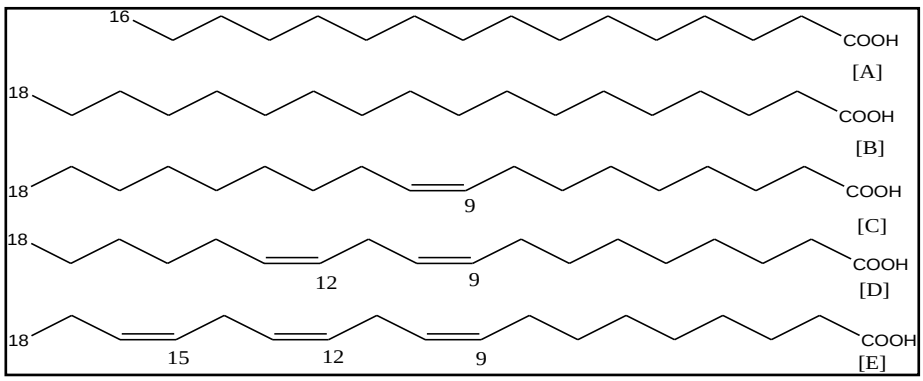

Figure 2.11: The structures of some typical fatty acids A: palmitic acid (16:0); B: stearic acid (18:0); C: oleic acid (18:1, ω9, Δ^9); D: linoleic acid (18:2, ω6, $\Delta^{9,12}$); E: α-linolenic acid (18:3, ω3, $\Delta^{9,12,15}$).

2.6.2. Fatty acid uptake and trafficking

In adipose tissue, fatty acids are liberated from triacylglycerol-rich lipoproteins through the action of lipoprotein lipase. Released fatty acids are bound by albumin and free fatty acids

present in the equilibrium are taken up by cells. From a physiological perspective, it would be highly preferable to regulate fatty acid entry into the cell to ensure fatty acid uptake when its extracellular concentration is low, to limit uptake when the extracellular concentration is high, to potentially select for specific fatty acid types, and to allow rapid adjustments in fatty acid provision to meet fluctuations in metabolic demands (Glatz, et al. 2010).

The solubility of fatty acids in aqueous solutions is low, i.e., in the range of 1–10nM (Vorum, et al. 1992). The ability of proteins to bind fatty acids dramatically increases the total amount of fatty acids that can be present in the aqueous phase. Albumin and cytoplasmic fatty acid-binding protein (FABPc) act as extracellular and intracellular buffers for fatty acids, respectively. For instance, albumin at a concentration of 300–600mM can accommodate up to 1–2mM fatty acids in plasma and interstitium (Richieri, et al. 1993), while FABPc inside the cell functions as a sink for incoming fatty acids can accommodate up to150–300mM fatty acids (Vork, et al. 1993; Richieri, et al. 1994). Scavenging of fatty acids by binding proteins play a crucial role to prevent the toxicity of free fatty acids and their intermediates in the cytosol by intracellular transport towards their sites of metabolic conversion (Schaap, et al. 1998).

There are different pathways for the uptake of FA through the cell membrane (Schwenk, et al. 2010) (Fig. 2.12). First, the amphipathic nature of the fatty acid molecule provides it with the biophysical properties to pass the phospholipid bilayer of the cell membrane by simple diffusion (Fig. 2.12, Path 1). Adsorption from the outer leaflet of the bilayer, flip-flop, and desorption of fatty acids functions to transfer protons across the membrane. Several studies showed that biological membranes do not form a barrier for fatty acids and that fatty acids can rapidly and spontaneously diffuse through phospholipid bilayers without the help of membrane proteins (Hamilton, 2007). A second possibility is that plasma membrane fatty acid-binding proteins (FABPpm), a family of small, highly abundant proteins that bind tightly and sequester free fatty acids (La Londe, et al. 1994), facilitate and regulate transmembrane fatty acid transport across the cell membrane and prevent destabilization of the membrane (Schwenk, et al. 2010). These fatty acid-binding proteins act as acceptors for fatty acids whereafter the fatty acids make their way through the cell membrane by simple diffusion (Fig. 2.12, Path 2). Furthermore, CD36 (88kDa; also referred to as ‘fatty acid translocase’) alone or together with plasma membrane fatty acid-binding protein (FABPpm) might serve as acceptors of albumin-derived extracellular FA to increase their local concentration at the cell surface and thus increase the number of FA-diffusion events (Stremmel, et al. 2001) (Fig. 2.12, Path 3). Additionally, fatty acid transport proteins (FATPs) are thought to accelerate the

rate of trans-membrane transport of FA, which are rapidly activated by plasma membrane acyl-CoA synthetase (ACS1) to form acyl-CoA esters (Fig. 2.12, Path 4). The colocalization of FATP1, FATP6, and CD36 within distinct areas of the plasma membrane makes an interaction likely (Gimeno, et al. 2003; Pohl, et al. 2000). Currently, it is not known to which extent an interaction of the CD36/FABPpm complex with one or more of the FATP species occurs. The mechanisms by which long-chain FAs enter mammalian cells may differ from one cell type to others. For example, the incorporation of FAs in fibroblasts depends on the structure of the FA: apparently, palmitic acid enters the cell by simple diffusion, while oleic acid (OA) uses saturable transport processes (Damyanova, et al. 2010). FATPs preferentially transport very-long- chain fatty acids VLC-FA (> C_{22}); and by action of their synthetase activity they directly convert them into very-long-chain acyl-CoA esters (Fig. 2.12, Path 5).

After being taken up, FAs are bound to cytoplasmic FA binding proteins (FABPc) (Haunerland, et al. 2004) that transport them to intracellular target locations such as mitochondria, peroxisomes, or the endoplasmic reticulum (Storch, et al. 2000). Once inside the cell, free fatty acids are minimally soluble in the aqueous cytoplasm. At high enough concentrations fatty acids exert a detergent-like effect that would disrupt membranes. In addition to these proteins, long-chain acyl-CoA synthetases (ACSLs) are involved in FA uptake by converting them into membrane impermeable acyl-CoAs in an ATP-dependent reaction, and thus facilitate the import of exogenous FAs by trapping them inside the cells (Duttaroy, 2009). The acyl-CoAs have numerous metabolic fates, including donating FAs for incorporation into TAGs and membrane phospholipids, as substrates for β-oxidation and protein acylation, and as ligands for transcription factors.

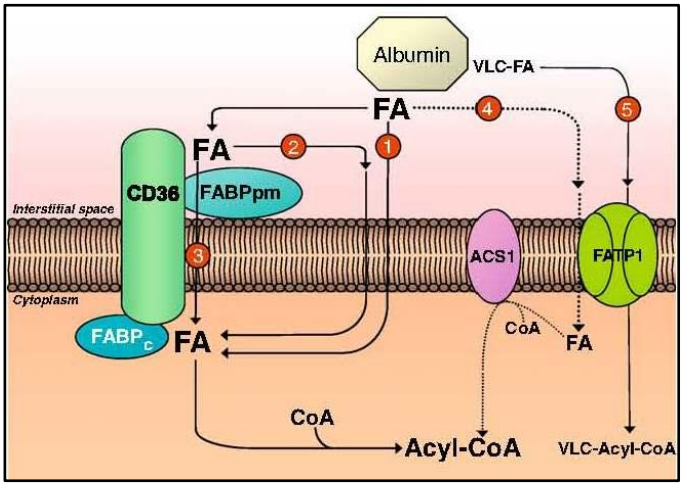

Figure 2.12: Proposed different models of fatty acid (FA) transport across the cell membrane (Schwenk, et al. 2010).

2.7. Cholesterol

In 1926, Heinrich Wieland was awarded the Nobel Prize in chemistry who suggested the structure of cholesterol and bile acids. Cholesterol (Fig. 2.13), is the most prevalent steriod in mammalian cells and serves as precursor of bile acids and steroid hormones. Vitamin D_3 is derived from 7-dehydrocholesterol, the immediate biosynthetic precursor of cholesterol. Cholesterol is a principal component of the plasma membranes of mammalian cells, and much smaller amounts of cholesterol are found in the membranes of intracellular organelles like mitochondria, Golgi complexes, and nuclear membranes. It intercalates among the phospholipids of the membrane, with its hydroxyl group at the aqueous interface and the remainder of the molecule within the leaflet. The relatively rigid fused ring system of cholesterol and the weakly polar group at the C-3 position have important consequences for the properties of plasma membranes. It stabilizes the liquid ordered phase of membranes and prevents phase transitions. Furthermore, most endocytic pathways like clathrin-dependent and clathrin-independent pathways are inhibited by the loss of cholesterol.

Cholesterol is also a component of lipoprotein complexes in the blood, and it is one of the constituents of plaques that form on arterial walls in atherosclerosis. Excess cholesterol is metabolized to oxysterols (Rock, 2008), which are not only blocking SREBP cleavage-activating protein (SCAP), but also facilitate proteolysis of SREBP and thereby down-regulate endogenous cholesterol synthesis and LDL receptor levels. Cholesterol has to be oxidized to oxysterols before it can leave the brain. In addition, oxysterols further reduce the cellular content of unesterified cholesterol by activating bile acid synthesis and cholesterol esterification. Rising cholesterol levels cause hydroxy-methylglutaryl-CoA (HMG-CoA) reductase to be ubiquitinated and degraded by the proteosome. They cause SREBP cleavage-activating protein (SCAP) to remain localized to the endoplasmic reticulum rather than translocating to the Golgi. Within endolysosomal compartements, cholesteryl esters can be hydrolized by acid lipase to fatty acids and free cholesterol required for membrane and lipoprotein formation and for hormone synthesis in adrenal cells. The free cholesterol binds to the NPC-2 protein, which transports and delivers the cholesterol to the NPC-1 protein, located in the perimeter membrane (Abdul-Hammed, et al. 2010). Deficiency of lysosomal acid lipase results in Wolman disease (Tanaka, 1995). Cholesterol, however, is insoluble in blood and is transported in the circulatory system bound to one of the varieties of lipoproteins. Therefore, in our study, we used albumin and low density lipoprotein as a carrier for cholesterol.

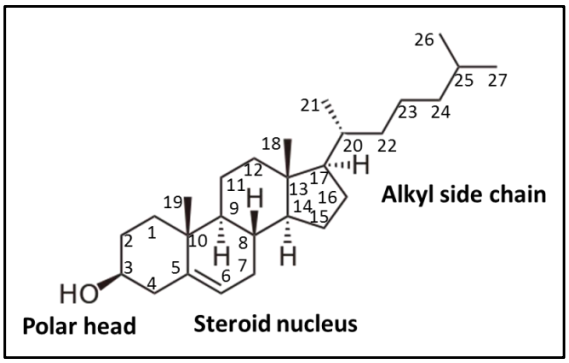

Figure 2.13: The structure of cholesterol, showing the rings of the steroid nucleus and carbon numbering.

2.7.1. Cholesterol and cholesteryl ester biosynthesis

Cholesterol is present in tissues and in plasma either as free cholesterol or combined with a long-chain fatty acid as a storage form. Both forms are transported in plasma by lipoproteins. More than half the cholesterol of the body is formed by synthesis (about 700 mg/d), and the average diet provides the remainder (Mayes, et al. 2003).

The cholesterol biosynthetic pathway (Fig. 2.14) starts from the cytosolic thiolase that catalyzes the Claisen condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA reacts with another molecule of acetyl-CoA in an aldol addition to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalyzed by cytosolic HMG-CoA synthase. The next step in this pathway is the rate-limiting step in cholesterol biosynthesis, in which HMG-CoA is reduced by two molecules of NADPH to produce mevalonate. The reaction is catalyzed by HMG-CoA reductase, a 97kDa glycoprotein that traverses the endoplasmic reticulum membrane with its active site facing the cytosol. A series of four reactions converts mevalonate to isopentenyl pyrophosphate and then to dimethylallyl pyrophosphate. Pyrophosphomevalonate decarboxylase phosphorylates the 3-hydroxyl group, which is followed by a trans-elimination of the phosphate and carboxyl groups to form the double bond in isopentenyl pyrophosphate. Isomerization of the double bond yields dimethylallyl pyrophosphate. Condensation of these two 5-carbon intermediates produces geranyl pyrophosphate, a further condensation of 5-carbon isopentenyl pyrophosphate forms farnesyl pyrophosphate. Then two molecules of farnesyl pyrophosphates condense to form squalene. Before ring closure occurs, squalene monooxygenase, an enzyme bound to the endoplasmic reticulum, converts squalene to squalene-2,3-epoxide. This reaction requires NADPH as coenzyme and O_2. A second ER membrane enzyme, 2,3-oxidosqualene:lanosterol cyclase, catalyzes the second reaction to form lanosterol, which involves the transfer of methyl groups from C_{14} to C_{13} and from C_8 to C_{14} as cyclization occurs. To convert lanosterol to cholesterol, another 20 steps are required that take place at the membranes of the endoplasmic reticulum and peroxisomes. They involve removal of the methyl groups on C_{14} and C_4 to form 14-

desmethyl lanosterol and then zymosterol. The double bond at C_8–C_9 is subsequently moved to C_5–C_6 in two steps, forming desmosterol. Finally, the double bond of the side chain is reduced, to produce cholesterol.

Cholesteryl esters are formed in the liver through the action of Sterol O-acyltransferase SOAT (formely called Acyl-CoA cholesterol acyl transferase ACAT). SOAT is the intracellular protein on the cytoplasmic face of the ER membrane, which catalyzes the transfer of a fatty acid from coenzyme A to the hydroxyl group of cholesterol, converting the cholesterol to a more hydrophobic and storage form. *In vivo*, SOAT plays an important physiological role in intestinal absorption of dietary cholesterol, and in control of the cellular free cholesterol pool that serves as substrate for bile acid and steroid hormone formation.

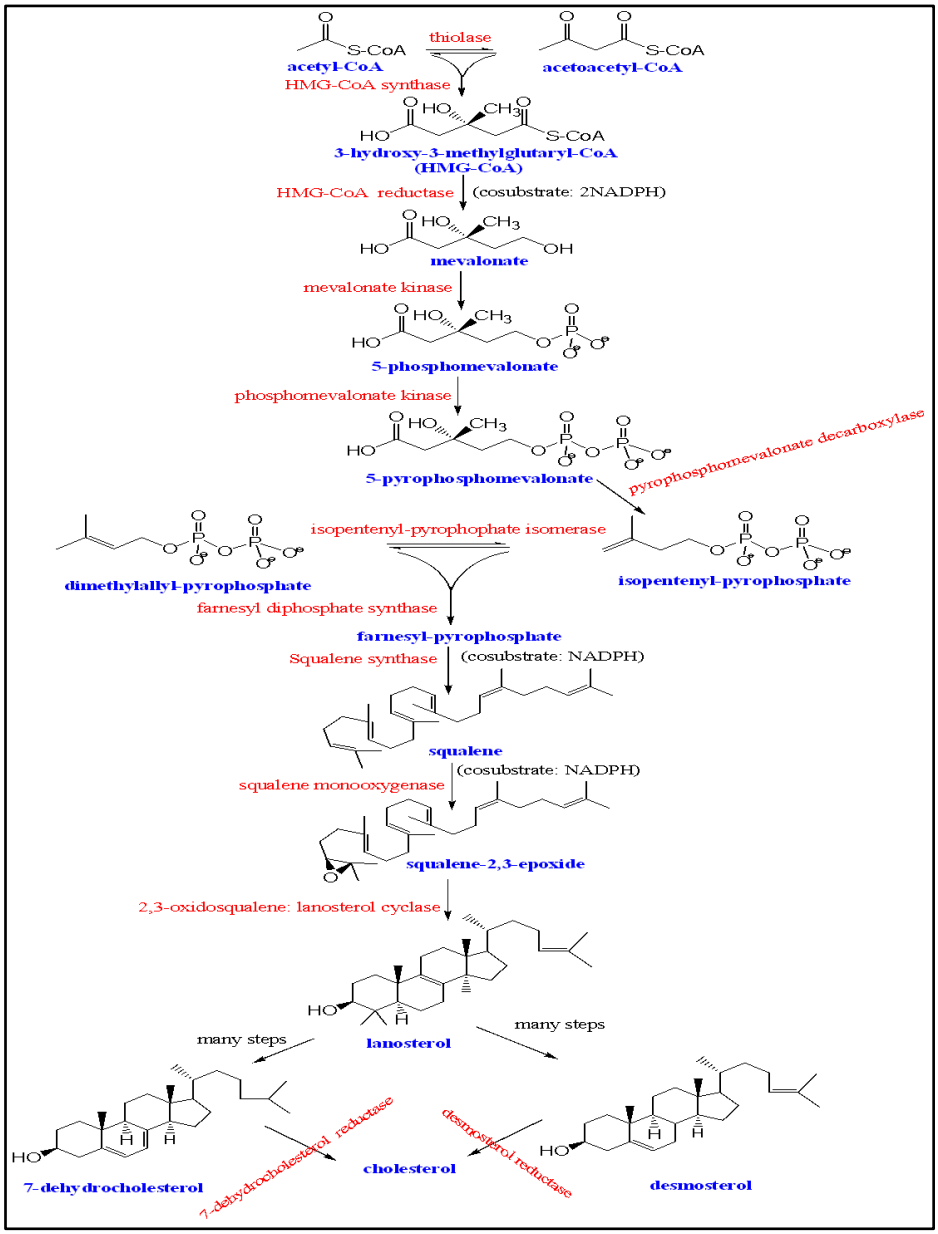

Figure 2.14: The biosynthetic pathway of cholesterol.

2.8. Di- and tri-acylglycerols

Diacylglycerols are the principal precursors of glycerophospholipids, consisting of two fatty acid chains covalently bound to a glycerol molecule through ester linkages. DAG can induce structural changes in the lipid bilayer, which may be responsible for the molecular signal transduction, such as activation of protein kinase C or to enhance membrane fusion processes. Switching off the signal generated by the formation of diacylglycerol is accomplished by diacylglycerol kinase, which converts the substrate into phosphatidic acid (Stathopoulos, et al. 1990). On the other hand, triacylglycerols are nonpolar, hydrophobic molecules, synthesized mainly in adipose tissue, liver, and intestine and serve as the principal energy storage molecule in eukaryotes. Although triacylglycerols are insoluble in water, mono-and diacylglycerols are able to form lyotropic liquid crystalline structures in water (Macierzanka, et al. 2006). Mammalian triacylglycerol synthesis is affected by a large number of factors. These include nutritional, hormonal, and pharmacological effects. A major function of triacylglycerols in mammalian cells is to allow the transport in the form of the serum lipoproteins and storage of acyl moieties in the body. The formation of triacylglycerols removes the potentially harmful fatty acids (Brindley, 1991). Moreover, plenty of evidence suggests that elevated triacylglycerol levels are an independent risk factor for cardiovascular disease (CVD) (Jacobson, et al. 2007; Botham, et al. 2007). Metabolism of triacylglycerols in animals requires lipoprotein lipase involved in uptake of FAs from plasma and hormone-sensitive lipase involved in release of fatty acids from lipid stores. Hormone-sensitive lipase is responsible for hydrolysis of the esters at positions 1 and 3 of the triacylglycerol, and monoacylglycerol lipase catalyzes hydrolysis of the remaining ester to yield a third free fatty acid and glycerol (Bernlohr, et al. 2002). Fatty acids are immediately bound to serum albumin and carried in the bloodstream to the liver, muscle, and other tissues for oxidation, while glycerol has to be shuttled back to the liver for oxidation or gluconeogenesis.

2.8.1. Di- and tri-acylglycerol biosynthesis

Diacylglycerol can originate from two sources (Fig. 2.16), either it is formed by phosphatidate phosphohydrolase (phosphatidic acid phosphatase) that converts phosphatidate (phophatidic acid) to 1,2-diacylglycerol (DAG), or it is synthesized by acylation of monoacylglycerol (MAG) by monoacylglycerol acyltransferase. Most of the activity of these enzymes resides in the endoplasmic reticulum of the cell, but some is found in mitochondria.

Phosphatidate phosphohydrolase is found mainly in the cytosol, but the active form of the enzyme is membrane-bound. It can be inhibited by some cationic amphiphilic drugs (Koul, et al. 1987). Furthermore, diacylglycerol can be transiently formed in cell membranes as a consequence of phospholipase C-type enzymes activated by a variety of hormones, growth factors, and neurotransmitters.

Diacylglycerol is a precursor for synthesis of triacylglycerol (TAG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). Triacylglycerol is formed (Fig. 2.16) by the esterification of a third fatty acid to the hydroxy group of diacylglycerol under the catalysis of diacylglycerol acyltransferases (DGAT-1, and DGAT-2) located in the endoplasmic reticulum (Kolter, Römpp Online). Their activities are especially high in lipogenic tissues like adipose and liver. In Caco-2 cells and rat intestinal mucosal membranes, 76% and 89% of the *in vitro* TAG synthesis are initiated from MAG, which is mediated by DGAT1, respectively. XP620 (Fig. 2.15) is an inhibitor for DGAT-1 investigated for the treatment of obesity (Cheng, et al. 2008). However, complete deletion of SOAT- or DGAT activity triggers FA-mediated cell death (Garbarino, et al. 2009).

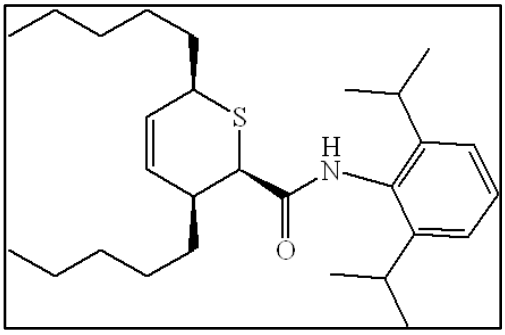

Figure 2.15: Structure of XP620

2.9. Phospholipids

Phospholipids form the essential milieu of cellular membranes and provide a barrier for entry of exogenous compounds into cells. Phospholipids act as precursors of second messengers such as diacylglycerol (DAG) and inositol-1,4,5-P_3. Phospholipids also function as storage of energy in the form of fatty acyl components. This function is probably quantitatively important only under extreme conditions such as starvation (Vance, 2002).

Phospholipids are divided into two main classes depending on whether they contain a glycerol backbone, glycerophospholipids or contain a sphingosyl backbone, sphingophospholipids.

2.9.1. Glycerophospholipids

Glycerophospholipids are members of the broader class of lipids known as phospholipids. Most of the phospholipids that are derived from glycerol are synthesized on the cytosolic side of the endoplasmic reticulum (ER) membrane from water-soluble cytosolic precursors.

2.9.2. Phosphatidic acid biosynthesis

The synthesis of phosphatidic acid (PA), the simplest negatively charged membrane phospholipid, is a common pathway that operates in nearly all organisms. PA is a key intermediate in the formation of glycerophospholipids (Wang, et al. 2003) and triacylglycerols (Mayes, et al. 2003).

In liver and kidney, glycerol kinase catalyzes the phosphorylation of glycerol to form L-glycerol-3-phosphate, which is then acylated at both the 1- and 2- positions to yield phosphatidic acid (or 1,2-diacylglycerol phosphate) (Fig. 2.16). First, the two molecules of acyl-CoA are formed by the activation of fatty acids by acyl-CoA synthetase and both glycerol and fatty acids must be activated by ATP before they can be incorporated into acylglycerols. The first acylation at position sn-1 is catalyzed by glycerol-3-phosphate acyltransferase, while 1-acylglycerol-3-phosphate acyltransferase catalyzes the second acylation at position sn-2. Commonly but not invariably, the fatty acid at C-1 is saturated and that at C-2 is unsaturated.

In eukaryotic systems, if the activity of glycerol kinase is absent or low as in muscle or adipose tissue, most of the glycerol 3-phosphate is formed from dihydroxyacetone phosphate by the action of glycerol-3-phosphate dehydrogenase. Again a specific acyltransferase adds the first acyl chain, followed by reduction of the backbone keto group by acyldihydroxyacetone phosphate reductase, using NADPH as the reductant. Enzymes on the cytosolic face of the ER membrane then catalyze the addition of different polar head groups. A phosphatase converts phosphatidic acid to diacylglycerol, as mentioned before.

2.9.3. Phospholipid biosynthesis

Phosphorylethanolamine and phosphorylcholine are formed by phosphorylation by ATP of ethanolamine or choline in the presence of the cytosolic enzymes ethanolamine kinase or choline kinase, respectively. The following reaction involves the rate limiting step, transfer of a cytidylyl group from cytidine triphosphate (CTP) to form cytidine diphosphates (CDP-ethanolamine or CDP-choline) and pyrophosphate. Pyrophosphate hydrolysis drives this

reaction forward. Finally, CDP-ethanolamine:1,2-diacylglycerol phosphoethanolamine transferase or CDP-choline:1,2-diacylglycerol phosphocholine transferase then links phosphoethanolamine or phosphocholine to the diacylglycerol backbone to form the neutral charged phospholipids, PE, and PC, respectively (Renooij, et al. 1981; Gibellini, et al. 2010). This sequence of reactions is known as the Kennedy-pathway. CDP-diacylglycerol derived from phosphatidic acid can also be used as a precursor for several other negatively charged phospholipids, including a phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL) (Hatch, 1994). The enzyme CDP-DAG synthase is largely microsomal, but is also found in the mitochondrial inner membrane (Zinser, et al. 1991).

However, an alternative pathway can convert phosphatidylethanolamine to phosphatidylcholine by methylation reactions involving S-adenosylmethionine (Shields, et al. 2003) catalyzed by PE-N-methyltransferase. This reaction has been sugested to play an important role in the transport of polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) from the liver to plasma and possibly other tissues (Wijk, et al. 2012). In mammals and yeast, PE can also be formed from PS by conversion of serine to ethanolamine through an indirect route, in which ethanolamine is formed by mitochondrial PS decarboxylase. In animal, PS is synthesized by PS-synthases I and II that exchange the choline and ethanolamine head groups of PC and PE for serine, respectively (Arikketh, et al. 2008; Vance, 2008).

Biosynthesis of inositol from glucose occurs in the brain, testes, and in other tissues to a lesser extent. The rate limiting step appears to be the synthesis of myo-inositol-3-phosphate from glucose-6-phosphate (Downes, et al. 1990). Inositol-3-phosphate is hydrolyzed to inositol by a phosphatase. Further phosphorylations can take place at different positions of myo-inositol (Roeber, 2002) and give rise to several phosphatidylinositol phosphates.

The biosynthesis of bis(monoacylglycero)phosphate (BMP), (Heravi, et al. 1999; Scherer, et al. 2010) involves hydrolysis of PG to 1-acyl-sn-glycero-3-phospho-rac-glycerol (LPG) by a lysosomal phospholipase A2 (PLA2). Sn-3:sn1' LPG is then acylated on the glycerol head group by a transacylase, using a phospholipid as the acyl donor, to form bis(monoacylglycero)phosphate that still retains the sn-3:sn-l' stereo configuration of the original PG and a lysophospholipid. The phosphoryl ester from sn-3 to the sn-l is reoriented by an enzymatic activity to yield sn-l:sn-l'-LPG. The final product, sn-1:sn-1'-BMP (Fig. 2.17), is formed upon acylation of the glycerol backbone of sn-1:sn-1'-LPG. BMP is not present in the limiting lysosomal membrane (Möbius, et al. 2003), but is required for sphingolipid degradation on inner membranes of the acidic compartments (Kolter, et al.

2010). In Lands' cycle (Lands, 1958), the rapid turnover of the sn-2 acyl moiety of glycerophospholipids by PLA2 liberates lysophospholipids and free FAs. The free FA released can be activated to acyl-CoA by acyl-CoA synthetase (ACS). Lysophospholipids are then converted to phospholipids in the presence of acyl-CoA by lysophospholipid Acyltransferases (LPLAT).

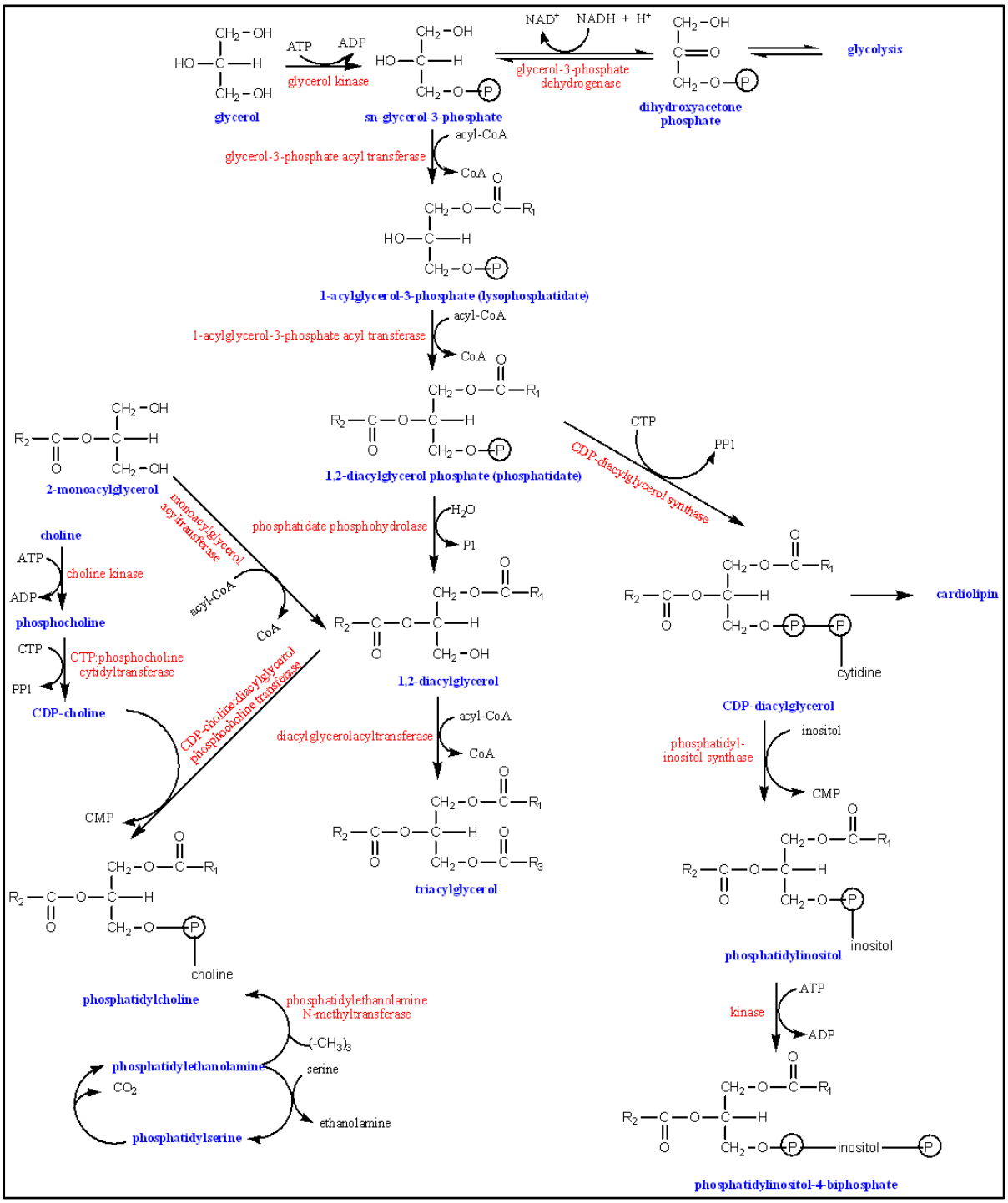

Figure 2.16: Synthesis of triacylglycerol and glycerolipids begins with the formation of phosphatidic acid, which may be formed from dihydroxyacetone phosphate or glycerol.

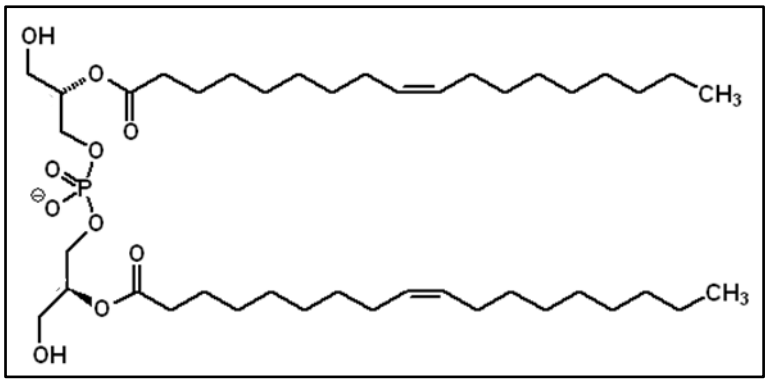

Figure 2.17: Structure of bis(monoacylglycero)phosphate. The acyl chain residues are on the sn-2 position of both glycerol backbones.

2.9.4. Sphingophospholipids

Sphingophospholipids represent another class of phospholipids frequently found in biological membranes that contain sphingosine ((2S, 3R)-2-aminooctadec-4-ene-1,3-diol) or another sphingoid base that forms the backbone of these lipids rather than glycerol in glycerophospholipids. The most common phospholipid in this class is the phosphorylcholine ester of an N-acylsphingosine (or ceramide) that is more commonly called sphingomyelin. Sphingomyelin represents a major lipid of certain membranes in brain and nervous tissue of higher animals. The lysosomal hydrolysis of sphingomyelin to ceramide and phosphocholine is catalyzed by acid sphingomyelinase, which cleaves the phosphodiester bond of sphingomyelin between ceramide and phosphorylcholine (Lansmann, et al. 2003). In humans, a rare genetic defect in the enzyme ASMase results in Niemann-Pick disease (Horinouchi, et al. 1995), which is associated with the accumulation of sphingomyelin in endosomes and lysosomes (Schuchman, 2010). Sphingomyelin accumulates in the brain, spleen, and liver. Type A of the disease becomes evident in infants, and causes mental retardation and early death.

Ceramide is the building block of other sphingolipids and glycosphingolipids and plays an important role in membrane signaling processes, and membrane fusion (Lopez-Montero, et al. 2005). Ceramides are known to be one of the main mediators of apoptosis in cells. Increasing the levels of ceramide by inhibition of ceramidases, which convert ceramide to sphingosine and free fatty acid, results in cell death (Strelow, et al. 2000). Likewise, inhibition of ceramide production by blocking the *de novo* synthesis pathway (fumonisin B1) or inhibiting neutral, (GW 4869), and acid (D606 and desipramine) sphingomyelinase activity, slows down apoptosis in response to a variety of factors including chemotherapeutic

agents, tumor necrosis factor-ß, angiotensin-II, and B-cell activation (ElBawab, et al. 2002; Takahashi, et al. 2006; Huang, et al. 2011).

2.9.5. Sphingolipid biosynthesis

In eukaryotic cells, sphingomyelin and glycosphingolipids are components of the outer leaflet of plasma membranes (Kolter, et al. 1999). The membrane-bound enzymes that catalyze the *de novo* biosynthesis of ceramide are localized on the cytosolic leaflet of the endoplasmic reticulum (Stiban, et al. 2008; Mandon, et al. 1992). The initial reaction of the *de novo* biosynthesis of sphingolipids (Fig. 2.18) is the condensation of the amino acid L-serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase (Hanada, 2003) to form 3-ketosphinganine. Reduction of the ketone product to sphinganine is catalyzed by 3-ketosphinganine reductase, with NADPH as a reductant. Sphinganine is acylated to form dihydroceramide by an enzyme family encoded by the lass-genes (longevity assurance genes) (Teufel, et al. 2009). These enzymes have different specificities for the fatty acid CoA thioesters and are generating different dihydroceramide species (Pewzner-Jung, et al. 2006). Dihydroceramide is then desaturated by dihydroceramide desaturase (Rother et al. 1992) to form ceramides (N-acylsphingosines). Ceramide is tranported with the aid of the transfer protein CERT (Hanada, 2006) from the ER to the Golgi apparatus or the trans-Golgi network. Glycosylation of the first hydroxyl group of ceramide by sugar nucleotides yields the cerebrosides galactosylceramide (GalCer) on the luminal site of the Golgi apparatus and glucosylceramide (GlcCer) on the cytosolic leaflet of the Golgi apparatus by galactosyltransferase and glucosyltransferase, respectively. GalCer is a major lipid of myelin, which makes up about 15% of the lipids of myelin sheath structures, whereas GlcCer is the major glycosphingolipid of extraneural tissues and is a precursor of most of the more complex glycosphingolipids. Sphingomyelin, which is both a phospho- and sphingolipid, is synthesized through the action of sphingomyelin synthase by transfer of phosphorylcholine from phosphatidylcholine to ceramide, liberating diacylglycerol. *De novo* sphingomyelin synthesis occurs mainly on the luminal face of the Golgi apparatus and to a lesser extent in the plasma membrane (Tafesse, et al. 2007). Further deacylation of sphingomyelin by sphingomyelin deacylase produces sphingosylphosphorylcholine (lysosphingomyelin). Phosphorylation of ceramide (another metabolic step) by ceramide kinase forms ceramide-1-phosphate (Bajjalieh, et al. 2000). In the salvage pathway, attachment of a fatty acid to the amino group of sphingosine or other related amino alcohols via an amide linkage gives rise to ceramides, which are catalyzed by ceramide synthase. Furthermore, ceramide can also be

formed by degradation of sphingomyelin and glycosphingolipids in lysosomes through the action of sphingomyelinases (Goni, et al. 2002) and glycosidases, respectively. On the other hand, catabolism of ceramide in lysosomes to sphingosine is catalyzed by acid ceramidase. Degradation of ceramide by ceramidases is enhanced by a variety of agents including cytokines, cell differentiating agents, death receptor ligands, cancer chemotherapeutic agents, and ionizing radiation (Sandhoff, et al. 2001). Sphingosine can be phosphorylated at the 1-position to sphingosine-1-phosphate (S1P). Phosphoethanolamine and hexadec-2-enal can be formed by cleavage of S1P via a pyridoxal phosphate dependent lyase.

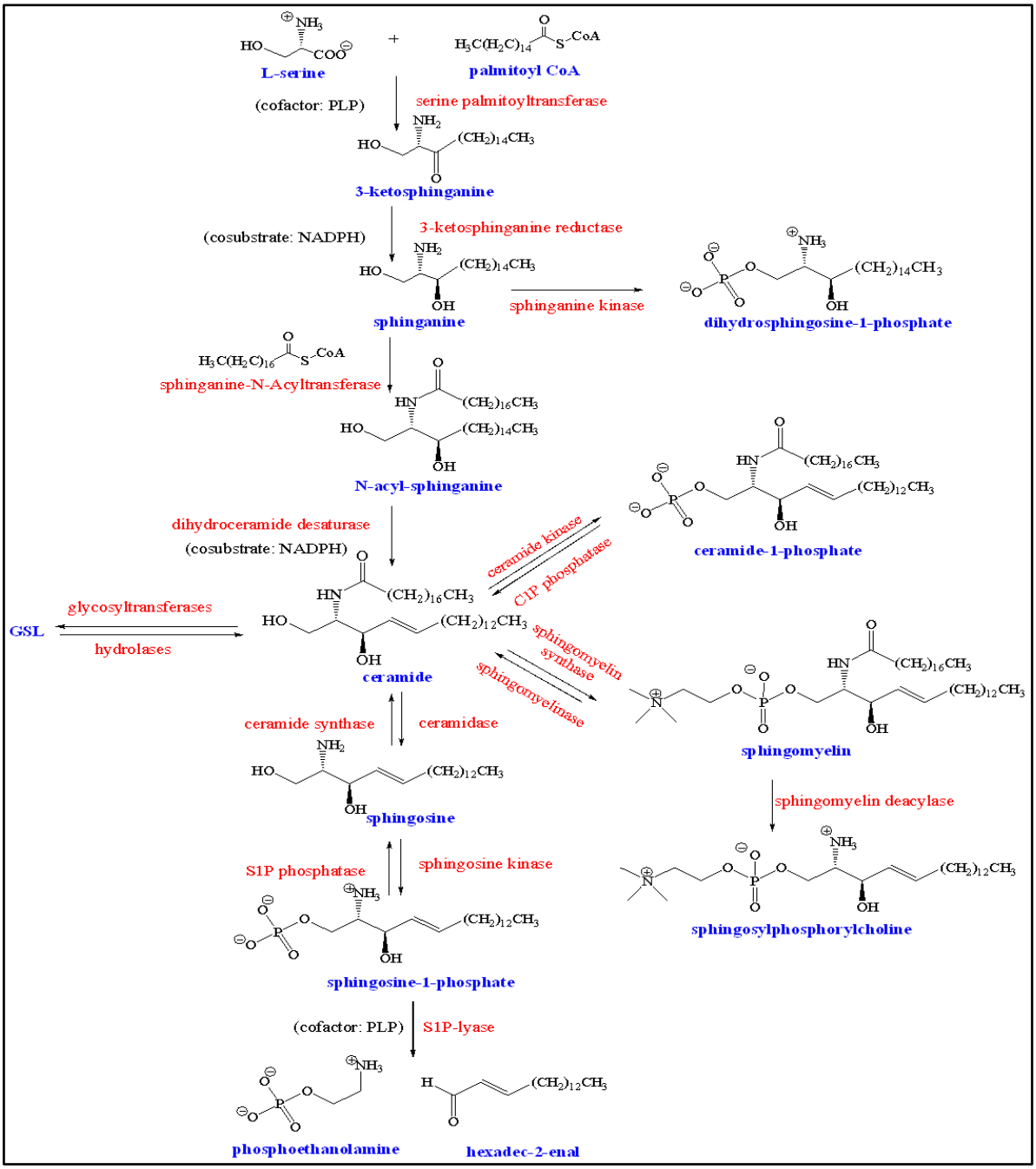

Figure 2.18: Biosynthesis pathway of sphingolipids in animals.

2.10. Cationic amphiphilic drugs (CADs)

Many lipids of exogenous origin enter the cell through the endolysosomal system. Endosomes and lysosomes are the intracellular sites through which the majority of nutrients enter the cell. Furthermore, the uptake and processing of many lipid classes is impaired in lysosomal storage diseases or in the presence of cationic amphiphilic drugs that interfere with endolysosomal lipid processing, and lead to drug-induced lipidoses (Kolter, et al. 2010; Anderson, et al. 2006; Nonoyama, et al. 2008). Long-term treatment of human patients or animals with cationic amphiphilic drugs (CADs) can induce the storage of lipids as a severe side-effect (Anderson, et al. 2006; Lüllmann-Rauch, et al. 1975; Lüllmann, et al. 1978). In the present work, the influence of five selected CADs on uptake and processing of phosphatidylcholine (PC) and sphingomyelin (SM) by different types of cultured cells is investigated. Furthermore, the influence of desipramine on uptake and processing of four C_{18} fatty acids by different cell types was studied.

Desipramine is a tricyclic antidepressant drug that induces premature degradation of acid sphingomyelinase, which is mainly localized in endosomes and lysosomes, and leads to endolysosomal storage of SM (Hurwitz, et al. 1994; Kölzer, et al. 2004). Desipramine interferes with the interaction of the positively charged protein with the negatively charged luminal membranes. This leads to the release of this enzyme from the surface of intraendosomal and intralysosomal membranes and its subsequent premature degradation. Drugs with such properties have been named functional inhibitors of acid sphingomyelinase (FIASMAs) (Kornhuber, et al. 2010). Also acid ceramidase activity is apparently reduced by desipramine in SCC-14A cells and the mouse fibroblast cell line L929 (Zeidan, et al. 2006), 5637 cells, and HeLa cells (Elojeimy, et al. 2006). In addition, desipramine increased cytoplasmic Ca^{2+} ion concentration in PC3 human prostate cancer cells (Huang, et al. 2007) and human osteosarcoma MG63 cells (Jan, et al. 2003) by causing Ca^{2+} release from the endoplasmic reticulum in a phospholipase C-independent fashion and by inducing Ca^{2+} influx. Furthermore, desipramine inhibits protein kinase C (PKC) activity in rat brain (Morishita, et al. 1997).

Imipramine is also a tricyclic antidepressant drug that acts as an apparent inhibitor of acid sphingomyelinase in cultured cells (Jensen, et al. 1999). Also imipramine induces phospholipidosis upon prolonged administration in animals (Lüllmann-Rauch, et al. 1975).

Chlorpromazine is a cationic amphiphilic phenothiazine derivative, and has been widely used as an antipsychotic neuroleptic drug. It antagonizes receptors for dopamine (D_2),

serotonin (5-HT_{2A}), histamine (H_1), and others, and due to its multiple side-effects, it had to be rejected from the market. Chlorpromazine also inhibits the formation of clathrin-coated pits (Nawa, et al. 2003) and disrupts clathrin-mediated endocytosis by inhibiting the relocation of clathrin and adaptor protein 2 complexes (Yao, et al. 2002) from the plasma membrane. It binds to the cytoplasmic tail of the cell surface receptors that recruit clathrin (Sorkin, 2004), and also inhibits LDL receptor recycling. It causes clathrin lattices to assemble on endosomal membranes and at the same time it prevents coated pit assembly at the cell surface of human fibroblasts (Wang, et al. 1993). Microscopy and transport studies revealed that chlorpromazine perturbs membrane properties of cellular organelles and leads to a blockage of the secretory pathway (De Filippi, et al. 2007). It can form stoichiometric 1:1-complexes with acidic phospholipids such as phosphatidic acid (PA) (Stuhne-Sekalec, et al. 1987) and phosphatidylinositol (PI) (Schwendener, 1988), but not with neutral glycerophospholipids such as phosphatidylcholine (PC). Previous studies have shown that chlorpromazine and other amphiphiles cause pronounced perturbation of membranes (Burack, et al. 1994) and induce the expression of a number of genes in both bacteria and mammalian cells (Vigh, et al. 1998). Since the activity of membrane-bound proteins (receptors, channels, and enzymes) can be dependent on the lipid composition of the membranes (Lee, 2003), CADs such as chlorpromazine might influence the activity of such proteins even without direct interaction between the protein and the CAD (Gjerde, et al. 2004; Chen, et al. 2005).

Chloroquine is a CAD used in the treatment of malaria, and acts as a potent inducer of lysosomal storage of phospholipids in both cell cultures and *in vivo* (Hostetler, et al. 1985). Chloroquine also inhibits the recycling of the LDL-receptor, but not of the scavenger receptor BI to the plasma membrane (Minahk, et al. 2008). Also chloroquine leads to an apparent inhibition of acid sphingomyelinase activity (Yoshida, et al. 1985). It has been suggested that chloroquine inhibits degradation of complex endocytosed molecules indirectly by inhibiting the transport from endosomes to lysosomes (Tolleshaug, et al. 1979; Berg, et al. 1980). Chloroquine (Kodavanti, et al. 1990), like Chlorpromazine (Pappu, et al. 1984) has been reported to inhibit lysosomal phospholipases A and C. Similar to desipramine, chloroquine and chlorpromazine were able to reduce apparent acid ceramidase activity (Elojeimy, et al. 2006), presumably according to the same mechanism as acid sphingomyelinase (Hurwitz, et al. 1994). Such drugs also interfere with P-glycoprotein involved in multidrug resistance (Jaffrezou, et al. 1995).

FTY720, is an immunosuppressant, and has been approved for the treatment of multiple sclerosis. It behaves as a prodrug of a functional antagonist of sphingosine phosphate receptors (Chun, et al. 2010). In addition, FTY720 shows multiple effects, e.g. as cannabinoid receptor antagonist (Paugh, et al. 2006), as cPLA2 inhibitor (Payne, et al. 2007), ceramide synthase inhibitor (Berdyshev, et al. 2009; Dawson, et al. 2011), or inhibits lysosomal acid sphingomyelinase but not neutral sphingomyelinase activity (Dawson, et al. 2011). It decreases cholesterol toxicity in primary human macrophages, and stimulates 27-hydroxycholesterol production (Blom, et al. 2010).

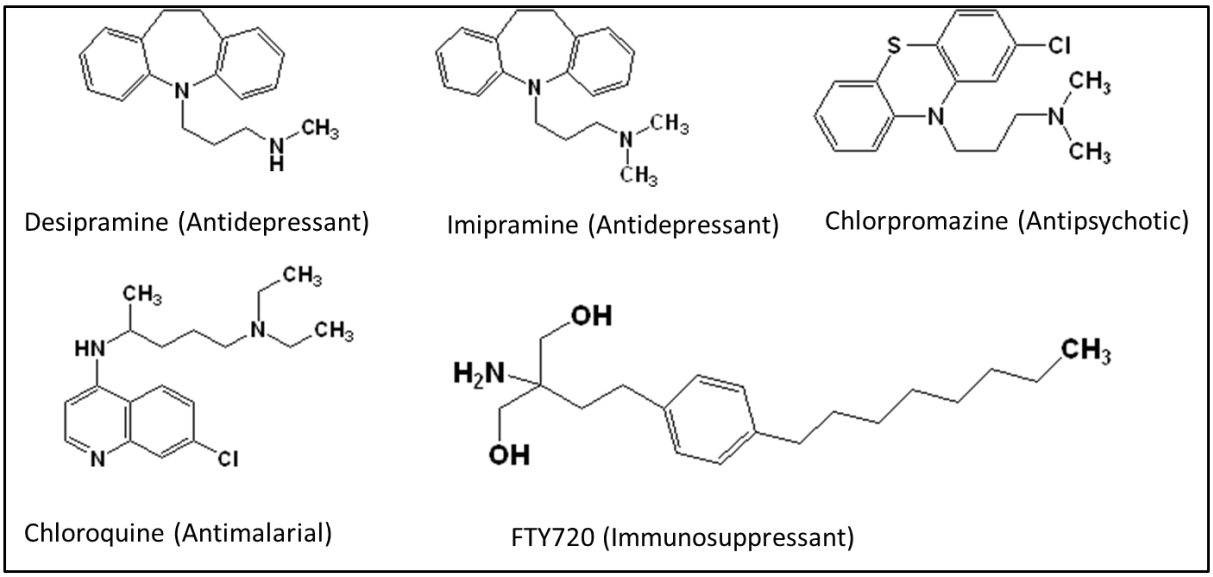

Figure 2.19: Structure of different cationic amphiphilic drugs

3. Aim and concept of the present study

The aim of this work is to characterize lipid entry pathways in cultured cells. Cultured cells of different types serve as model systems of reduced complexity for living cells in their physiological surroundings. Although lipids of different classes have been applied to different types of cultured cells before, a systematic study is missing that compares quantitative differences between different cell types towards uptake and processing of different lipids. We were interested in this question also to determine the impact of lipid delivery methods, and of different amphiphilic drugs on cellular lipid entry processes.

We investigated the capacity of four different cell types to incorporate and metabolize various lipids including complex lipids with different fatty acid moieties from the culture medium such as fatty acids (FAs), triacylglycerols (TAGs), cholesterol esters (CE), cholesterol (Chol), phosphatidylcholine (PC), and sphingomyelin (SM).

In this work, also two different lipid delivery methods have been compared. Uptake and processing of exogenous lipid probes were determined that were either added to cultured cells in complex with bovine serum albumin (BSA), or as components of low density lipoprotein (LDL) particles. Based on our results, it will be possible in the future to select the optimal cell type, delivery method, and read-out to evaluate the impact of the endolysosomal system on uptake and processing of a certain lipid.

We analyzed the role of Niemann-Pick disease, type A (NPA) on the uptake and processing of [^{14}C]cholesterol and [^{14}C]phosphatidylcholine compared to normal fibroblasts.

We also investigated the influence of representative cationic amphiphilic drugs, desipramine, imipramine, chlorpromazine, chloroquine, and FTY720 on the processing of selected lipids and their ability to induce phospholipidosis in cultured cells. For the first time, we could demonstrate a drastic and cell type-specific effect of the antidepressant desipramine on uptake and metabolism of fatty acids.

4. Results

4.1. Uptake and processing of C-18 fatty acids in cultured cells

Uptake and processing of four different FAs with C_{18} chain length were investigated. They differ in the degree of unsaturation (18:0, 18:1, 18:2, or 18:3), and were applied in concentrations of 10µM. They were added to the different types of cultured cells in complex with bovine serum albumin (BSA) (Pütz, et al. 1995). After different incubation time ranging from 15 - 120min, their cellular uptake and processing into different lipid classes were determined. All fatty acid that have been tested are readily taken up by cultured cells and incorporated into both nonpolar and polar lipid fractions. In addition, the impact of a representative CAD, desipramine, on this process was investigated.

4.1.1. Fatty acid uptake and processing

Fibroblasts, macrophages, A431 cells, and HepG2 cells were cultured as described in the materials and methods section (Chapter 6). 1-[^{14}C]FAs (stearic acid, oleic acid, linoleic acid, and linolenic acid) were delivered to cultured cells as BSA-complexes in concentrations of 10µM for 15, 30, and 120min (Table 4.1).

The FAs were added as complexes with BSA to mimic the physiological situation, to avoid possible toxic effects of the free acids, and to avoid addition of a solvent that had to be used to increase their solubility. The molar ratio between FA and albumin in the medium has been found to influence the uptake kinetics of FAs in rat hepatocytes (Sorrentino, et al. 1989). The molar FA/BSA ratio was maintained at 1:1; the physiological ratio rarely exceeds 2:1 (Spector, 1986). We used a maximal value of 2h as incubation time since elongation and desaturation rates, which are slow compared to the esterification rates, can be neglected within this time frame. For example, 80% of radiolabeled palmitic acid added to cultured hamster fibroblasts underwent no desaturation or elongation within 2h incubation time (Maziere, et al. 1982). 80-90% of the FAs metabolically incorporated into phosphoglycerides had not been altered in chick embryo fibroblasts (Daniel, et al. 1980) and human fibroblasts (Spector, et al. 1979). In our experiments, the cell viability in the presence of FA showed no significant indications of decreased cell integrity and amount of cellular protein.

4.1.2. Differences between fatty acids uptake of different structure

Stearic acid (SA), oleic acid (OA), linoleic acid (LA) and linolenic acid (LOA) were added to four different types of cultured cells, human fibroblasts, macrophages, A431 cells, and HepG2 cells. The different FAs were taken up to a similar degree by macrophages, fibroblasts, and HepG2 cells. In A431 cells, however, uptake of LA and OA was higher than of SA and LOA. From the FAs present in the medium, (1-15) % were taken up after 15min, and (5-44) % after 120min incubation time by the four cell types.

In A431 cells and HepG2 cells, when cultured with FAs for 120min incubation time, the lowest amount was observed for SA, and the highest for LA and LOA, respectively. The highest amounts were observed for OA, and the lowest for SA and LOA in macrophages and fibroblasts, respectively (Table 4.1).

4.1.3. Differences between cell types

FA uptake differed between the investigated cell types: the average uptake of FA during 15, 30, and 120min incubation time is shown in Table 4.1. In HepG2 cells, uptake of LOA was highest, followed by that of LA. Compared to the other cells types (fibroblasts, macrophages, and HepG2 cells), uptake of the saturated and unsaturated FAs from the medium was highest by A431 cells (123 *p*mol/mg protein for SA, and 154 *p*mol/mg protein for LA), followed by fibroblasts. In the other cell types, uptake was almost in the same range. No significant differences were noted in cellular FA uptake among 18:0, 18:1, 18:2, and 18:3 in all investigated cell types.

Hence, uptake of 1-[^{14}C]FAs depends both on the investigated cell type and the identity of the FAs. Uptake was not linear with time over 120min (Fig. 4.1, Fig. 4.2), but appears to follow saturation kinetics. The results are in agreement with a report on the uptake of SA, OA, LA, and LOA by HepG2 cells (Dokko, et al. 1998), when 0.25-1.0mM of FA-albumin complexes with a molar ratio of 4:1 during a 4h incubation period were applied.

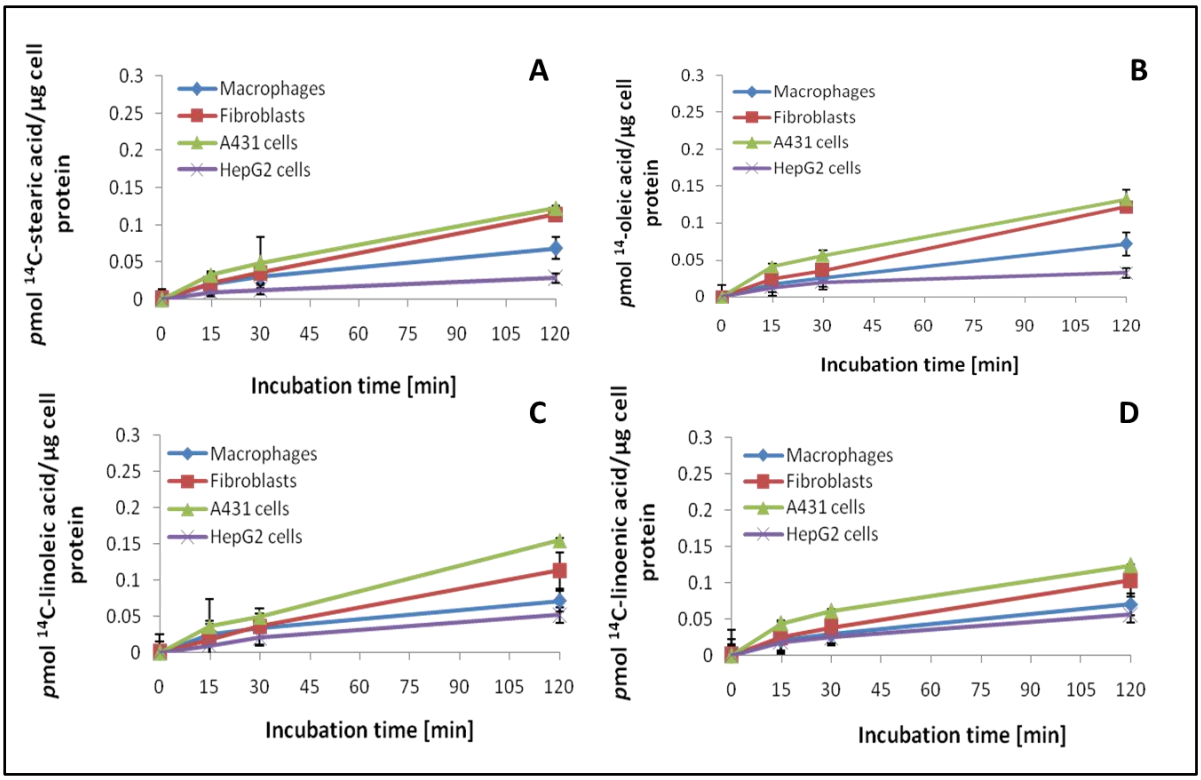

Figure 4.1: Uptake of FA (10µM) delivered in complex with BSA (1:1) by cultured cells. A. stearic acid, B. oleic acid, C. linoleic acid, and D. linolenic acid. The uptakes were measured 15, 30, and 120min after addition of the FA-BSA complexes. These diagrams were determined by scintillation counting of the lipid extract after different incubation time.

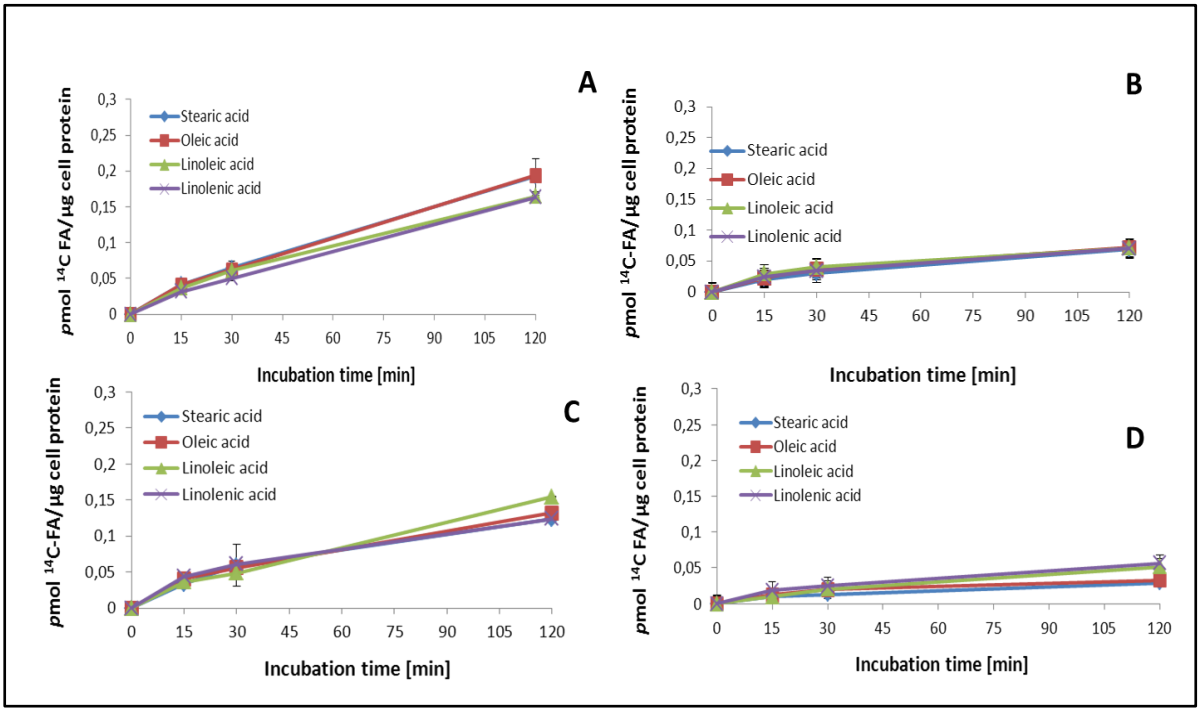

Figure 4.2: Uptake of different FA by A. fibroblasts, B. macrophages, C. A431 cells, D. HepG2 cells. These diagrams were determined by scintillation counting of the lipid extract after different incubation time.

4.1.4. Utilization of fatty acids

Incorporation of radiolabeled FAs into different lipid classes was investigated after lipid extraction and separation of the polar and nonpolar lipid fractions (Chapter 6). Lipid classes were separated by thin layer chromatography (TLC; Chapter 6). The incorporation of FAs into lipid classes after 120min incubation time of four cell types is shown in table 4.2. The incorporation of FAs into the various lipid classes differed considerably among the cell types, and with the identity of the FA added to the cells.

Table 4.1: FA uptake by four different types of cultured cells.

Cellular lipid uptake (*p*mol/ mg of protein)	**Fibroblasts**			**Macrophages**			**A431 cells**			**HepG2 cells**		
	15min	30min	120min	15min	30min	120min	15min	30min	120min	15min	30min	120min
Stearic acid	**22.1**	**35.7**	**114**	**20.3**	**30.3**	**69.1**	**33.1**	**49.1**	**123**	**9.8**	**13.0**	**28.7**
Oleic acid	**23.6**	**35.3**	**122**	**16.6**	**25.1**	**71.5**	**40.7**	**56.2**	**132**	**12.5**	**19.6**	**32.4**
Linoleic acid	**18.1**	**36.0**	**113**	**24.2**	**33.1**	**70.9**	**36.3**	**48.8**	**154**	**9.5**	**20.5**	**51.6**
Linolenic acid	**24.8**	**38.5**	**103**	**20.4**	**28.1**	**70.2**	**43.6**	**61.1**	**124**	**18.5**	**24.7**	**56.1**

Table 4.2: Metabolic incorporation of exogenously added FAs into lipid classes.

Incorporation of FA into cellular lipids (*p*mol /mg of protein) after 2h incubation	**Fibroblasts**				**Macrophages**				**A431 cells**				**HepG2 cells**			
	ST	**OA**	**LA**	**LOA**	**ST**	**OA**	**LA**	**LOA**	**ST**	**OA**	**LA**	**LOA**	**ST**	**OA**	**LA**	**LOA**
Cholesterol ester	**0.75**	**0.39**	**1.43**	**3.07**	**6.0**	**2.3**	**0.1**	**0.1**	**2.1**	**1.9**	**2.7**	**2.4**	**0.2**	**0.2**	**0.1**	**0.4**
TAG	**2.17**	**0.96**	**1.25**	**2.75**	**20.0**	**8.6**	**0.1**	**0.1**	**7.7**	**9.6**	**7.0**	**3.8**	**2.2**	**2.6**	**4.4**	**8.8**
FA	**12.8**	**2.48**	**3.89**	**10.4**	**9.8**	**6.8**	**0.5**	**0.6**	**13.4**	**9.6**	**7.0**	**6.3**	**5.1**	**5.5**	**5.5**	**11.4**
DAG	**4.05**	**8.42**	**7.13**	**7.23**	**5.6**	**3.3**	**0.6**	**0.8**	**3.6**	**12.9**	**24.6**	**24.1**	**1.4**	**2.5**	**3.1**	**4.0**
Cer	**1.71**	**1.15**	**1.17**	**1.41**	**1.0**	**0.4**	**0.2**	**0.3**	**0.9**	**0.7**	**1.5**	**2.6**	**0.2**	**0.2**	**0.3**	**0.4**
Glccer	**0.77**	**2.19**	**1.20**	**1.15**	**0.1**	**0.9**	**0.3**	**0.3**	**0.6**	**2.5**	**2.8**	**3.8**	**0.1**	**0.6**	**0.6**	**0.6**
PE, BMP	**11.4**	**5.88**	**8.24**	**12.1**	**3.9**	**3.8**	**8.9**	**19.1**	**6.1**	**5.8**	**6.2**	**4.8**	**4.7**	**3.8**	**4.2**	**6.2**
PG	**8.96**	**22.9**	**4.44**	**4.03**	**0.9**	**1.9**	**2.3**	**3.1**	**20.8**	**28.7**	**10.7**	**9.9**	**1.4**	**3.1**	**2.4**	**2.4**
PC, PA	**38.7**	**44.8**	**55.4**	**45.2**	**10.8**	**17.3**	**29.8**	**18.9**	**22.3**	**33.2**	**49.9**	**29.9**	**4.9**	**5.5**	**15.9**	**15.1**
PI	**7.41**	**5.18**	**7.50**	**7.15**	**6.4**	**4.7**	**9.4**	**12.8**	**10.9**	**7.3**	**15.3**	**7.2**	**2.8**	**1.4**	**2.6**	**2.5**
SM	**10.7**	**6.98**	**5.28**	**3.97**	**3.4**	**3.5**	**4.1**	**4.2**	**9.2**	**9.3**	**12.7**	**9.9**	**1.6**	**0.9**	**2.6**	**1.9**
Sum	**99.5**	**101**	**96.9**	**98.6**	**68.2**	**59.5**	**56.3**	**60.7**	**98.2**	**121**	**140**	**109**	**25.2**	**26**	**41.3**	**54.0**

Fibroblasts, macrophages, A431 cells, and HepG2 cells were cultured as described in the materials and methods section in serum-free medium supplemented with 10µM [^{14}C]-FAs (added as a BSA-FA complex; the BSA/FA ratio was 1:1). After different incubation time (15, 30, and 120min), cells were harvested, and lipids were extracted. FAs incorporated into nonpolar and polar lipids were determined as *p*mol of lipid per mg of protein.

4.1.5. FA processing in macrophages

When macrophages were cultured in the presence of the FAs, a major incorporation of FAs into TAG and CE was observed. 1-[^{14}C]SA and 1-[^{14}C]OA were predominantly incorporated into TAG, DAG and CE. On the other hand, the incorporation of 1-[^{14}C]LA and 1-[^{14}C]LOA into TAG, DAG and CE was significantly lower when compared to SA and OA (Fig. 4.3B). The major FAs incorporated in the nonpolar lipid fraction of macrophages were OA and SA; they comprised 44 % and 60 %, respectively, of the total FAs that where incorporated into the nonpolar lipid fractions (TAG, DAG, and CE), or were found as free FAs.

In the same cell types, LA and LOA (Fig. 4.4B) were preferentially incorporated into polar lipids (PLs). OA was only modestly incorporated into polar lipids, and SA showed the lowest incorporation into polar lipids compared to the other FAs.

In the PL fraction of macrophages cultured in the presence of FA-BSA complexes, the major incorporated FAs were LA and LOA. They each comprised 94.1% and 94.4%, respectively, of the total FAs incorporated into PLs and sphingolipids (Table 4.2). FAs are selectively incorporated into polar lipids depending on the degree of unsaturation (SA<OA<LA<LOA). The results show that in macrophages, more unsaturated FAs are predominantly incorporated into PLs, while more saturated FAs are incorporated more into LD lipids.

4.1.6. FA processing in HepG2 cells

The liver is the key organ in lipid metabolism. Therefore, we investigated uptake and metabolism of FAs also in HepG2 cells, which are widely used as a model system for hepatic lipid metabolism. These cells are human hepatoma cells and known to exert a number of human liver functions, including lipid uptake, synthesis, VLDL assembly and VLDL secretion (Lin, et al. 1995).

When incubated with different FAs in medium, the incorporation of 1-[^{14}C]FA into TAG and DAG increased with incubation time. Delivery of SA, OA, LA, and LOA as BSA complex resulted in incorporation of 2.2, 2.6, 4.4, and 8.8 *p*mol/mg protein into TAG after 120min incubation (Fig. 4.3D), respectively. Furthermore, when HepG2 cells were incubated with radiolabeled C_{18}-FAs of different structure, the level of FAs incorporated into polar lipids was much greater for unsaturated FAs than for saturated FAs. Both LA and LOA were markedly incorporated into polar lipids of HepG2 cells. Delivery of OA, LA, and LOA as BSA complex resulted in incorporation of 15.6, 28.8, and 29.3 *p*mol/mg protein into PLs

after 120min incubation (Fig. 4.4D), respectively. We observed a positive relationship between the number of double bonds and the incorporation both into nonpolar and polar lipids.

The results show that in HepG2 cells, the incorporation of FAs into cellular lipids is increased with increasing number of double bonds. The uptake values also show (table 4.1) that more unsaturated FAs are taken up compared to saturated FAs.

4.1.7. FAs processing in fibroblasts

Human fibroblasts readily take up FAs and incorporate them into cellular polar and nonpolar lipids (Spector, 1986; Rosenthal, et al. 1981). When human skin fibroblasts were cultured in the presence of FAs, only a minor incorporation of FAs into nonpolar lipids was observed compared to polar lipids. Within the nonpolar lipid fraction in fibroblasts (Fig. 4.3A), but also in A431 cells (Fig. 4.3C), we observed the predominant incorporation into DAG after 120min incubation. An exception was SA in A431 cells, where a predominant incorporation into TAG was detected after 120min incubation. The major FAs incorporated into the nonpolar lipid fraction of fibroblasts was LOA, which comprised 24% of the total LOA that where incorporated into the nonpolar lipid fractions (TAG, DAG, and CE), or were recovered as free LOA.

In the PL fraction of fibroblasts (Fig. 4.4A), the major incorporated FA was OA. It comprised 87% of the total OA incorporated into PLs and sphingolipids (ceramide, glucosylceramide, and sphingomyelin). From this value, 50% and 25% of OA incorporated into the polar lipids were incorporated into PC and PG, respectively.

The results show that in fibroblasts, the incorporation of SA, OA, LA, and LOA into nonpolar lipids occurred predominantly into DAG, and the incorporation of OA into PG is increased compared with other FAs.

4.1.8. FA processing in A431 cells

After 120min incubation, incorporation of OA, LA, and LOA into nonpolar lipids of A431 cells occurred predominantly into DAG, while SA was more incorporated into TAG than DAG (Fig. 4.3C). Within the polar lipid fraction, these FAs have been preferentially incorporated into PC (Fig. 4.4C). LA was more incorporated into the polar lipids than the other FAs, it comprised 72%, followed by OA which comprised 71% of total FAs

incorporated into the polar lipids. Moreover, OA and SA were more incorporated into PG than LA and LOA.

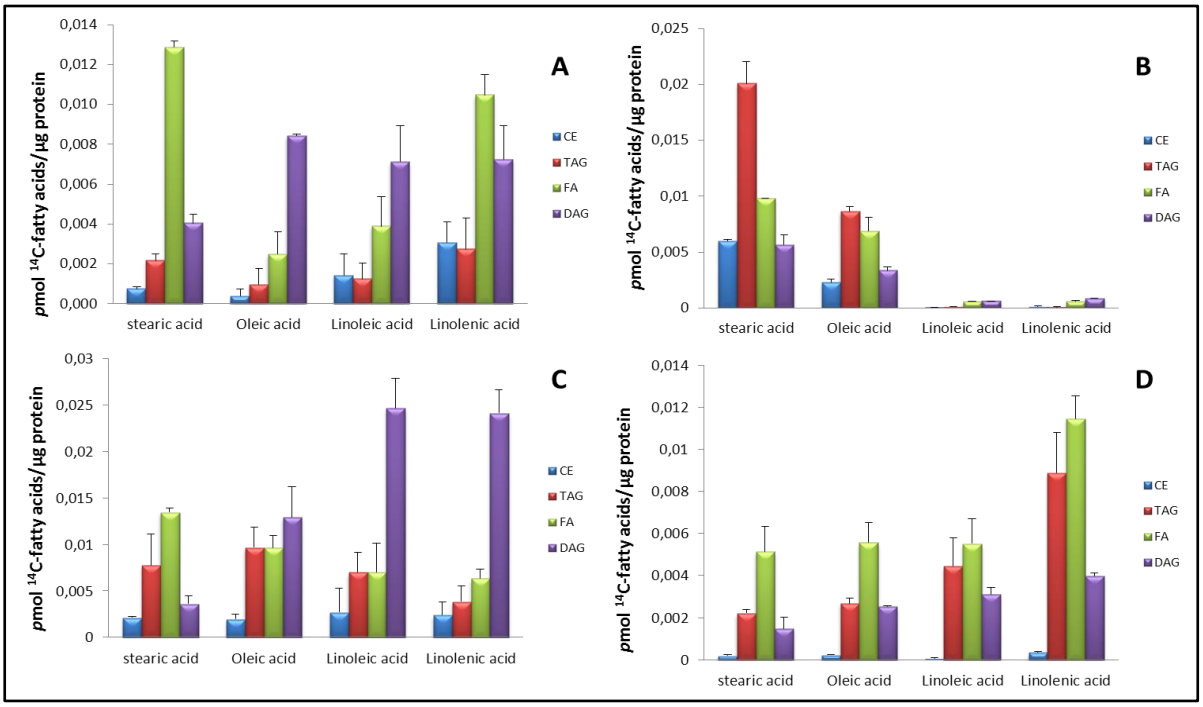

Figure 4.3: Incorporation of FAs into cellular nonpolar lipids after incubation of the cells with 10µM 1-[^{14}C]-FAs delivered as BSA complex in a 1:1 molar ratio for 120min in the culture medium, A. fibroblasts, B. macrophages, C. A431 cells, and D. HepG2 cells. Separation of lipid classes was achieved by using the solvent system (nHexane/Diethylether/Acetic acid 70:30:1 by vol.). Radioactivity found in the lipid extract of different cells was set equal 100%. Radioactivity was analyzed by phosphoimager analysis. All data are given as mean values of more than two different experiments.

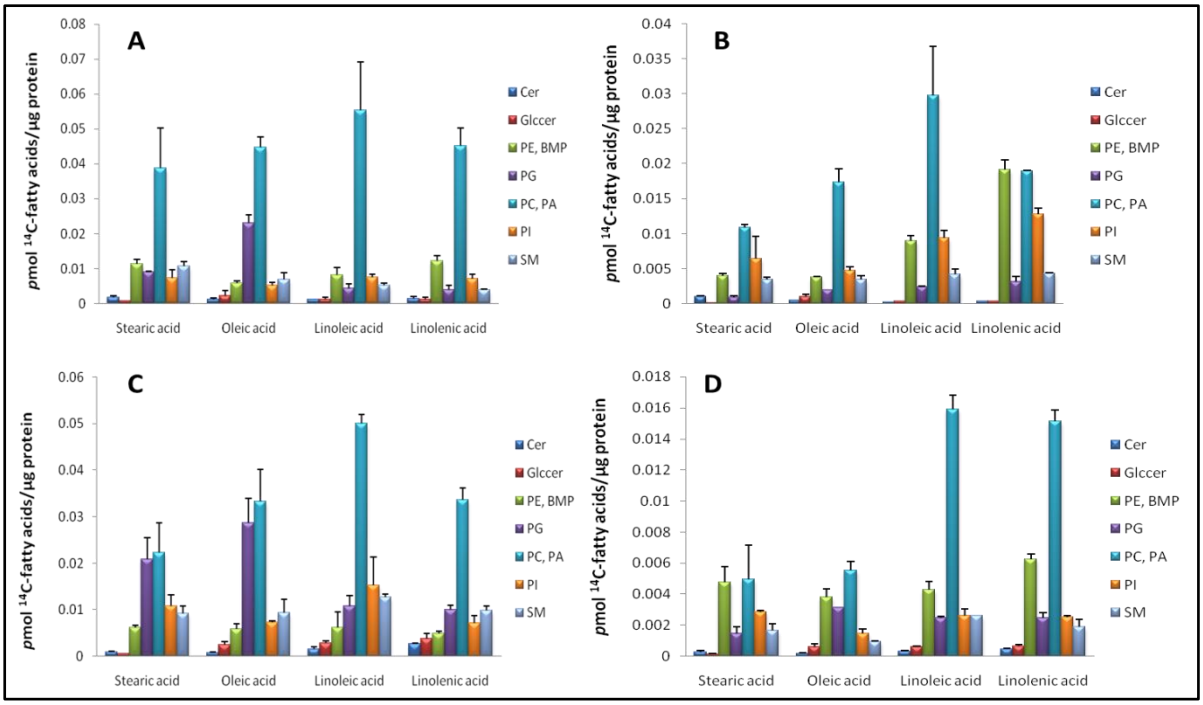

Figure 4.4: Incorporation of FAs into cellular polar lipids after incubation of the cells with 10µM 1-[^{14}C]-FAs delivered as BSA complex in a 1:1 molar ratio for 120min in the culture medium, A. fibroblasts, B. macrophages, C. A431 cells, and D. HepG2 cells. Separation of lipid classes was achieved by using the solvent system (chloroform/methanol/water 65:25:4 by vol.). Radioactivity found in the lipid extract of different cells was set equal 100 %. Radioactivity was analyzed by phosphoimager analysis. All data are given as mean values of more than two different experiments.

4.1.9. FA processing in different cell types

Fig. 4.5 shows the incorporation of FAs in cellular PLs, nonpolar lipids, and the recovery of free FAs in *p*mol/µg protein.

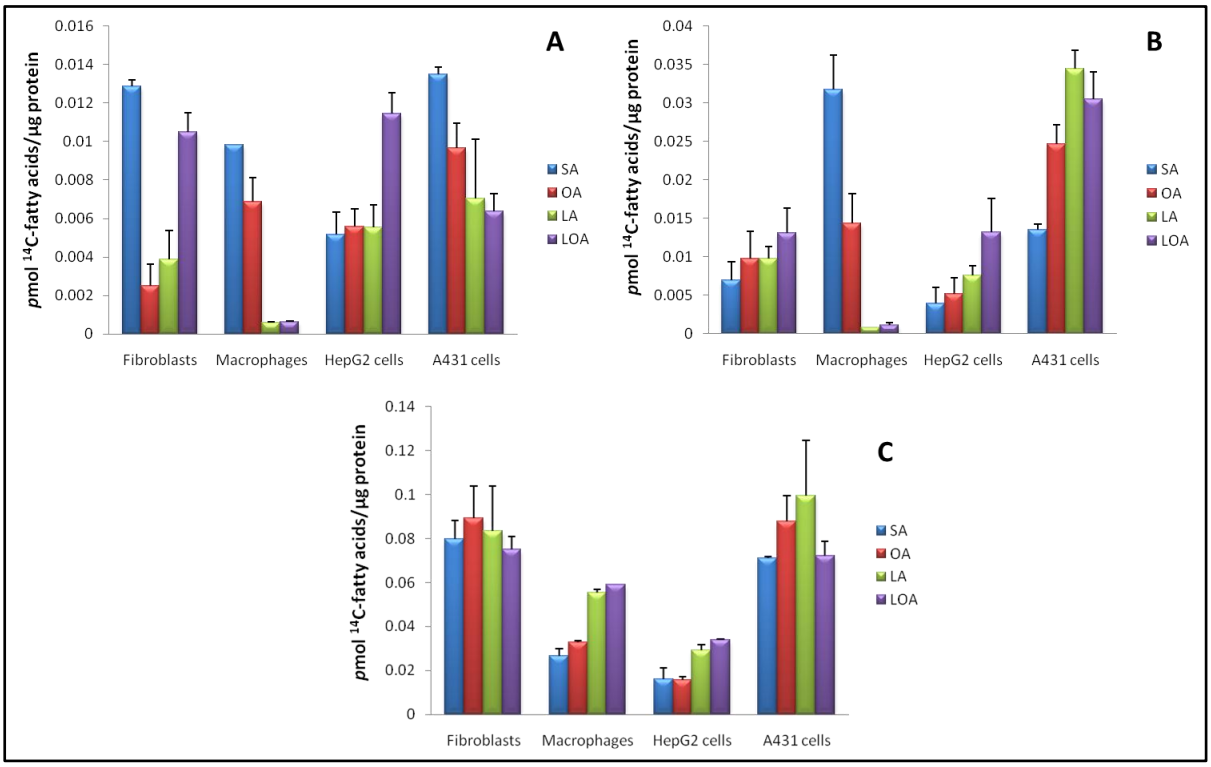

Figure 4.5: Incorporation and/or recovery of 1-[^{14}C] 18:0, 18:1n-9, 18:2n-6, and 18:3n-3 FA into cellular lipid classes given as *p*mol ^{14}C-FAs/µg protein. Free FA (A), nonpolar lipids (B), and polar lipids (C). Nonpolar lipids include diacylglycerol, triacylglycerol and sterol ester. Polar lipids includes ceramide, glucosylceramide, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, sphingomyelin, bis(monoacylglycero)phosphate, and phosphatidic acid.

In fibroblasts and A431 cells, all FAs were mainly esterified to polar lipids (0.07 - 0.1 *p*mol/µg protein), whereas (0.007 - 0.034 *p*mol/µg protein) was incorporated into the neutral lipid fraction, and (0.0025 - 0.013 *p*mol/µg protein) were recovered as free FAs.

In A431 cells, LA was mainly incorporated into cellular nonpolar lipids, followed by LOA, OA, and SA. The incorporation into polar lipids was not significantly different between the FAs, ranging from 0.07 - 0.1 *p*mol/µg protein.

In fibroblasts, the recovery of 18:0 as FFA was significantly higher than other FAs, followed by LOA, LA, and OA. The incorporation into neutral and polar lipids was not significantly different between the FAs.

In macrophages, SA and OA, but not LA and LOA were mainly esterified into nonpolar lipids (> 0.014 *p*mol/µg protein), whereas in polar lipids, LA and LOA, but not SA and OA were mainly incorporated (0.055 *p*mol/µg protein), and the lowest amounts were recovered as free FA.

In HepG2 cells, LOA was mainly incorporated into both, cellular neutral and polar lipids, followed by LA, OA, and SA. This is in agreement with a previous report in *atlantic salmon hepatocytes* (Stubhaug, et al. 2005) where the main proportion of [1-^{14}C]FAs taken up was incorporated into cellular lipids in order 16:0<18:1<18:2<18:3.

4.1.10. Pulse-chase studies

In order to compare fate of a FA after their initial incorporation into different lipids, four types of cultured cells were pulsed with medium containing 10µM of BSA-[^{14}C]oleic acid at 37 ^{0}C and 4 ^{0}C. After 2h incubation time, radiolabeled oleic acid was removed, followed by incubation with medium containing 10% FCS (chase) for a variable period of time (24, 48, 72, 122, and 144h) at 37 ^{0}C, as described in chapter 6.

In macrophages, the pulse–chase experiments showed that, after 24h, radioactivity was largely confined to TAG. By the end of the chase period, substantially more radiolabeled oleic acid accumulated in TAG compared to the other lipids (Fig. 4.6B). In the same cell type (Fig. 4.7B), more labeled FA was incorporated into PG after 72h. At 4 ^{0}C, the labeled oleic acid was much less incorporated into TAG after 24h than at 37 ^{0}C (data not shown).

When cultured fibroblasts were incubated in the presence of 10µM of BSA-[^{14}C]oleic acid at 37 ^{0}C for 2h, in the nonpolar lipid fractions, labeled oleate was preferentially incorporated into DAG (Fig. 4.6A), and most of the oleate incorporated into phospholipids was in PC and PG (Fig. 4.7A). During the chase period, more of the labeled oleate was found in TAG instead of DAG, with relatively little accumulation of free FA after 48h incubation. In polar lipids, there was incorporation of labeled oleate mainly in PC, PA, and PG using 2h pulse. In the chase experiment, the labeled oleate in PC and PA decreased after 24h, with an increase of label in PG, GlcCer, and PI. At 4 ^{0}C, free FA was significantly decreased after 2h incubation with increased incorporation in TAG (data not shown).

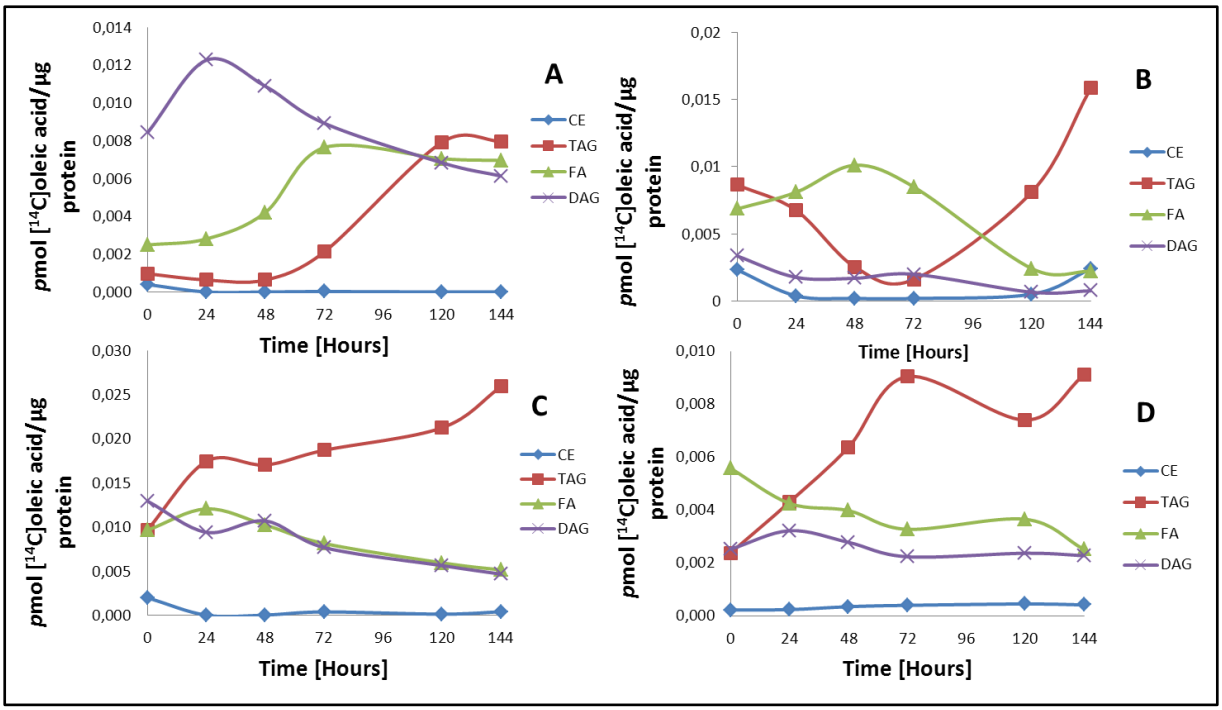

Figure 4.6: Pulse-chase experiments with [^{14}C]oleic acid at 37 ^{0}C . Incorporation of [^{14}C]oleic acid into different nonpolar lipids after different chase times are shown A. fibroblasts; B. macrophages; C. A431 cells; and D. HepG2 cells. Cells were either collected for lipid extraction after 2h labeling (pulse), or washed and incubated without labeled OA in medium containing 10% FCS for different incubation time (chase).

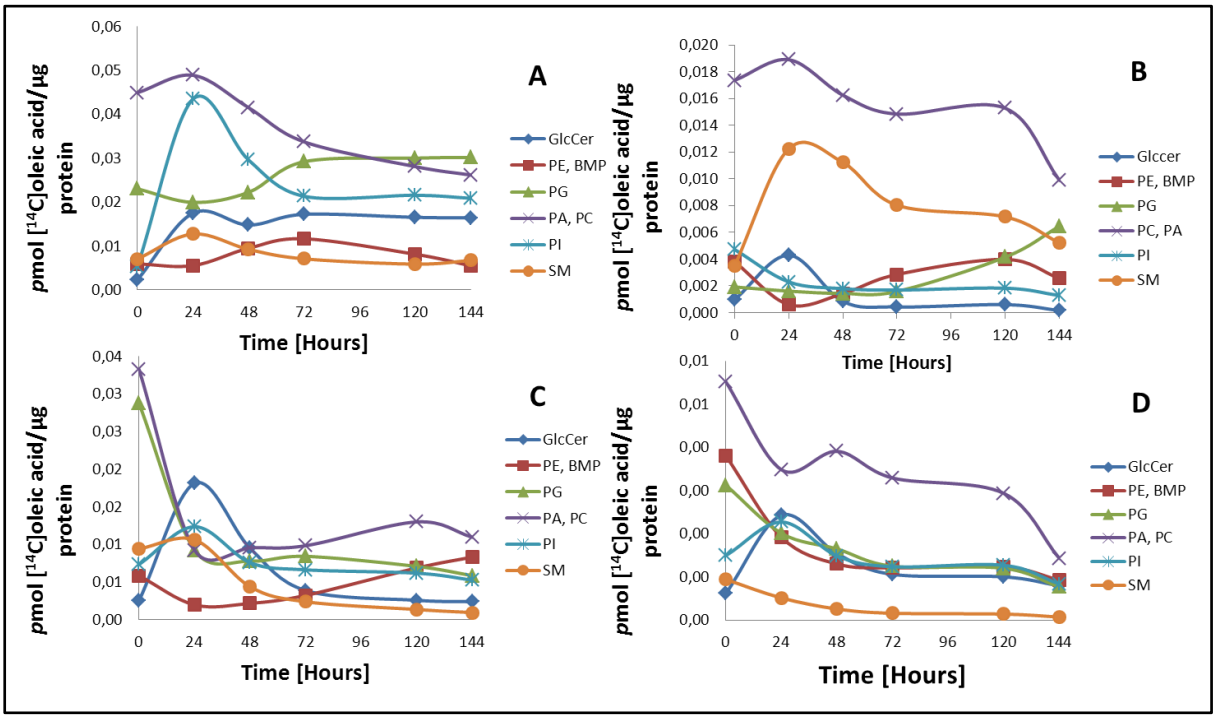

Figure 4.7: Pulse-chase experiments with [^{14}C]oleic acid at 37^{0}C . Incorporation of [^{14}C]oleic acid into different polar lipids after different chase times are shown A. fibroblasts; B. macrophages; C. A431 cells; and D. HepG2 cells. Cells were either collected for lipid extraction after 2h labeling (pulse), or washed and incubated without labeled OA in medium containing 10% FCS for different incubation time (chase).

In A431 cells, the [^{14}C]oleic acid was incorporated predominantly into phospholipids. During the chase period, the labeled oleate in PC, PA, and PG decreased after 24h, with an increase of lable PE, GlcCer, and PI. However, after 24h chase, the amount of radioactivity in the DAG fraction decreased. By the end of the chase period, substantially more radiolabeled oleic acid accumulated into TAG compared to the other lipids (Fig. 4.6C). At 4 ^{0}C, free FA was significantly decreased after 24h incubation with increased incorporation of TAG (data not shown).

In HepG2 cells, the incorporation of [^{14}C]oleate into TAG increased during the chase period (Fig. 4.6D). The incorporation of labeled oleate into polar lipids increased during the 24h chase in PI and GlcCer (Fig. 4.7D). Incorporation of label oleate into TAG during the 24h chase was accompanied by concomitant decrease in radioactivity in the phospholipid.

In summary, most FAs were initially incorporated in polar lipids, presumably via Lands' cycle. After longer chase time, they end up on the TAGs.

4.1.11. The effect of cationic amphiphilic drug, desipramine on FAs uptake and processing

CADs are known to interfere with the processing of complex lipids within the endolysosomal system. There are no data on the impact of CADs on FA processing in the literature. Although it is not expected that endosomes and lysosomes are involved in FA uptake and metabolism, we investigated the impact of the tricyclic antidepressant, desipramine, on uptake and processing of SA, OA, LA, and LOA. Desipramine is known to cause phospholipidosis in humans and animals by inducing premature degradation of acid sphingomyelinase.

Surprisingly, in the presence of 10μM and 20μM desipramine in the medium, the incorporation of 1-[^{14}C]FA into TAGs was drastically reduced in cultured A431 cells (Fig. 4.8) and macrophages (Fig 4.9), and slightly in HepG2 cells (data not shown). The small effect on processing of FAs in HepG2 cells may be attributed to the low uptake of desipramine (Fig. 4.13). In A431 cells, the incorporation of SA, OA, LA, and LOA into TAG was decreased by 97, 83, 94, and 93% when treated with 20μM desipramine, respectively. In macrophages, the effects of desipramine are less than in A431 cells. It decreases the incorporation of SA, OA, LA, and LOA into TAG by 77, 73, 66, and 45%, respectively. Desipramine also affects the incorporation of FAs into DAG, but to a lesser extent. In A431 cells, the incorporation of SA, OA, LA, and LOA into DAG was decreased 74, 29, 49, and

63%, when treated with 20µM desipramine, respectively. In macrophages, the incorporation into DAG was decreased by only 17, 19, 42, and 44%, respectively.

In contrast to the other cell types, the incorporation of 1-[^{14}C]FA into TAGs was drastically increased in cultured fibroblasts treated with 10µM and 20µM desipramine (Fig. 4.10). The incorporation of SA, OA, LA, and LOA into TAG was increased by 83, 80, 87, and 88% in the presence of 20µM desipramine, respectively. Furthermore, the incorporation into DAG also increased by 65, 77, 73, and 67% in presence of 20µM desipramine, respectively.

In contrast to the nonpolar lipid fractions of A431 cells, in the polar lipid fractions, incorporation of linoleic acid was drastically increased into PC, PE, and PG in the presence of 10µM and 20µM desipramine in the medium (Fig. 4.11). The other fatty acids were only slightly incorporated into polar lipids of A431 cells.

In fibroblasts, incorporation of all FAs into PI and ceramide was significantly increased in the presence of desipramine (Fig. 4.12). In macrophages, the incorporation of SA and OA into PC, PG, and SM was increased slightly in the presence of 10µM and 20µM desipramine, but the incorporation into PE was not affected. Furthermore, in the same cell type, the incorporation of LA and LOA into PC, PG, PE, and SM was not affected (data not shown).

The cell viability in the presence of 10µM and 20µM desipramine was not affected and ranged from 88 to 100% of untreated cells (Fig. 4.38). Therefore, the reduced incorporation of FAs into TAG and DAG in the presence of desipramine in A431 cells and macrophages cannot be attributed to toxicity.

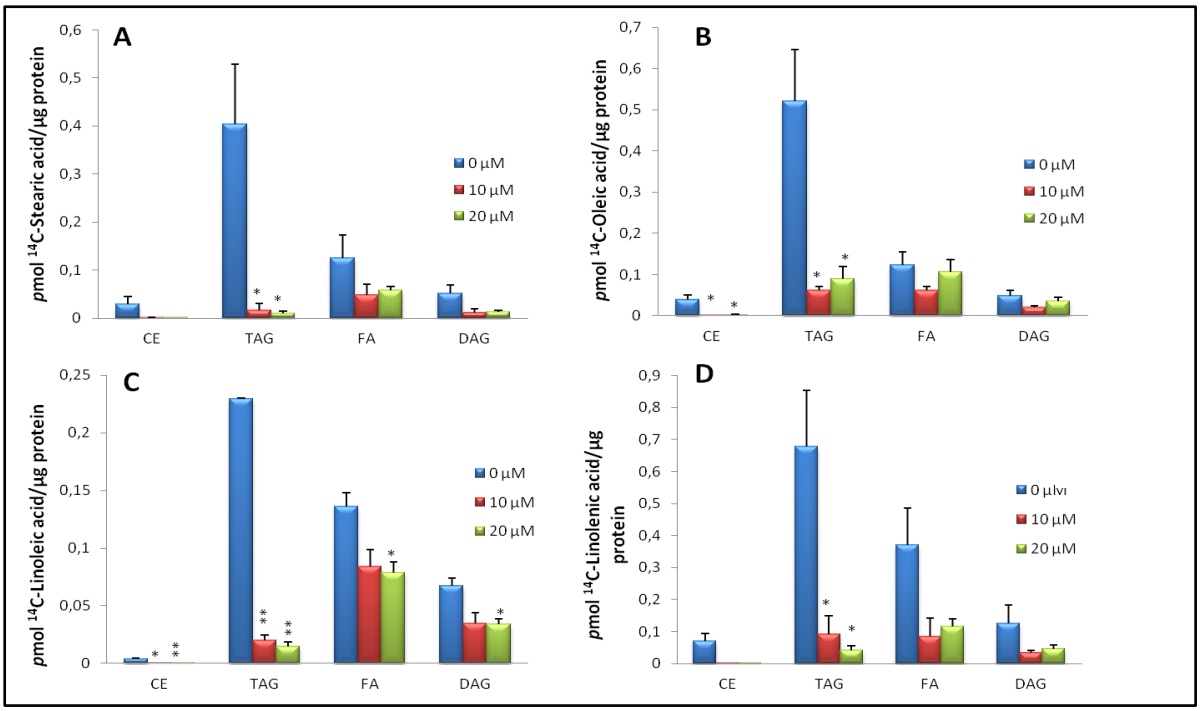

Figure 4.8: Incorporation of A. [^{14}C]stearic acid, B. [^{14}C]oleic acid, C. [^{14}C]linoleic acid, and D. [^{14}C]linolenic acid into nonpolar lipids of A431 cells in the absence and presence of two different concentrations of desipramine. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

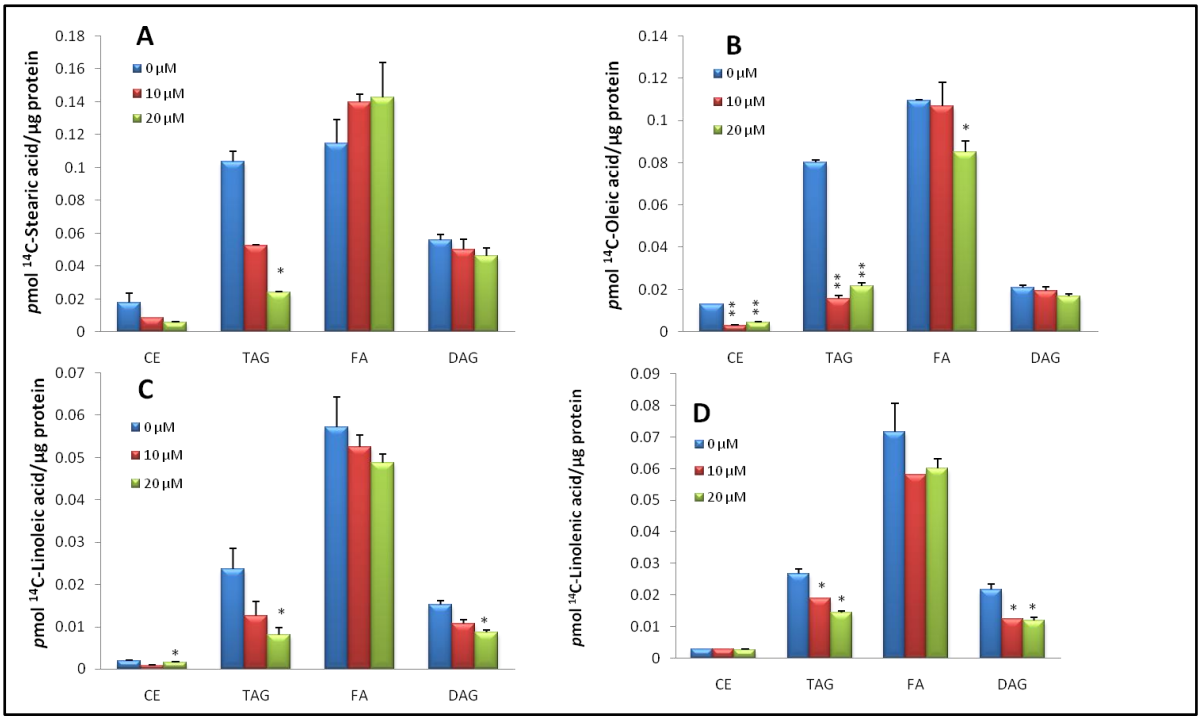

Figure 4.9: Incorporation of A. [^{14}C]stearic acid, B. [^{14}C]oleic acid, C. [^{14}C]linoleic acid, and D. [^{14}C]linolenic acid into nonpolar lipids of macrophages in the absence and presence of two different concentrations of desipramine. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

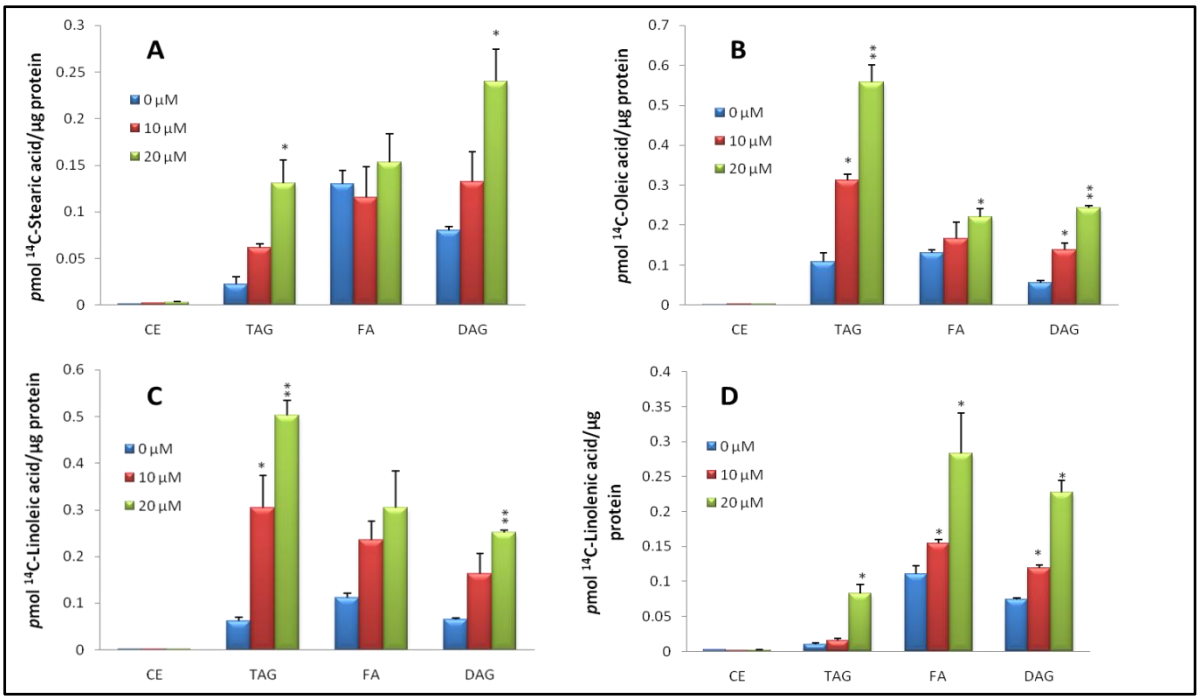

Figure 4.10: Incorporation of . [^{14}C]stearic acid, B. [^{14}C]oleic acid, C. [^{14}C]linoleic acid, and D. [^{14}C]linolenic acid into nonpolar lipids of fibroblasts in the absence and presence of two different concentrations of desipramine. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

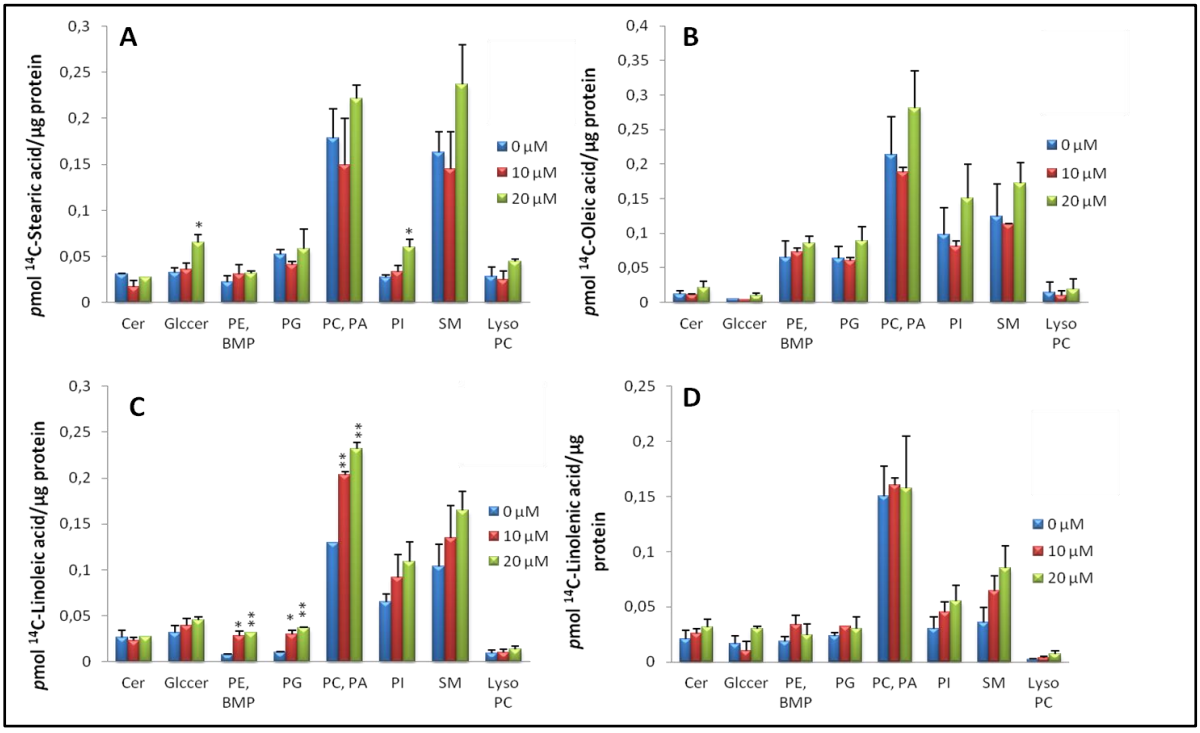

Figure 4.11: Incorporation of A. [^{14}C]stearic acid, B. [^{14}C]oleic acid, C. [^{14}C]linoleic acid, and D. [^{14}C]linolenic acid into polar lipids of A431 cells in the absence and presence of two different concentrations of desipramine. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

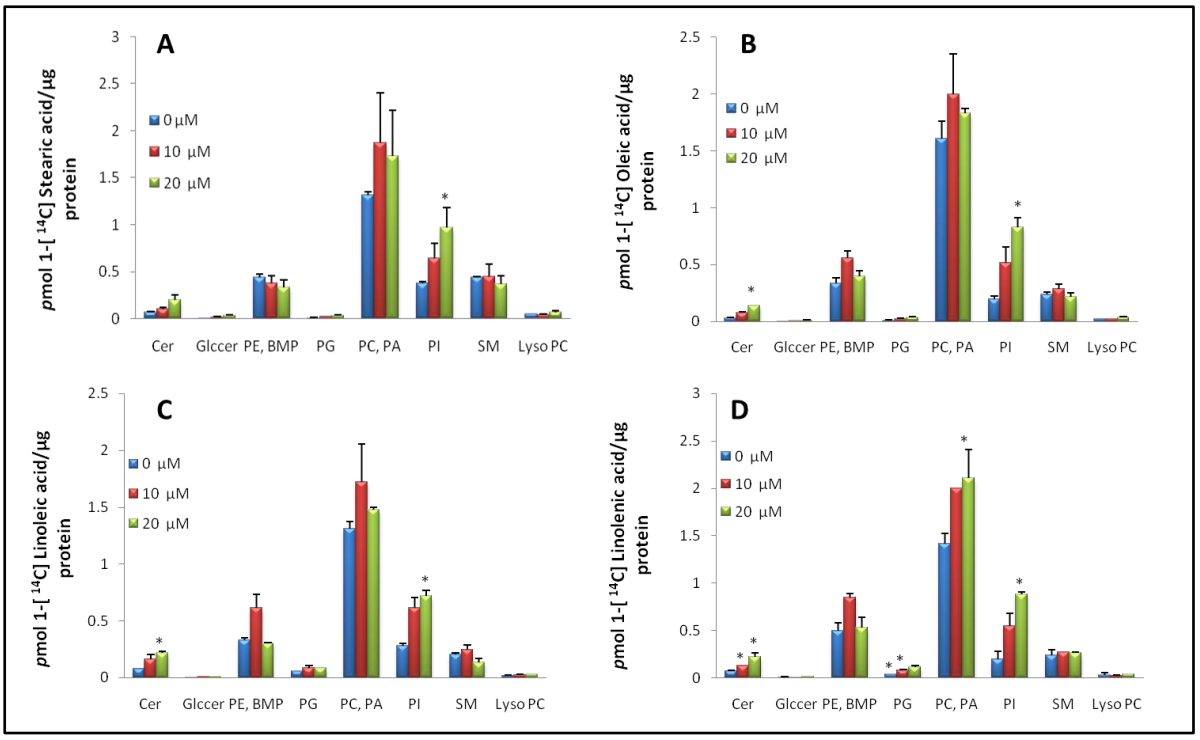

Figure 4.12: Incorporation of A. [^{14}C]stearic acid, B. [^{14}C]oleic acid, C. [^{14}C]linoleic acid, and D. [^{14}C]linolenic acid into polar lipids of fibroblasts in the absence and presence of two different concentrations of desipramine. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

Figure 4.13 shows the uptake of [^{3}H]-desipramine into four different cell types after 48h incubation time. The uptake of [^{3}H]-desipramine by A431 cells and HepG2 cells was slightly decreased, but only with 40µM desipramine. Uptake of [^{3}H]-desipramine into macrophages was slightly increased. In contrast, the uptake was significantly decreased in the presence of 20µM and 40µM desipramine by fibroblasts. The cell viability in the presence of 40µM desipramine was reduced 28% compared to untreated cells (Fig. 4.38); while the amount of cellular protein was reduced by 8%. Therefore, the reduced incorporation of FAs into TAG and DAG in the presence of desipramine in macrophages and A431 cells cannot be attributed only to toxicity.

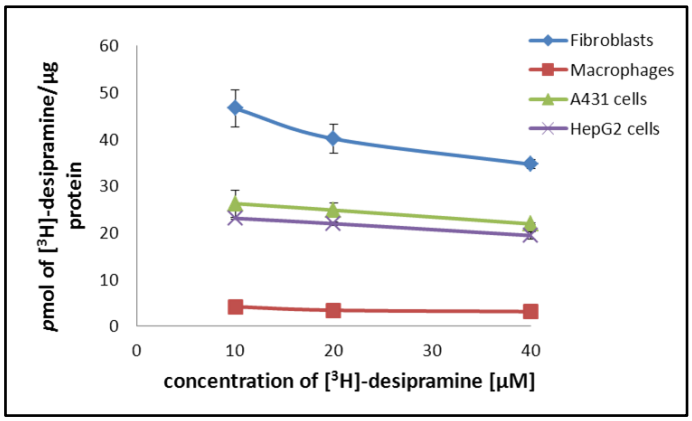

Figure 4.13: Uptake of [^{3}H]-desipramine into different cultured cells

4.2. Analysis of two lipid delivery methods towards lipid uptake and processing

As probes for lipid uptake and processing via the endolysosomal system, suitably labeled complex lipids can be added to cells. Lipids like cholesterol, cholesterol ester, and triacylglycerol are nearly insoluble in blood. In the circulatory system, they are transported by lipoproteins, and are also found in complex with albumins. Therefore, we used albumin and low density lipoprotein as carriers for TAG, CE, and cholesterol in the medium of cultured cells. This should mimic the physiological situation in which these lipids enter cells via endocytosis and other pathways. TAG and CE are then degraded by lysosomal acid lipase, and the FA moiety is liberated and degraded or re-utilized for lipid biosynthesis and protein modification.

In this part, the effects of two different ways for lipid delivery, either with albumin or lipoprotein complexes, were examined in different cell types. We used cholesterol, cholesterol ester, and triacylglycerol labeled with [^{14}C]oleic acid, since oleic acid is one of the major FAs of cholesterol esters and triacylglycerol (http://lipidlibrary.aocs.org/Lipids/cholest/index.htm).

To study possible differences in uptake and metabolism in dependence of the lipid delivery method, we used [4-^{14}C]cholesterol, [1-^{14}C]cholesteryloleate, and [1-^{14}C]trioleoylglycerol in complex with LDL or BSA.

4.2.1. Cholesteryl ester uptake and processing

[1-^{14}C]Cholesteryloleate (1µM) was added to four different types of cultured cells, human fibroblasts, macrophages, A431 cells, and HepG2 cells, either as a complex with LDL, or with BSA. As shown in figure 4.14, the uptake of both complexes, [^{14}C]CE-LDL and [^{14}C]CE-BSA, by fibroblasts is higher than by the other cell types. Compared to [^{14}C]CE-BSA, the uptake of [^{14}C]CE-LDL is 4-fold higher in fibroblasts and A431 cells, 3-fold higher in macrophages, and 2-fold higher in Hep-G2 cells during an 24h incubation at 37 °C.

The incorporation of the oleate moiety derived from CE into lipids of the nonpolar lipid fraction of different cell types is illustrated in figure 4.15. There were significant differences in the incorporation of 1-[^{14}C]oleate into total neutral lipids (except [^{14}C]CE) between the types of the cells incubated with [^{14}C]CE and LDL as shown in Fig. 4.15A. After 24h incubation, the incorporation of cholesterol ester-derived [^{14}C]oleate into nonpolar lipids remained largely unchanged in fibroblasts and macrophages. In HepG2 cells and A431 cells,

CE-LDL derived 1-[^{14}C]oleate is more incorporated into TAG than in the other cell types. When compared to BSA, delivery via LDL leads to an increased formation of metabolic products derived from the fatty acid part. Also the dependence of the cell type is visible. This result indicates that the differences in metabolism of cholesteryloleate depend on the delivery method. If the function of the endolysosomal system is assayed by processing of [1-^{14}C]cholesteryloleate, it should be delivered as LDL-complex and the incorporation of labeled oleic acid into TAG, PE, and PC should be chosen as read-out.

The incorporation of [^{14}C]oleate derived from CE delivered as LDL-complex into lipid classes of the nonpolar lipid fraction in dependence of incubation time and cell type is shown in figure 4.16. In HepG2 cells, after 24h of incubation at 37 °C, the major fraction of CE-derived [^{14}C]oleate is incorporated into TAG (Fig. 4.16B) and, to a lesser extent, into phospholipids (Fig. 4.21B). A small fraction of label is recovered as non-esterified fatty acid and found in diacylglycerol. In HepG2 cells, the incorporation of the CE-derived [^{14}C]oleate into TAG was 3-fold higher than in macrophages. Furthermore, in macrophages, the re-incorporation into and/or recovery of cholesteryl[^{14}C]oleate is 2-fold higher than in HepG2 cells.

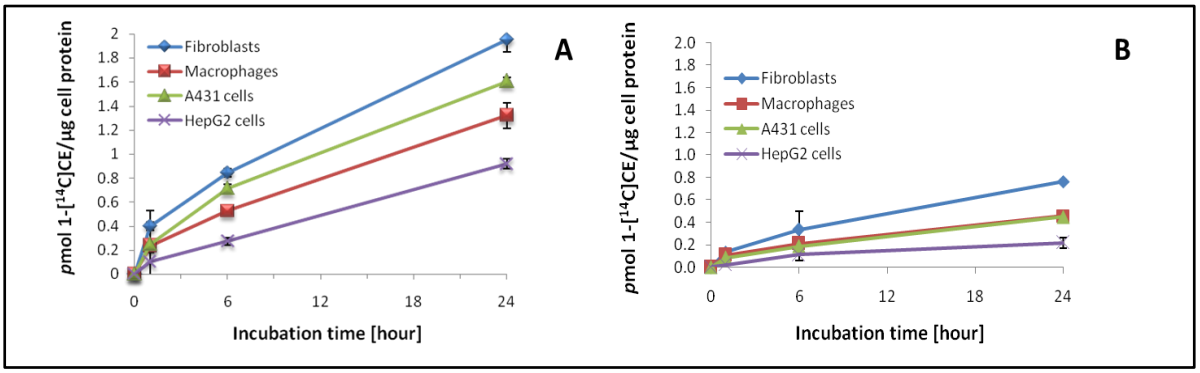

Figure 4.14: Uptake of [^{14}C]CE (1µM) delivered as complex with LDL (A) or BSA (B) by four types of cultured cells. The uptakes were measured 1, 6, and 24h after addition of the CE-complexes. Uptake was determined by scintillation counting of the lipid extract after different incubation time.

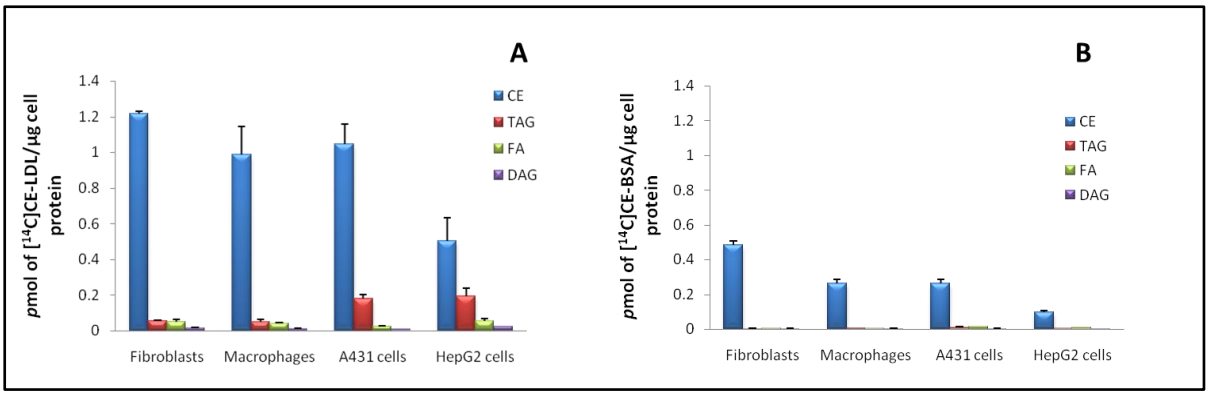

Figure 4.15: A. Incorporation of CE-derived [^{14}C]oleate delivered as LDL-complex into lipids of the nonpolar lipid fraction. B. Incorporation of CE-derived [^{14}C]oleate delivered as BSA-complex into lipids of the nonpolar lipid fraction. In both cases, CE-was present in 1µM concentration in the medium; incubation time: 24h. Lipid classes were separated using the solvent system (nHexane/Diethylether/Acetic acid 70:30:1 by vol.).

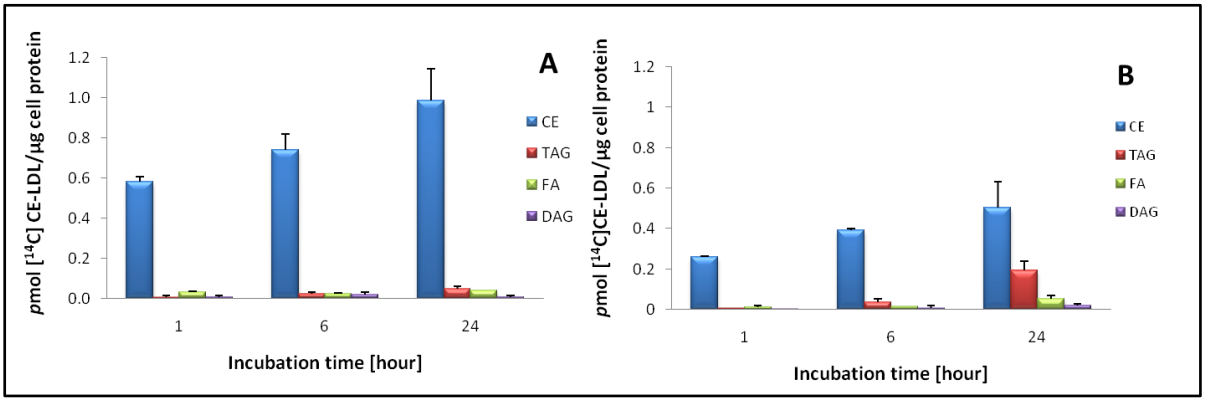

Figure 4.16: Incubation of different types of cultured cells with 1µM [^{14}C]CE-LDL complex in the culture medium for 1, 6, and 24h, A. macrophages, and B. HepG2 cells. Lipid classes were separated by tlc using the solvent system (nHexane/Diethylether/Acetic acid 70:30:1 by vol.). Radioactivity found in the lipid extract of different cells was set equal 100%. Radioactivity was analyzed by phosphoimager analysis.

4.2.2. Cholesterol uptake and processing

[4-^{14}C]cholesterol was added to four different types of cultured cells, human fibroblasts, macrophages, A431 cells, and HepG2 cells either as a complex with LDL or with BSA in a concentration of 1µM. When compared to [4-^{14}C]cholesterol delivered as BSA-complex, the uptake of [4-^{14}C]cholesterol delivered as LDL-complex is 3-fold higher in macrophages, 2.5-fold higher in fibroblasts, Hep-G2 cells, and A431 cells. Furthermore, uptake of [4-^{14}C]cholesterol delivered as LDL by fibroblasts is higher than by the other cell types, and uptake of [4-^{14}C]cholesterol delivered as BSA-complex by A431 cells is higher than by the other cell types (Fig. 4.17).

The incorporation of the [4-^{14}C]cholesterol delivered as LDL-complex into CE of macrophages is higher than in the other cell types, as shown in figure 4.18A. Compared to [4-^{14}C]cholesterol delivered as BSA complex, the incorporation of [4-^{14}C]cholesterol into CE after 24h incubation time is 10-fold higher when [4-^{14}C]cholesterol is delivered as LDL-complex. The ability of [4-^{14}C]cholesterol delivered as LDL-complex to be incorporated in cholesteryloleate of macrophages can be related to atherosclerosis. If the function of the endolysosomal system is assayed by transport of [4-^{14}C]cholesterol, it should be delivered as LDL-complex and the incorporation of labeled cholesterol into CE of macrophages should be chosen as read-out.

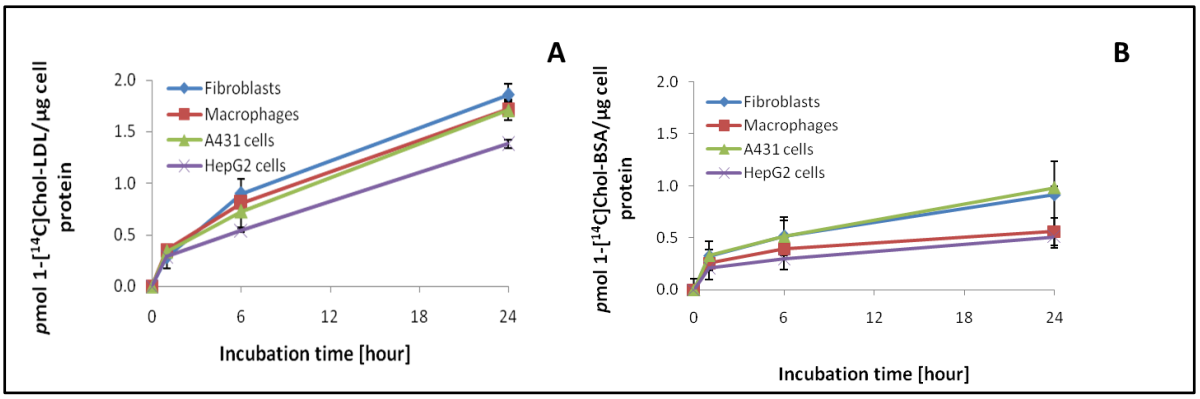

Figure 4.17: Uptake of [4-^{14}C]cholesterol (1µM) delivered as complex with LDL (A) or BSA (B) by four types of cultured cells. The uptakes were measured 1, 6, and 24h after addition of the Chol-complexes. Uptake was determined by scintillation counting of the lipid extract after different incubation time.

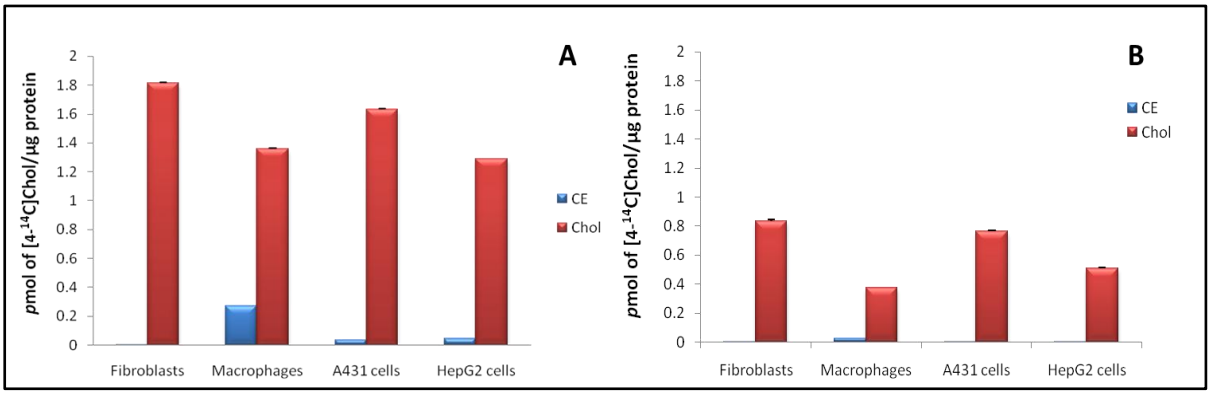

Figure 4.18: A. Incorporation of [4-^{14}C]cholesterol delivered as LDL-complex into lipids of the nonpolar lipid fraction. B. Incorporation of [4-^{14}C]cholesterol delivered as BSA-complex into lipids of the nonpolar lipid fraction. In both cases, [4-^{14}C]cholesterol was present in 1µM concentration in the medium; incubation time: 24h. Lipid classes were separated using the solvent system (nHexane/Diethylether/Acetic acid 70:30:1 by vol.).

4.2.3. Triacylglycerol uptake and processing

[1-^{14}C]Trioleoylglycerol was added to four different types of cultured cells, human fibroblasts, macrophages, A431 cells, and HepG2 cells, either as a complex with LDL or with BSA, in a concentration of 1µM in the culture medium. As shown in figure 4.19, the uptake of [^{14}C]TAG-LDL and [^{14}C]TAG-BSA by fibroblasts is higher than by the other cell types. Compared to [^{14}C]TAG-BSA, the uptake of [^{14}C]TAG-LDL is 4-fold higher in A431 cells, Hep-G2 cells, and macrophages, and 3-fold higher in fibroblasts.

When TAG is delivered as LDL-complex, TAG-derived [^{14}C]oleic acid is mainly incorporated into neutral lipids. When macrophages are exposed to 1µM TAG-LDL–complex, the incorporation of TAG-derived [^{14}C]oleate into CE is higher compared to the

other investigated cell types (Fig. 4.20A). In addition, the incorporation of [^{14}C]oleate into CE after 24h incubation time is 11-fold higher when TAG is delivered as LDL-complex than when delivered as BSA complex (Fig. 4.20C, Fig. 4.20D). Incorporation of TAG-derived [^{14}C]oleate into DAG was 10- fold lower than into TAG, suggesting that DAG might be an intermediate in the process of FA-incorporation into TAGs. On the other hand, the labeled oleic acid derived from TAG-BSA was incorporated only slightly into the nonpolar lipid fractions of the four investigated cell types (Fig. 4.20B). In cultured cells exposed to 1µM TAG-BSA–complex, levels of free [^{14}C]oleic acid were higher in fibroblasts than that of the other cell types (Fig. 4.20B). If the function of the endolysosomal system is assayed by processing of TAG-derived [^{14}C]oleate, it should be delivered as LDL-complex and the incorporation of labeled oleic acid into PC (Fig. 4.21A) should be chosen as read-out.

We demonstrated that lipid complexes with LDL show higher uptake and processing compared to lipid complexes with albumin. We also demonstrated that the uptake of [^{14}C]CE-LDL, [^{14}C]Chol-LDL, and [^{14}C]TAG-LDL in fibroblasts is higher compared to other cell types.

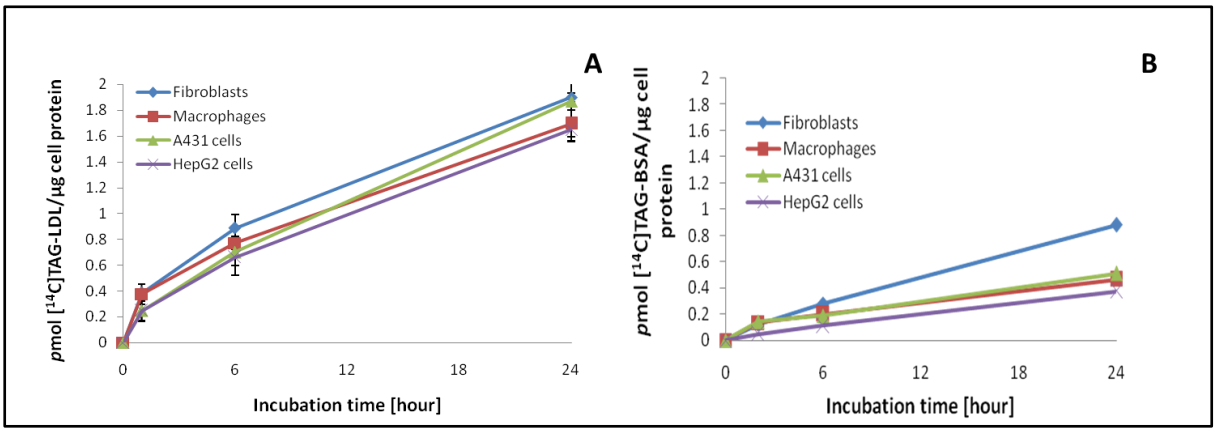

Figure 4.19: Uptake of [1-^{14}C]trioleoylglycerol (1µM) in complex with A. LDL, B. BSA by cultured cells. The uptakes were measured 1, 6, and 24h after addition of the trioleoylglycerol-complexes. These diagrams were determined by scintillation counting of the lipid extract after different incubation time.

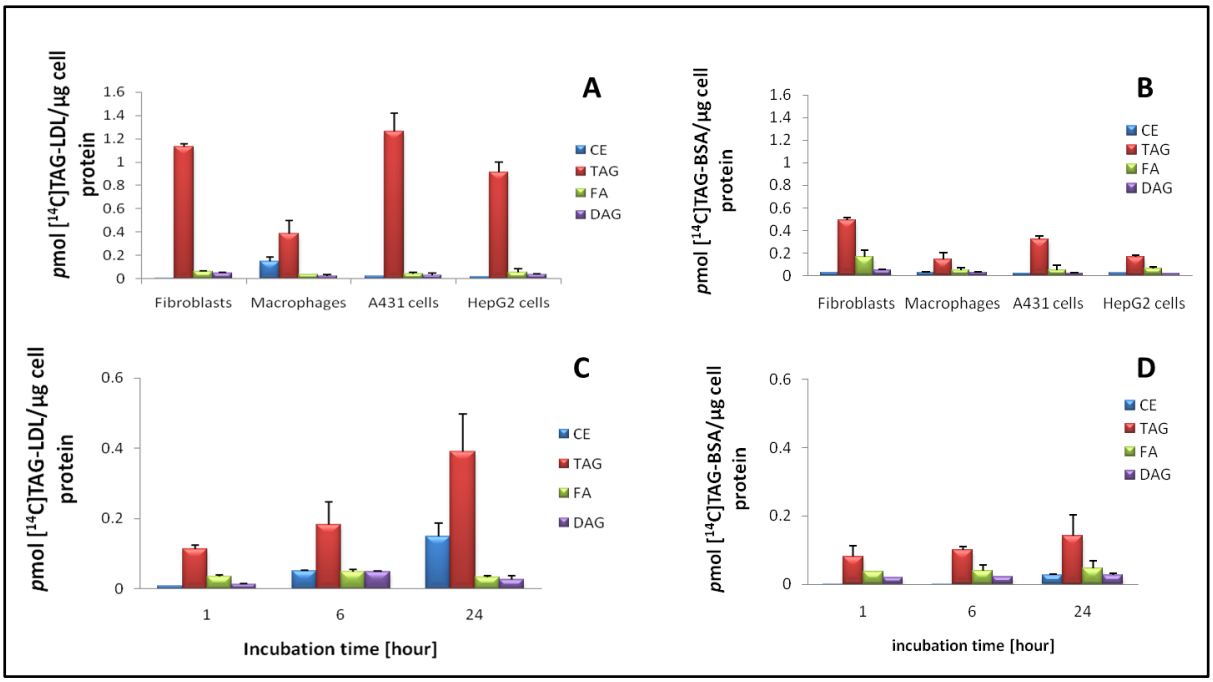

Figure 4.20: Incubation of cells with A. [^{14}C]TAG-LDL , B. [^{14}C]TAG-BSA complexes (1µM) (upper panel) in the culture medium for 24h. Incubation of macrophages with C. [^{14}C]TAG-LDL, D. [^{14}C]TAG-BSA (bottom panel) for 1, 6, and 24h. Lipid classes were separated using the solvent system (nHexane/Diethylether/Acetic acid 70:30:1 by vol.). Radioactivity found in the lipid extract of different cells was set equal 100%. Radioactivity was analyzed by phosphoimager analysis.

The polar lipid pattern that arise from the utilization of CE- and TAG-derived [^{14}C]oleic acid were also compared (Fig. 4.21). Both complex lipids were applied as LDL- and BSA-complexes with an incubation time of 24h and a concentration of 1µM. Our results demonstrate that even though the oleate in cholesteryl oleate and trioleoylglycerol was delivered in the same carrier as LDL particles, oleic acid-incorporation into polar lipids leads quantitatively to different pattern.

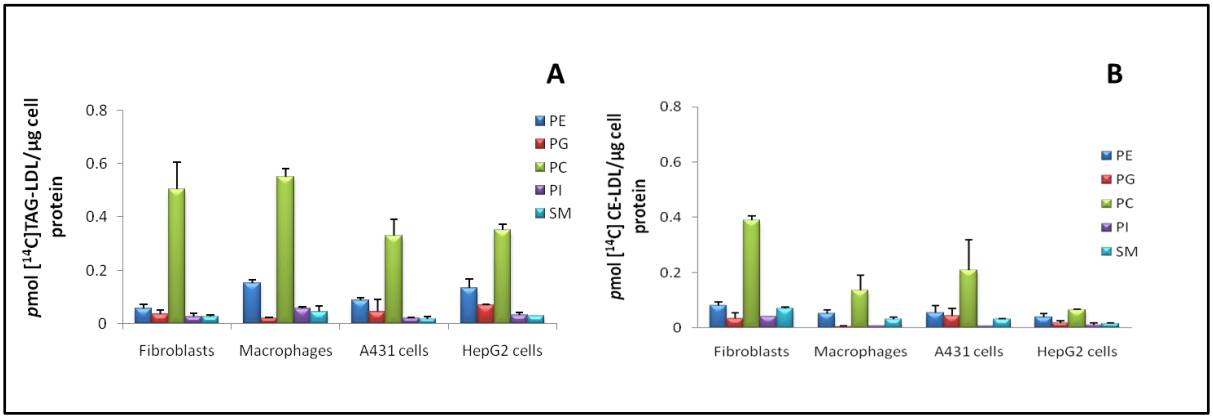

Figure 4.21: illustrates the incorporation of A. 1µM TAG-LDL-derived [^{14}C]oleate B. 1µM CE-LDL-derived [^{14}C]oleate into polar lipids of different cell types after 24h incubation. Lipid classes were separated using the solvent system (chloroform/methanol/water 65:25:4 by vol.).

4.3. Analysis of lipid processing in Niemann-Pick disease, type A cells (NPA) compared to normal fibroblasts

NPA is one of the lysosomal storage disease in which sphingomyelin accumulates in cells and tissues. To assess the role of the endolysosomal system in the processing of LDL-associated [^{14}C]cholesterol and LDL-associated [^{14}C]phosphatidylcholine, human fibroblasts and fibroblasts from patients with NPA disease, were investigated after 6h and 24h incubation.

Incubation with LDL-associated [^{14}C]cholesterol shows that there were no detectable differences in cholesterol uptake between normal fibroblasts and NPA disease after 6h incubation. Whereas, after 24h incubation, cholesterol uptake increased 21% in NPA cells compared to normal cells as shown in figure 4.22A.

Incubation with LDL-associated [^{14}C]phosphatidylcholine shows that [^{14}C]phosphatidylcholine is largely hydrolyzed in both normal fibroblasts and NPA disease cells. Some of the labeled fatty acids that results from lysosomal hydrolysis are effluxed into the media, and other incorporated into polar and nonpolar lipids (Groener, et al. 1996). After 6h and 24h incubation, levels of [^{14}C]phosphatidylcholine that are presumably trapped in lysosomes increased by 20%, and 25%, respectively, compared to normal cells (Fig. 4.22B). We also observed that the PC-derived FA is incorporated into polar lipids or recovered as free fatty acid to a lesser extent in NPA disease cells compared to normal cells after 24h incubation time. We found that the relative levels of labeled fatty acids and polar lipids in NPA disease cells after 24h were 10% and 8% lesser than that in normal fibroblasts, respectively, as shown in figure 4.23. In summary, these observations indicate an impaired processing of lipids via the endolysosomal system in NPA disease.

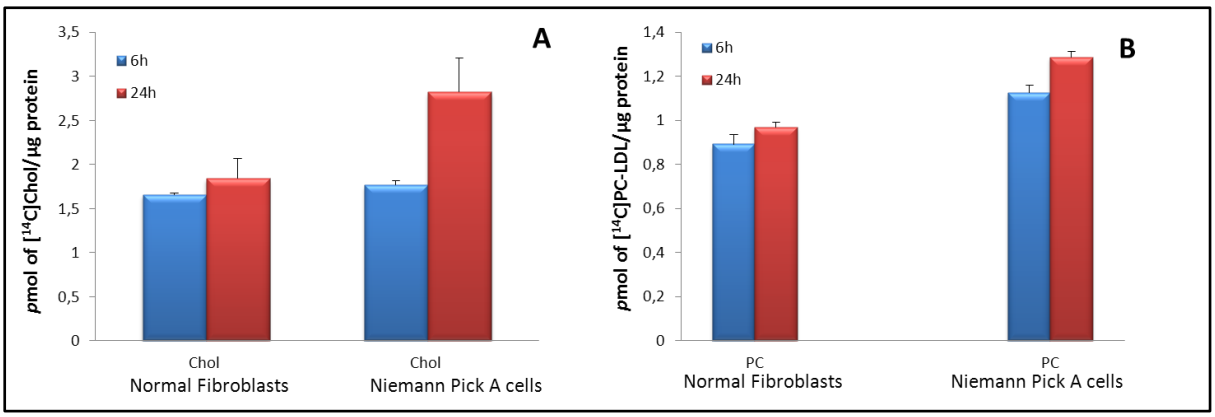

Figure 4.22: Cells were incubated for 6h and 24h with 1µM A. LDL-associated [^{14}C]cholesterol; B. LDL-associated [^{14}C]phosphatidylcholine in normal fibroblasts and NPA disease cells. Normal fibroblasts and NPA disease cells were grown as described under experimental procedures (Chapter 6).

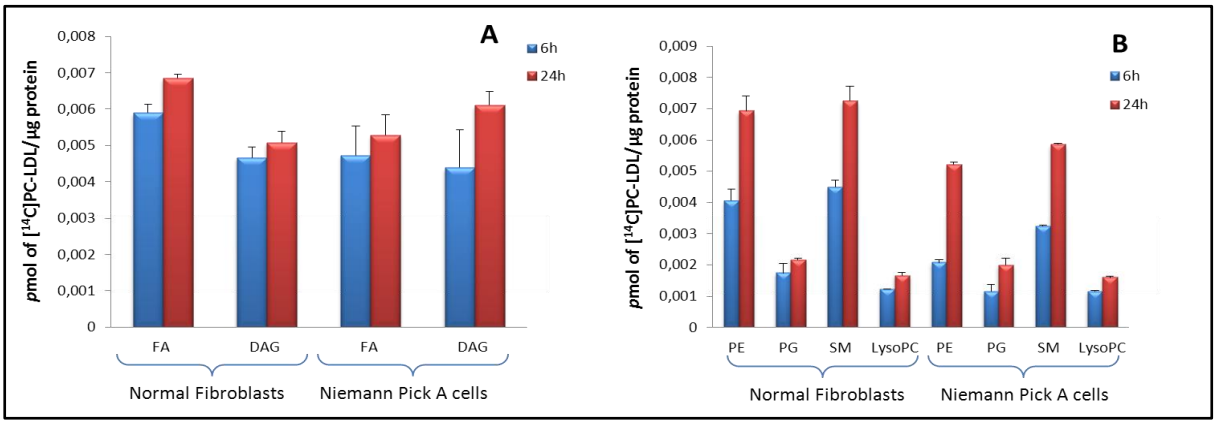

Figure 4.23: Cells were incubated for 6h and 24h with 1µM LDL-associated [^{14}C]phosphatidylcholine. The figure shows the incorporation of PC-derived [^{14}C]palmitate in A. nonpolar lipids, and B. polar lipids in normal fibroblasts and NPA disease cells. Normal fibroblasts and NPA disease cells were grown as described under experimental procedures (Chapter 6).

4.4. Effect of cationic amphiphilic drugs on uptake and processing of choline-containing phospholipids by different types of cultured cells

Phospholipidosis induced by CADs impairs cellular function to an unknown extent. The best investigational example for a molecular effect of CADs is the premature degradation of acid sphingomyelinase, which leads primarily to SM-accumulation. Therefore, uptake and processing of PC containing [^{14}C]palmitic acid (Table 4.3) and SM containing [^{14}C]stearic acid (Table 4.4) as lipid probes was determined in four different types of cultured cells. To target the endolysosomal system, the lipid probes were delivered to the cells as part of LDL particles. In the absence of additional drugs added to the culture medium, both probes were taken up to a similar extent by macrophages, fibroblasts, A431 cells, and HepG2 cells as shown in figure 4.24.

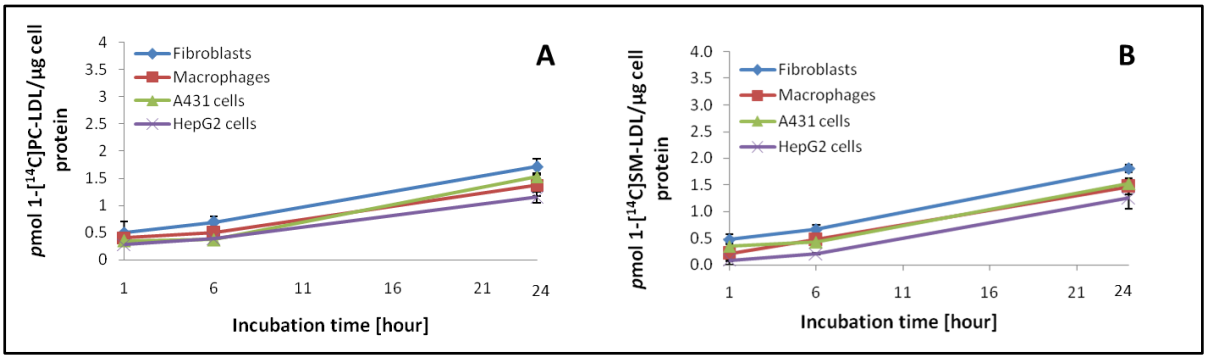

Figure 4.24: Uptake of A. [^{14}C]-PC, B. [^{14}C]-SM delivered as complex with LDL by four types of cultured cells. Concentration = 1µM. The uptakes were measured 1, 6, and 24h after addition of the SM-complexes. Uptake was determined by scintillation counting of the lipid extract after different incubation time.

4.4.1. Differences between cell types

The impact of the different CADs on uptake and processing of the SM- and the PC-probe was investigated in the four different cell types. The average uptake of the SM-probe in the presence of 0, 20, and 40µM CADs is shown in tables 4.3, and that of the PC-probe in table 4.4.

Table 4.3: Cellular SM-probe uptake by four different types of cultured cells.

Cellular SM-probe uptake (*p*mol/µg of protein)	**Fibroblasts**			**Macrophages**			**A431 cells**			**HepG2 cells**		
	0µM	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**
Desipramine	**1.70**	**1.69**	**1.70**	**1.09**	**0.77**	**0.70**	**1.17**	**0.85**	**0.77**	**0.82**	**0.29**	**0.27**
Imipramine	**1.78**	**1.70**	**1.66**	**1.45**	**1.41**	**1.36**	**1.37**	**1.31**	**1.19**	**1.09**	**1.07**	**1.07**
Chlorpromazine	**1.73**	**1.49**		**1.35**	**1.34**		**1.35**	**1.17**		**1.16**	**1.06**	
FTY720	**1.82**	**1.62**		**1.30**	**0.78**		**1.36**	**0.94**		**0.87**	**0.73**	
Chloroquine	**1.79**	**1.74**	**1.63**	**1.42**	**1.35**	**1.30**	**1.46**	**1.42**	**1.40**	**1.07**	**1.01**	**0.90**

Table 4.4: Cellular PC-probe uptake by four different types of cultured cells.

Cellular PC-probe uptake (*p*mol/µg of protein)	**Fibroblasts**			**Macrophages**			**A431 cells**			**HepG2 cells**		
	0µM	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**	**0µM**	**20µM**	**40µM**
Desipramine	**1.60**	**1.62**	**1.56**	**0.97**	**0.42**	**0.38**	**1.22**	**1.15**	**0.94**	**0.79**	**0.47**	**0.42**
Imipramine	**1.76**	**1.72**	**1.65**	**1.34**	**1.21**	**1.08**	**1.52**	**1.50**	**1.49**	**1.11**	**1.09**	**1.07**
Chlorpromazine	**1.90**	**1.41**		**1.26**	**1.11**		**1.46**	**1.15**		**0.87**	**0.81**	
FTY720	**1.82**	**1.72**		**1.30**	**1.10**		**1.42**	**1.34**		**0.97**	**0.93**	
Chloroquine	**1.82**	**1.70**	**1.68**	**1.42**	**1.29**	**1.20**	**1.59**	**1.52**	**1.55**	**1.01**	**0.88**	**0.73**

Fibroblasts, macrophages, A431 cells, and HepG2 cells were cultured as described in the materials and methods section in serum-free medium supplemented with 1µM of the [^{14}C]SM-probe (Table 4.3) and the [^{14}C]PC-probe (Table 4.4). Both were added as lipid-LDL complexes. After treatment with different CADs in different concentrations (0, 10, 20 and 40µM) and incubation for 24h, cells were harvested and lipids were extracted. The incorporation of the labeled FA derived from the lipid probe into nonpolar and polar lipids was determined as *p*mol of lipids per µg of protein. Cell viability was not significantly influenced by 10µM or 20µM CADs as shown in figure 4.38.

4.4.2. Impact of desipramine on SM and PC uptake

The uptake of the SM- and the PC-probe in terms of *p*mol per µg cell protein was dependent on the identity and the concentration of the investigated CADs, on the incubation time, and on the cell type. In cultured cells that were pre-incubated for 24h with 0, 10, 20, and 40µM desipramine, the uptake of the lipid probes per µg protein decreased with increasing concentrations of desipramine in the culture medium. This decrease in lipid uptake was visible after 24h and was dose dependent. In cultured cells pre-treated with different concentrations of desipramine (Fig. 4.25), the uptake of [^{14}C]PC and [^{14}C]SM was significantly decreased in macrophages and HepG2 cells compared to in the control cells. From the SM probe or PC probe present in the medium, 23% and 22% were taken up without treatment with DMI, 21% and 15% were taken up after treatment with 20µM DMI, and 19% and 10% after treatment with 40µM DMI, respectively.

4.4.3. Effect of desipramine on SM and PC processing

When macrophages were cultured in the presence of different concentrations of desipramine, 10µM desipramine had only a slight effect on [^{14}C]SM processing. 10µM desipramine led to a decrease in labeled total neutral lipids and free fatty acids by 24% and 18%, respectively. 40µM desipramine led to a decrease in labeled total neutral lipids and free fatty acids by 39% and 55%, respectively. TAG, CE, and DAG were reduced by 47%, 80%, and 13%, respectively. Treatment of macrophages with 20µM desipramine resulted in an increased level of the exogenously added SM probe consistent with the effect of desipramine on premature degradation of acid sphingomyelinase, and a reduced intracellular capacity to release the radiolabeled fatty acid from the incorporated lipid probe. At 10 and 20µM desipramine, levels of labeled ceramide also increased 1.5-fold compared to control cells (Fig. 4.26B). This is in agreement with the reported induction of degradation of acid ceramidase by desipramine and other CADs (Elojeimy, et al. 2006). These results support the dual effects of desipramine on both acid sphingomyelinase and acid ceramidase also in this cell type. Incubation with 40µM desipramine led to a 7-fold increase in labeled SM compared to untreated cells.

The effect of desipramine on the incorporation of [^{14}C]PC-derived palmitic acid into PLs of macrophages is shown in figure 4.27B. In cultured cells treated with 10µM desipramine, PC-derived [^{14}C]palmitic acid was incorporated approximately to the same extent into complex

cellular lipids as in the control. A slight increase of labeled PC was observed in the presence of 20µM desipramine, whereas a significant increase of labeled PC, PA, and lysoPC was visible in the presence of 40µM desipramine. Labeling of PA and LysoPC was increased 2.5- and 2-fold, respectively.

In macrophages and HepG2 cells, after treatment with 10µM and 20µM desipramine, the incorporation of [^{14}C]PC-derived palmitic acid into[^{14}C]TAG appeared to be higher than that of other nonpolar lipids. After treatment with 40µM desipramine, the reverse was observed. In A431 cells, the incorporation of [^{14}C]PC-derived palmitate into TAG was decreased slightly, but increased slightly in fibroblasts treated with 40µM desipramine (data not shown). Furthermore, 40µM desipramine inhibits significantly the incorporation of [^{14}C]PC-derived palmitate into total neutral lipids and free fatty acids of macrophages by 33% and 28%, respectively. TAG, CE, and DAG were reduced by 39%, 21%, and 23%, respectively. These data suggest that only higher concentration (40µM) of desipramine significantly inhibit the incorporation of [^{14}C]PC-derived palmitate into TAG of cultured macrophages, A431 cells and HepG2 cells, but not of fibroblasts.

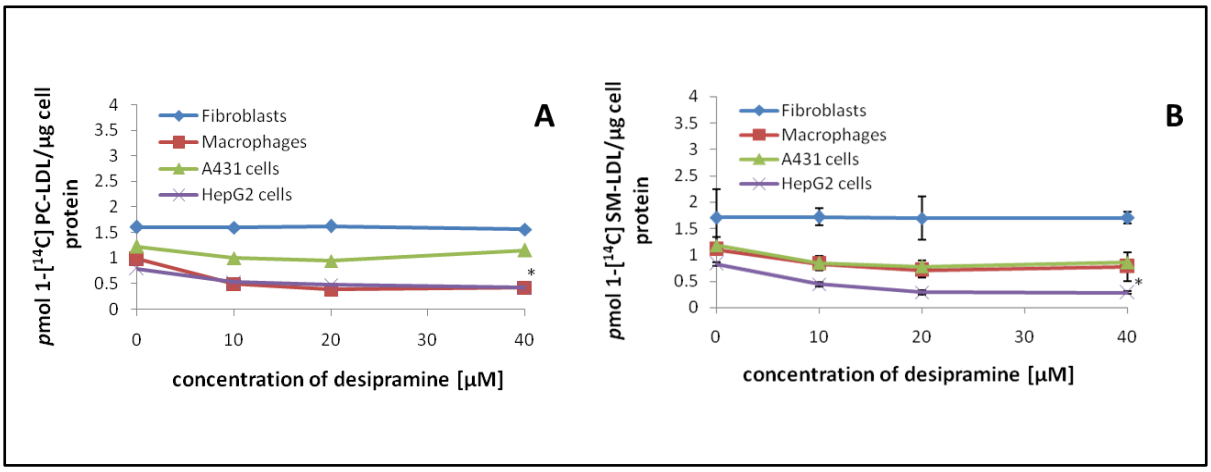

Figure 4.25: Uptake of labeled A. PC, B. SM administered as part of LDL particles by cultured cells after 24h incubation time in the absence and presence of different concentrations of desipramine. Cells were pre-incubated with different concentration of desipramine for 24h before the addition of the complexes. Uptake was determined by scintillation counting of the lipid extract, so that the given values represent the sum of unchanged phospholipid and hydrophobic metabolites derived from it. Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

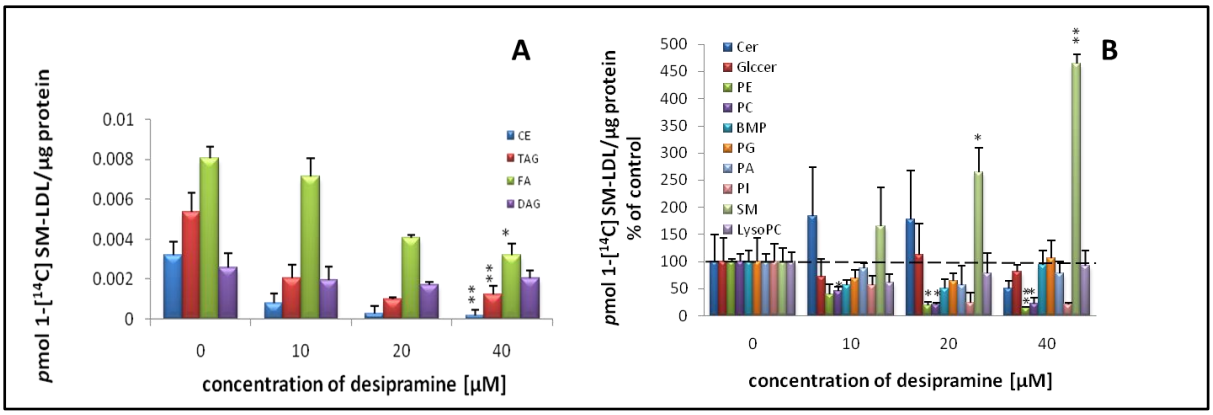

Figure 4.26: Effect of desipramine on the incorporation of SM-derived 1-[^{14}C]stearic acid into nonpolar lipids (left) and polar lipids (right) of macrophages after 24h incubation time (pre-incubation with desipramine for 24h before the addition of the complexes). Statistical significance between desipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

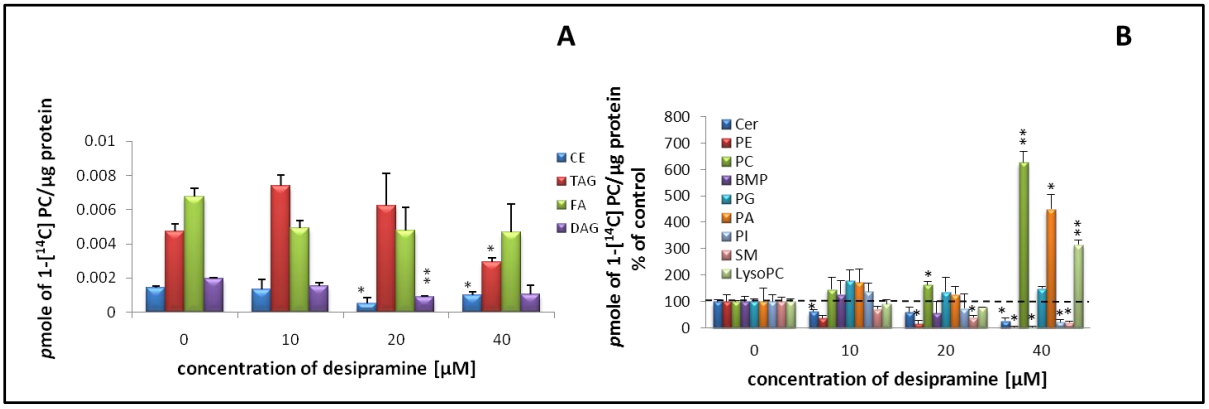

Figure 4.27: Effect of desipramine on the incorporation of PC-derived 1-[^{14}C]palmitic acid into nonpolar lipids (left) and polar lipids (right) of macrophages after 24h incubation time (pre-incubation with different concentrations of desipramine 24h before the addition of the complexes). Statistical significance between desipramine-treatment and matched control is determined using statistical student's t-test: * represents $p < 0.05$, and ** represents $p < 0.005$.

4.4.4. Impact of chlorpromazine on SM and PC uptake

Chlorpromazine has been demonstrated to inhibit the formation of clathrin-coated pits (Nawa, et al. 2003) and acts as an inhibitor of receptor-mediated endocytosis. Therefore it should also reduce the uptake of lipids delivered as part of LDL particles via the LDL-receptor. We examined the role of chlorpromazine in four cell types and measured the uptake of PC and SM as part of LDL complexes. Uptake of the PC- and SM-probe delivered as LDL complex was determined in the presence of (0, 10, and 20µM) of chlorpromazine. Concentrations of more than 20µM reduced the cell viability and amount of cellular protein (Fig. 4.38A). As shown in figure 4.28A, in the presence of 20µM chlorpromazine no big effects of chlorpromazine on lipid uptake were detected in macrophages and HepG2 cells. Independent

of this, uptake of LDL-associated PC and LDL-associated SM was lower in fibroblast and A431 cells compared to other cell types.

4.4.5. Effect of chlorpromazine on SM and PC processing

The incorporation of SM-derived [^{14}C]stearic acid into nonpolar lipids was depressed markedly in the presence of chlorpromazine (Fig. 4.29A). This includes the incorporation into DAG, which is the intermediate for the synthesis of TAG, PC and PE and is formed primarily by the action of phosphatidic acid phosphohydrolase, an enzyme that can be inhibited by CADs (Leli, at al. 1987; Pappu, et al. 1984). Also levels of labeled TAG were decreased by 65%. Furthermore, chlorpromazine had an inhibitory effect on the synthesis of PC and other phospholipids from [^{14}C]SM-Stearate. In macrophages, SM-derived [^{14}C]stearic acid was predominantly recovered as SM, which comprised 95% of the total labeled phospholipids of the polar lipid fraction. These data suggest that chlorpromazine may act by inhibiting CTP:phosphocholine cytidylyltransferase (Fig. 5.3), as shown by Pelech, et al. 1984, and decrease the incorporation of [^{14}C]SM-derived stearate precursors into PC and PE as seen in our data (Fig. 4.29B).

When PC-derived [^{14}C]palmitic acid was added to the cells as part of LDL-complexes, chlorpromazine at 10μM did not alter lipid metabolism significantly. However, at 20μM there was a slightly decreased incorporation of PC-derived [^{14}C]palmitate into neutral lipids of macrophages. The incorporation of [^{14}C]palmitic acid derived from PC into TAG and DAG decreased 22%, and 25%, respectively (Fig. 4.30A). Level of PC-derived palmitic acid increased with increasing concentration of chlorpromazine (Fig. 4.30B). Labelling of PE, Cer, and SM decreased in response to the same dose. In the presence of 20μM chlorpromazine, the level of labeled PC increased 50% after 24h incubation. On the other hand, the incorporation of PC-derived [^{14}C]palmitate into neutral lipids of A431 cells was decreased by 22% on treatment with 20μM chlorpromazine. This concentration has no significant effects on the incorporation of [^{14}C]palmitate into neutral lipids of HepG2 cells (data not shown).

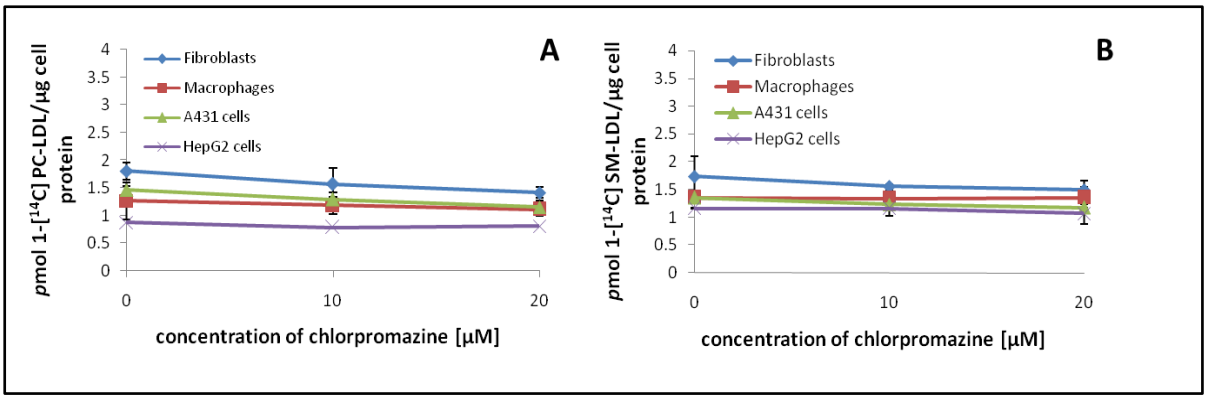

Figure 4.28: Uptake of labeled A. PC, B. SM administered as part of LDL particles into the lipid fraction of cultured cells after 24h incubation time. Cells were pre-incubated with different concentrations of chlorpromazine for 24h before the addition of the complexes. Uptake was determined by scintillation counting of the lipid extract, so that the given values represent the sum of unchanged phospholipid and hydrophobic metabolites derived from it.

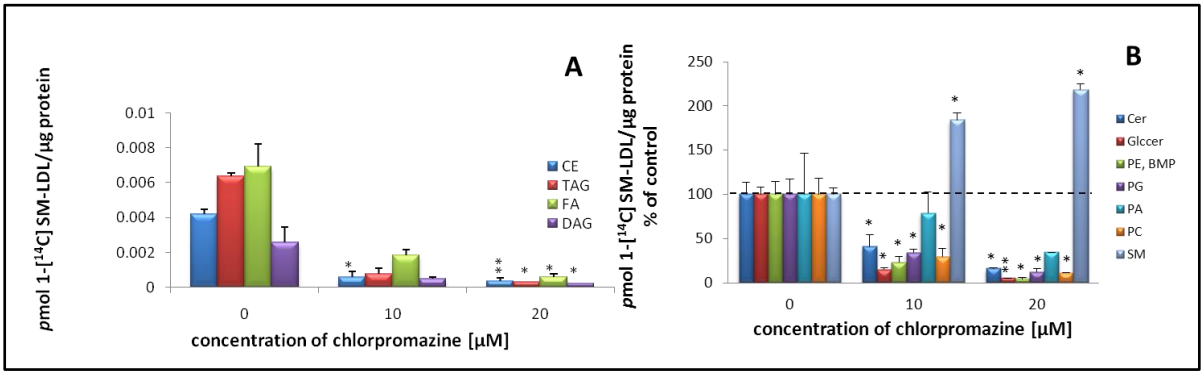

Figure 4.29: Effect of chlorpromazine on the incorporation of SM-derived 1-[^{14}C]stearic acid into nonpolar lipids (left) and polar lipids (right) of macrophages after 24h incubation time (pre-incubation with different concentrations of chlorpromazine for 24h before the addition of the complexes). Statistical significance between chlorpromazine treated and matched controls is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

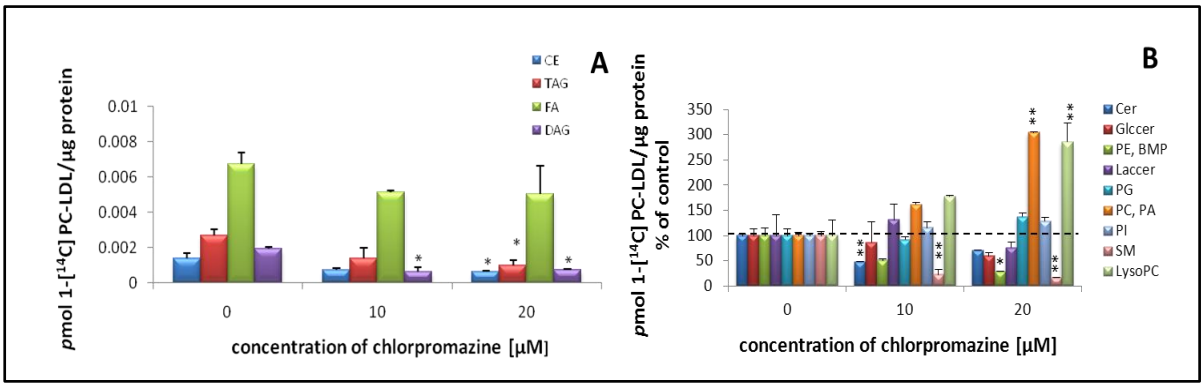

Figure 4.30: Effect of chlorpromazine on the incorporation of PC-derived 1-[^{14}C]palmitic acid into nonpolar lipids (left) and polar lipids (right) of macrophages after 24h incubation time (pre-incubation with different concentrations of chlorpromazine for 24h before the addition of the complexes). Statistical significance between chlorpromazine treated and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

4.4.6. Impact of imipramine on SM and PC uptake

Figure 4.31 shows the uptake of radiolabeled PC and SM after 24h incubation with imipramine in different types of cultured cells. In macrophages, increasing concentrations of imipramine in the culture medium cause an decreased uptake of PC by the cells, which is observed in the other cell types to a much lesser extent (Fig. 4.31A). The uptake of radiolabeled SM delivered as part of LDL particles in the absence and in the presence of 10, 20 and 40μM imipramine by macrophages, fibroblasts, and HepG2 cells is shown in figure 4.31B. At higher concentrations of imipramine in the culture medium, uptake of SM was slightly decreased in A431 cells.

4.4.7. Effect of imipramine on SM and PC processing

Cultured cells were pre-incubated with medium containing 0, 10, 20 and 40μM imipramine for 24h and then treated with SM labeled by [^{14}C]-stearic acid, still in the presence of drug for 24h. We observed an increase of labeled phospholipids per μg of protein during imipramine exposure. This phospholipid accumulation appeared after 24h and was dose dependent. The effect of imipramine on the incorporation of SM-derived [^{14}C]stearic acid into nonpolar and polar lipids of macrophages is shown in figure 4.32A and figure 4.33A. In the presence of 10μM imipramine in the culture medium, the incorporation of SM-derived [^{14}C]stearic acid into phospholipids was nearly the same as in control cells. However, a significant increase of label in acidic phospholipids was observed after treatment with 20μM imipramine. The highest incorporation was observed into phosphatidic acid. It can be assumed that this is due to a decreased degradation of phosphatidic acid in the presence of imipramine, since labeling of PC and PE were two times lower. The incorporation of SM-derived [^{14}C]stearic acid into neutral lipids and recovery of free fatty acid was decreased by 45% and 67%, respectively, when macrophages were treated with 40μM imipramine.

The incorporation of PC-derived [^{14}C]palmitic acid into cholesteryl ester and diacylglycerol were lower in macrophages pre-treated with 40μM imipramine compared to control cells, i.e. 1.4 and 1.1 vs 0.2 and 0.5 *p*mol/mg protein in CE and DAG, respectively. No significant change in the incorporation of PC-derived [^{14}C]palmitic acid into TAG pre-treated with 40μM imipramine was observed in comparison to control cells (Fig. 4.32B). Furthermore, the incorporation of PC- derived [^{14}C]palmitic acid into neutral lipids and its recovery as free fatty acid was decreased by 15% and 27%, respectively, when cells were treated with 40μM imipramine. There were no significant differences in the incorporation of the labeled fatty

acid derived from PC in phospholipids between control cells and macrophages pre-treated with 10µM imipramine. The incorporation of PC-derived [^{14}C]palmitic acid into PC was increased by 16% in the presence of 40µM imipramine (Fig. 4.33B).

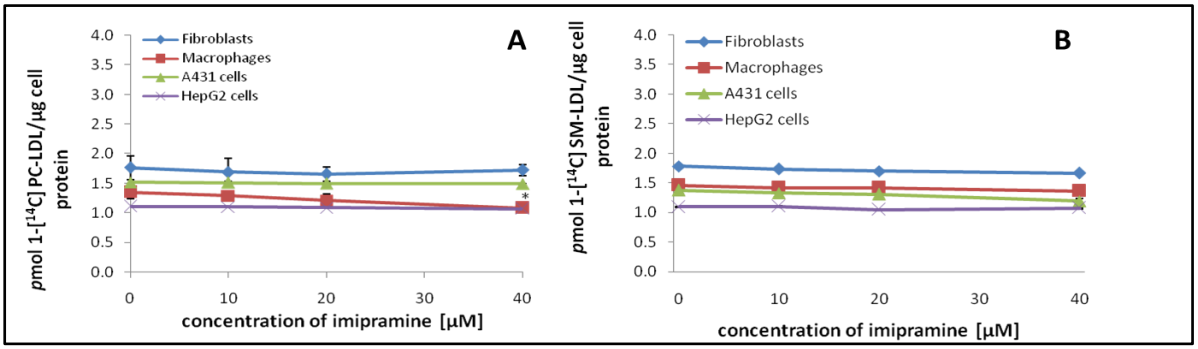

Figure 4.31: Uptake of labeled A. PC, B. SM. Both lipids were administered as part of LDL particles. Labelling of the lipid fraction of cultured cells after 24h incubation time with imipramine was determined in the absence and presence of different concentrations of imipramine. Cells were pre-incubated with different concentrations of imipramine for 24h before the addition of the complexes. Uptake was determined by scintillation counting of the lipid extract, so that the given values represent the sum of unchanged phospholipid and hydrophobic metabolites derived from it. Statistical significance between imipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

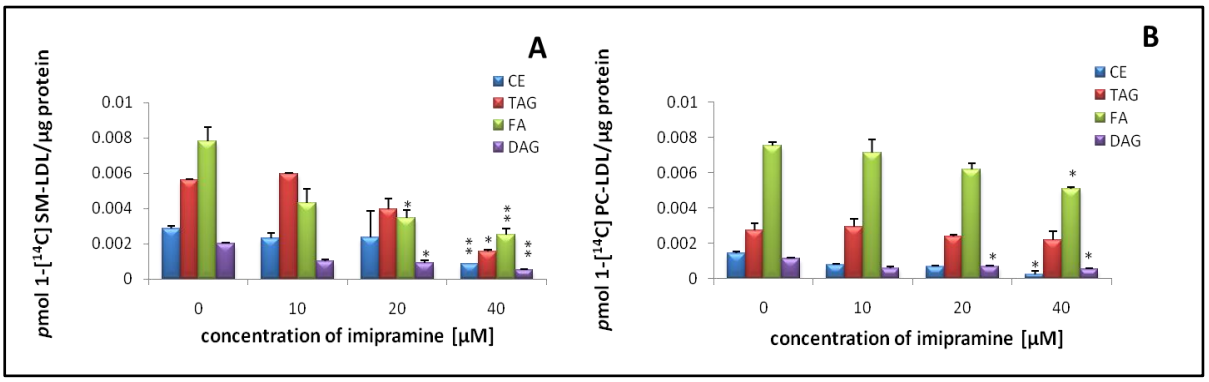

Figure 4.32: Effect of imipramine on the incorporation of SM-derived [^{14}C]stearic acid (left) and PC-derived [^{14}C]palmitic acid (right) into nonpolar lipids of macrophages after 24h incubation time (pre-incubation with different concentrations of imipramine for 24h before the addition of the complexes). Statistical significance between imipramine-treated and matched controls is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

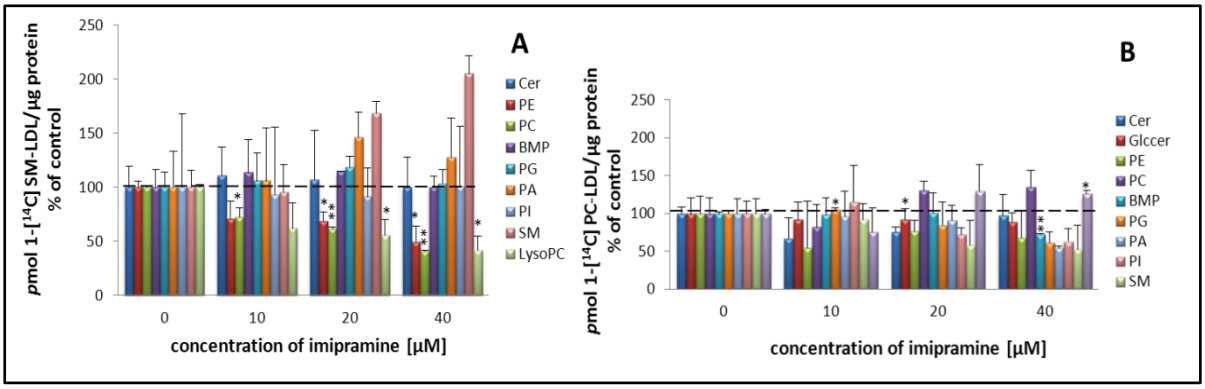

Figure 4.33: Effect of imipramine on the incorporation of SM-derived [^{14}C]stearic acid (left) and PC-derived [^{14}C]palmitic acid (right) into polar lipids of macrophages after 24h incubation time (pre-incubation with different concentrations of imipramine for 24h before the addition of the complexes). Statistical significance between imipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

4.4.8. Impact of chloroquine on PC and SM uptake

Chloroquine is known to cause inhibition of LDLR recycling (Minahk, et al. 2008). This offers the possibility to investigate the importance of LDLR on the uptake of radiolabeled PC and SM delivered as LDL particles. The four cell types were cultured in the presence of 1µM of the lipid probe and different concentrations of chloroquine. PC uptake assays revealed a significant reduction in [^{14}C]PC uptake by macrophages and HepG2 cells (Fig. 4.34A). Analysis of [^{14}C]PC uptake by fibroblasts treated with chloroquine showed a slight reduction in uptake in the presence of 40µM chloroquine (Fig.4.34A). This result is in agreement with previous result which suggest that PC as part of LDL-particles can be delivered to mouse hepatocytes by an LDLR independent pathway (Minahk, et al. 2008). Furthermore, the uptake of [^{14}C]SM by macrophages, fibroblasts, and HepG2 cells was slightly decreased in the presence of chloroquine compared to control cells. Chloroquine has no significant effects on lipid uptake of both the PC and the SM-probe by A431 cells (Fig. 4.34).

These data also supports the previous results which represent that chloroquine may disturb LDLR recycling pathways.

4.4.9. Effect of chloroquine on PC and SM processing

The addition of chloroquine induced a decrease in the incorporation of fatty acids released from PC or SM into TAGs in macrophages (Fig. 4.35A, Fig. 4.35B).

When macrophages were incubated with [^{14}C]PC in the absence of chloroquine, (69%, 11%, and 14%) of the radioactivity was found in phospholipids, neutral lipids, and free fatty acid, respectively. PC represents about 50% of the radioactivity of the total phospholipids. When macrophages were incubated with [^{14}C]SM in the absence of chloroquine, (64%, 14%, and 18%) of the radioactivity was found in phospholipids, neutral lipids, and free fatty acid, respectively. Here, SM represents about 70% of radioactivity of the phospholipids. When macrophages were incubated for 24h in the presence of 40µM chloroquine, (82%, 4%, and 8%) and (76%, 8%, and 12%) of the radioactivity was found in phospholipids, neutral lipids, and free fatty acid after labeling with both, the PC- and the SM-probe, respectively (Fig. 4.35). PC represents about 58% of radioactivity of the total phospholipids after labeling with

the PC-probe, while SM represents about 78% of radioactivity of the total phospholipids after labeling with the SM-probe. The effects of 40µM chloroquine to decrease the incorporation into neutral lipids were also observed with other cell types, when LDL associated PC and LDL associated SM probes were used (data not shown).

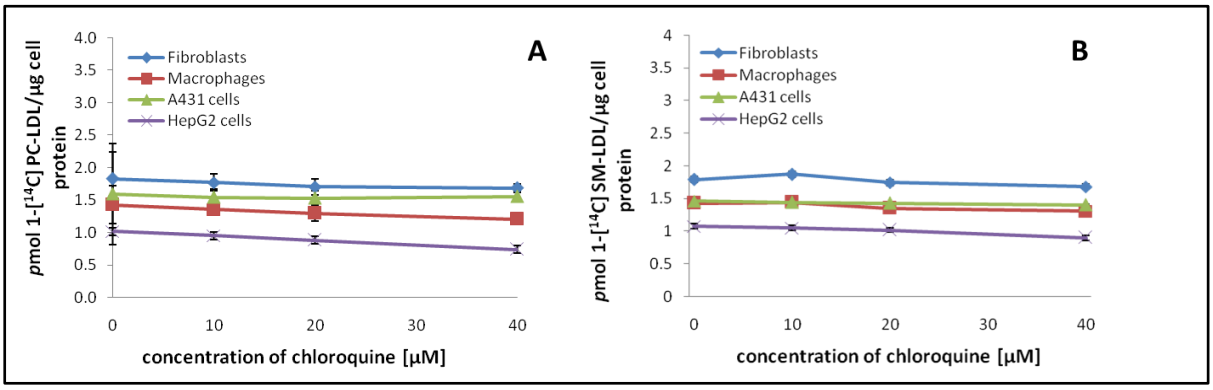

Figure 4.34: Uptake of labeled A. PC, B. SM. Both lipids were administered as part of LDL particles. Concentration = 1µM. Cells were pre-incubated with different concentrations of chloroquine for 24h before the addition of the complexes. Uptake was determined after 24h incubation by scintillation counting of the lipid extract, so that the given values represent the sum of unchanged phospholipid and hydrophobic metabolites derived from it. Statistical significance between chlorpromazine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

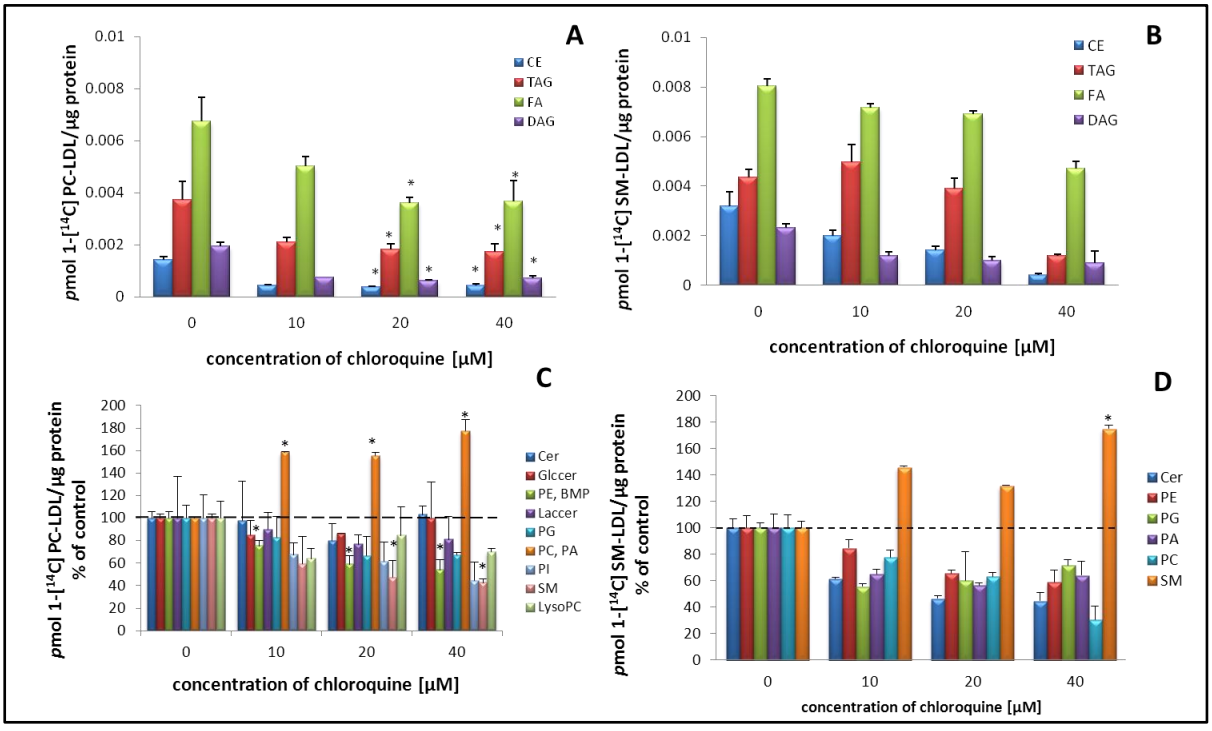

Figure 4.35: Effect of chloroquine on the incorporation of PC-derived 1-[^{14}C]palmitic acid (A, C) and SM-derived 1-[^{14}C]stearic acid (B, D) into nonpolar lipids (upper panel) and polar lipids (bottom panel) of macrophages after 24h incubation time (pre-incubation with different concentrations of chloroquine for 24h before the addition of the complexes). Statistical significance between chloroquine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

4.4.10. Impact of FTY720 on PC and SM uptake

FTY720 has been demonstrated that lead to an apparent inhibition of acid sphingomyelinase (Dawson, et al. 2011) and all six ceramide synthase isozymes (Berdyshev, et al. 2009). We examined the role of FTY720 on uptake and processing of PC and SM in four cell types. Both phospholipids were delivered as part of LDL-particles in concentration of 1μM in the presence of different concentrations of FTY720 (0, 10, and 20μM). Concentrations of more than 20μM FTY720 reduced cell viability and amount of cellular protein. In the presence of FTY720, the uptake of both, [^{14}C]SM and [^{14}C]PC was slightly decreased in fibroblasts and HepG2 cells, and the uptake of [^{14}C]SM was significantly decreased in macrophages and A431 cells compared to in the control cells, as shown in figure 4.36.

4.4.11. Effect of FTY720 on PC and SM processing

When cultured macrophages were incubated with [^{14}C]PC or [^{14}C]SM in the presence of FTY720 for 24h, the effect of FTY720 on processing of the lipid probes was dependent on its concentration. At 10μM, FTY720 did not alter levels of labeled metabolites significantly. However, at 20μM concentration, FTY720 produces a significant increase in the incorporation of 1-[^{14}C]stearic acid derived from SM into GlcCer and LacCer (Fig. 4.37D). In macrophages treated with 1μM LDL associated SM-stearate, 10μM FTY720 led to a decrease in labeled neutral lipids and PLs by 10% and 18%, respectively. While, 20μM FTY720 led to a decrease in labeled neutral lipids and PLs by 42% and 24%, respectively. Free stearic acid was increased by 34% when treated with 20μM FTY720 compared to control. Treatment of macrophages with 20μM FTY720 resulted in an increased level of the exogenously added SM probe consistent with the effect of FTY720 on premature degradation of acid sphingomyelinase.

The incorporation of PC-derived [^{14}C]palmitic acid into cholesteryl ester and triacylglycerol were decreased in macrophages by 21%, and 34% pre-treated with 20μM FTY720 compared to control cells, respectively. Free palmitic acid was increased by 38% when treated with 20μM FTY720 compared to control cells (Fig. 4.37A). There were no significant differences in the incorporation of the labeled palmitic acid derived from PC in polar lipids between control cells and macrophages pre-treated with 10μM FTY720. The level of PC-derived palmitic acid was increased in the presence of 20μM FTY720. The incorporation of the

labeled palmitic acid into PC was increased by 31% in the presence of 20µM FTY720 (Fig. 4.37C).

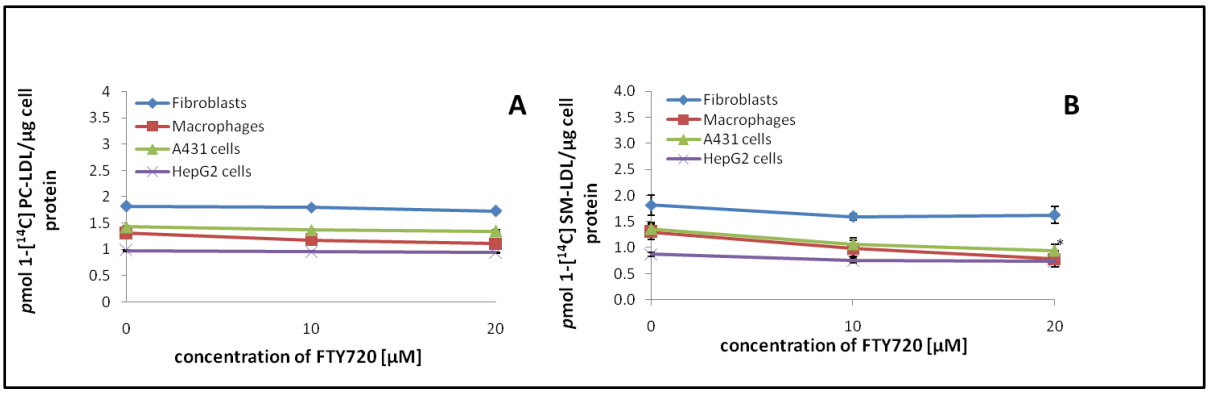

Figure 4.36: Uptake of 1µM labeled A. PC, B. SM, administered as part of LDL particles by cultured cells after 24h incubation time. Cells were pre-incubated in the absence and presence of different concentrations of FTY720 for 24h before the addition of the complexes. Uptake was determined by scintillation counting of the lipid extract, so that the given values represent the sum of unchanged phospholipid and hydrophobic metabolites derived from it. Statistical significance between chlorpromazine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$.

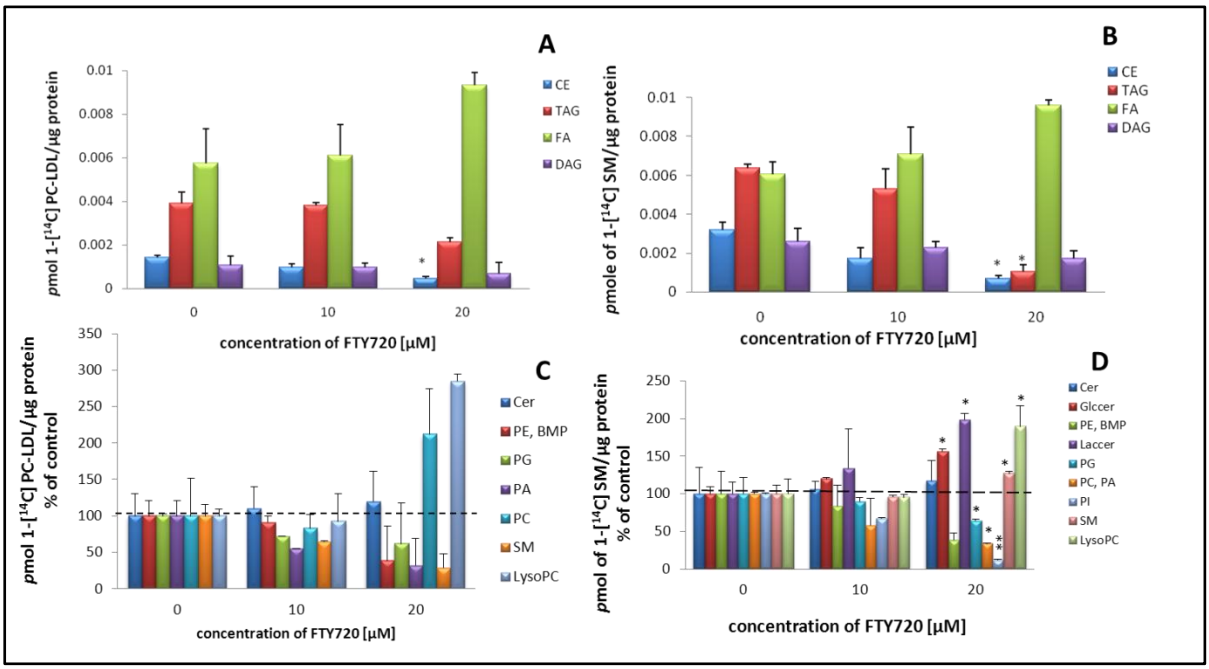

Figure 4.37: Effect of FTY720 on the incorporation of PC-derived [^{14}C]palmitic acid (A, C) and SM-derived [^{14}C]stearic acid (B, D) into nonpolar lipids (upper panel) and polar lipids (bottom panel) of macrophages after 24h incubation time (pre-incubation with different concentrations of FTY720 for 24h before the addition of the complexes). Statistical significance between imipramine treatment and matched control is determined using statistical student's t-test. * represents $p < 0.05$, and ** represents $p < 0.005$.

In summary, we demonstrated that lipid-LDL complexes are taken up at a higher rate in fibroblasts compared to other cell types. On the other hand, Chol-BSA complexes are taken up at a higher rate in A431 cells compared to CE-BSA, TAG-BSA which is taken up at a higher rate in fibroblasts. We also demonstrated that when macrophages were treated with 20µM CADs, [^{14}C]SA derived from SM was mainly recovered as SM (1.3 *p*mol/µg protein) when treated with chlorpromazine, followed by desipramine, which comprised (0.9 *p*mol/µg protein). [^{14}C]palmitic acid derived from PC was mainly recovered as PC (1.1 *p*mol/µg protein) when treated with chlorpromazine, followed by FTY720, which comprised (1.0 *p*mol/µg protein). Therefore, chlorpromazine has the strongest negative impact on sphingomyelinase action and phosphatidylcholine degradation.

4.4.12. Analysis of cell viability

Analysis of cell viability in different cultured cells is one of the important means of evaluating *in vitro* drug effects in cytotoxicity essays. Our conditions were not accompanied by a loss of cell viability as determined by total cell protein. One of the traditional methods of cell viability analysis is the CellTiter Blue (CTB) assay. As shown in figure 4.38, in the CellTiter Blue assay, a concentration of 40µM FTY720 and chlorpromazine show a decrease in cell viability of more than 50 %. Therefore, effects of these concentrations of the drugs on lipid metabolism cannot be considered.

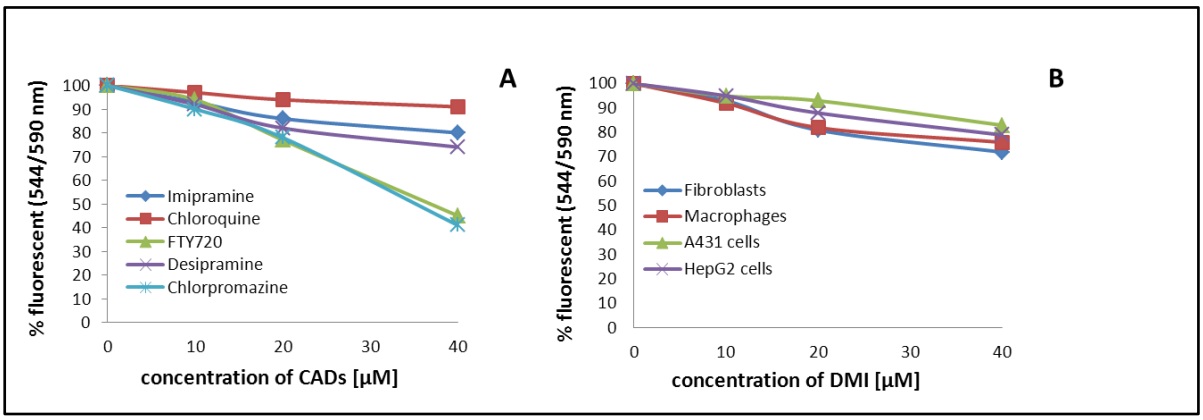

Figure 4.38: Cell viability assay in A: macrophages (CADs concentrations dependence), and B: different cell types in the presence of desipramine.

4.4.13. Analysis of lipid binding to LDL

The lipid-protein complexes were examined by gel chromatography. Separation by gel-filtration chromatography is based on differences in the size of the analyte molecules

(molecules with less access to the pore volume elute first, while the smallest molecules elute last). Lipid-LDL complexes were isolated through a sephacryl S-300 (pore size: 10-3000kDa), column size (2 x 20cm) and equilibrated with 10mM Tris-HCl (pH 7.5) containing 0.3mM NaCl, and 0.3mM EDTA as a running buffer, and the column was eluted with the same buffer. In gel chromatography column runs, more than 78% of the lipid radioactivity and more than 90% of the protein were recovered.

Figure 4.39 showed that in the [^{14}C]PC–LDL complex, the aggregation state was different from the pure [^{14}C]PC. The original homogeneous distribution of [^{14}C]PC aggregates changed to heterogeneous distribution after interacted with LDL molecules. This experiment was designed to show that [^{14}C]PC molecules readily bound with ApoB100 in LDL molecules to form [^{14}C]PC–LDL complex separated differentially from the sole [^{14}C]PC under identical experimental conditions.

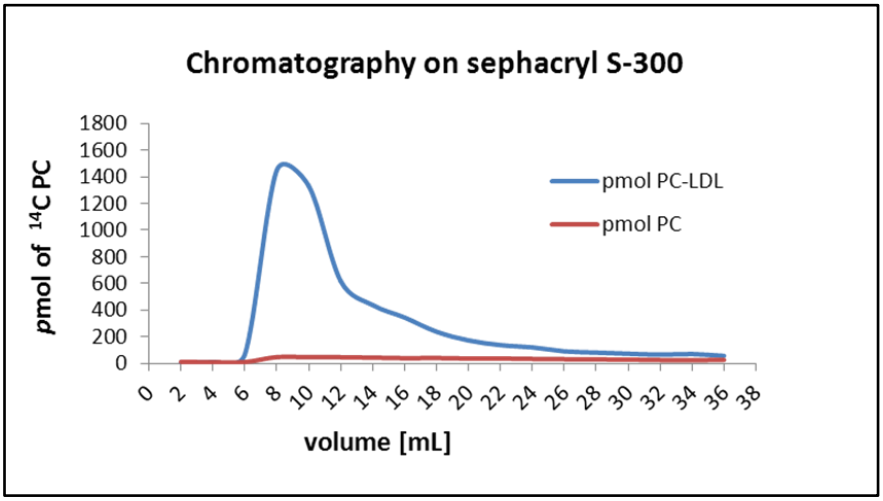

Figure 4.39: Gel chromatographic elution profile of [^{14}C]-PC-LDL complex and [^{14}C]-PC on Sephacryl S-300.

5. Discussion

5.1. Uptake and processing of C-18 fatty acid in cultured cells

As far as it is known, fatty acids are taken up without contribution of the endolysosomal system, and fatty acid probes themselves are not suitable to assay lipid uptake and processing via this compartment. Nevertheless, a series of labeled FAs of different structure (saturated, ω-3, ω-6, and ω-9) were used to get insight into their metabolic utilization in different types of cultured cells. The resulting incorporation pattern can be compared to those that arise from hydrolyses of FA-labeled complex lipids in the lysosomes. Based upon the data reported before in cultured keratinocytes (Schürer, et al. 1988), FAs were applied in a concentration of 10µM, at that concentration the uptake of all fatty acids was in the same range. At a lower concentration (0.1µM), the uptake of linoleic acid is reported to significantly exceed that of other FAs including stearic acid, oleic acid, and palmitic acid (Schürer, et al. 1988). In contrast, the uptake of stearic acid was significantly lower than that of the other FAs at concentrations of more than 10µM (Schürer, et al. 1988). In these and in our experiments, the reason for this might be linked to G-protein coupled receptors. For example OA binds to GPR43 (Briscoe, et al. 2003), and ω3-unsaturated FAs to GPR120 (Oh, et al. 2010; Ichimura, et al. 2012). Furthermore, in Huh-7 cells, OA stimulates rapid (15min) LD formation through activating the GPR120 receptor (Wakelam, et al. 2012).

To mimic the physiological situation, we applied the FAs in complex with BSA (Pütz, et al. 1995). The decision to use radiolabeled FAs is based on previous results from a series of tests with FA probes that were labeled with azide groups as chemical reporter groups or with stable isotopes. Both methods were not sensitive enough in our hands to determine the whole set of metabolic products on a background of endogenous lipids with mass spectrometry as readout. These control experiments with exogenously added radiolabeled FAs revealed that the metabolism of these lipid probes varies in part considerable with the number of Z-double bonds present in the probes, and with the cell type. Therefore, it is not possible to draw conclusions on the complete FA entry pathway with only one lipid probe and cell type.

The dissociation constants for various FA from the FA-BSA complexes were dependent upon the type of fatty acid and not dependent on the FA/BSA ratio (Demant, et al. 2002). The values of the rate of FA dissociation from BSA increase in the order stearic acid < oleic acid < palmitic acid ≈ linoleic acid < arachidonic acid at 37 °C (Demant, et al. 2002).

Although it is not expected that endosomes and lysosomes are involved in FA uptake and metabolism, the impact of a cationic amphiphilic drug (CAD), desipramine (DMI), on uptake and processing of SA, OA, LA, and LOA that is known to cause phospholipidosis in humans and animals by inducing premature degradation of endosomal acid sphingomyelinase were also analyzed. Furthermore, there are no data on the impact of DMI on FA processing in the literature.

5.1.1. FA uptake in cultured cells

In chapter 4, the basal uptake of different FAs by four different types of cultured cells was determined. Both, cell type and FA structure, influence FA uptake by confluent cultured fibroblasts, macrophages, A431 cells, and HepG2 cells. The cellular uptake of FAs is a regulated process; and unsaturated FAs have shown to induce formation of lipid droplets, presumably by activation of the G-protein coupled receptor GPR120 (Rohwedder, et al. 2011; Oh, et al. 2010; Ichimura, et al. 2012) and to stimulate FA uptake . For example, in placental trophoblast cells, FA uptake increases by 20–50%, when the cells were pre-incubated for 24h with 100mM long chain polyunsaturated FAs (LCPUFA). These FAs were preferentially incorporated into cellular phospholipids. Stimulation of FA uptake by LCPUFA corresponds to the increased expression of long-chain acyl-CoA synthetase (ACSL), indicating that acyl-CoA synthetase activity may be required for stimulation of FA uptake by LCPUFAs in this and in other cell types.

The nature of the binding of exogenously FA to cultured cells may be of similar nature of binding of exogenously added gangliosides reported previously (Saqr, et al. 1993). It was demonstrated that exogenous gangliosides are present in aqueous solution either as monomers, aggregates (oligomers), or micelles. These are incorporated into cells but are mainly adherent to the surface membrane. This form represents the major portion of exogenous lipids bound to the plasma membrane, and is loosely associated with the cell surface. Another part is attached to binding proteins as monomers or micelles that can only be removed by trypsination, or as monomers inserted into hydrophobic regions of the membrane. Therefore, exogenously added FAs and lipids which are generally recovered from the lipid extract of cells might contain a fraction, that is only adhered to the cells.

5.1.2. FA processing in HepG2 cells

The human hepatoma cell line (HepG2) is one of particular interest as the liver is the primary site for the synthesis of fatty acids. The HepG2 cell line has been shown to exhibit many functions of differentiated human liver cells, through the expression of secretory proteins such as albumin, transferrin and fibrinogen (Dixon, et al. 1993) and retains many hepatic metabolic functions, including lipoprotein and apolipoprotein synthesis and cholesterol metabolism (Javitt, 1990). We used the HepG2 cell line in this study as a model of fatty acid uptake and processing by liver cells.

In HepG2 cells, SA, OA, LA, and LOA are taken up at almost identical rates, and no significant differences in cellular uptake were noted among these FAs. These results are in agreement with (Dokko, et al. 1998). In the same cell type, we observed that the more double bonds the FAs contained, the more they are incorporated into LD lipids. In HepG2 cells, we observed that both saturated, and to a greater extent, unsaturated C_{18}-FAs, especially LOA, are efficiently incorporated in TAG, DAG, and CE. This is in agreement with previous work (Fujimoto, et al. 2006), which demonstrated that FAs with hydrocarbon chains of C_{12} - C_{18} (saturated and unsaturated long chain FAs) were accumulated in TAG and CE, which were concentrated in LDs. Moreover, hepatocytes and hepatic stellate cells play a central role in retinoid (Fig. 5.1A) or vitamin A ((2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic acid) storage and metabolism (Vogel, et al. 2000). To maintain plasma retinol levels, hepatocytes are responsible for the uptake of postprandial retinyl esters and are responsible for secretion of retinol bound to retinol-binding protein, since hepatocytes are essentially involved in maintaining circulating levels of retinol. Furthermore, the long-chain FA binding protein (L-FABP) binds a broad range of hydrophobic ligands, including FAs, and has a preference for unsaturated FAs as opposed to saturated FAs (McArthur, et al. 1999). L-FABP overexpression markedly increased the rate of FA uptake in hepatoma cells (Wolfrum, et al. 1999), and high level expression of L-FABP not only stimulated FA uptake, but also increased intracellular esterification of exogenously supplied FAs (Murphy, et al. 1996). Culture of three different hepatoma cell lines (HepG2; PLC/PRF 5; Mz-Hep-1) using [^{3}H]OA in the presence of various concentrations of albumin showed that cellular uptake of [^{3}H]OA over the initial 30sec incubation period was maximal, and the uptake of FAs by human hepatoma cell lines is, at least in part, mediated by a membrane FA binding protein (Stremmel, et al. 1989). These data suggest a significant role of fatty acid in regulating the incorporation into polar and nonpolar lipids by HepG2 cells.

The LD formation was effectively blocked by a long-chain acyl-CoA synthetase (ACSL) inhibitor, triacsin C (Fig. 5.1B); therefore, long chain FAs and ACSLs are significant factors for LD formation in hepatocytes. Also caveolae, but not clathrin-coated pits, have been implicated in uptake and intracellular trafficking of LCFA in HepG2 cells (Pohl, et al. 2002). Inhibition of caveolae formation by filipin III resulted in a reduction of [^{3}H]OA and [^{3}H]SA uptake by 54%, and 44%, respectively.

In our experiments, all FAs were more incorporated into polar lipids (PLs), especially into PC than into the other PLs, while the ratio of labeled PC/PE with SA and OA was less than with the other FAs. This is in agreement with previous work (Marra, et al. 1992), which demonstrated that when SK-Hep-1 cells were incubated with [^{14}C]LA, the unaltered FA was mainly incorporated into PC. Examination of the total FA of HepG2 cells revealed a high level of OA (Woldseth, et al. 1993). The ability of HepG2 cells to incorporate 0.66µM of different saturated and unsaturated FAs from the cultured medium was determined for following 1-[^{14}C]FAs: 16:0, 18:0, 18:2(n-6), 18:3(n-3), and 20:3(n-6) (Angeletti, et al. 1995). These results are in agreement with our results.

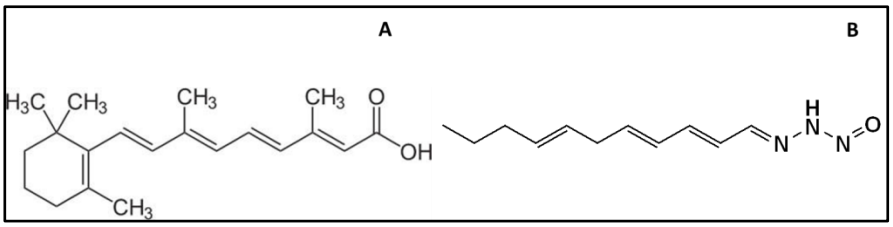

Figure 5.1: Chemical structure of A. retinoic acid, B. triacsin C.

5.1.3. FA processing in macrophages

The phospholipid FA compositions of cellular membranes alter their fluidity and induce changes in cell signaling leading to altered gene expression and lipid mediator production (Calder, 2010). In macrophages, the phospholipid FA composition influences processes such as apoptosis, production of cytokines, release of inflammatory mediators, and NO production. In macrophages, there is a strong positive correlation between phagocytosis and the content of polyunsaturated FA (Calder, et al. 1990). Macrophages enriched with polyunsaturated FAs showed 25-55% enhancement of phagocytic capacity. We observed that SA and OA are markedly incorporated in nonpolar lipids. Among unsaturated FAs, OA was more incorporated in nonpolar lipids, followed by LA and LOA. In contrast, we observed that LA and LOA are markedly incorporated in polar lipids compared to SA and OA. Furthermore, within the time frame used in our study, labeling of PC was always greater than that of PE,

except for LOA, which was incorporated into PE to a larger extent than into the other PL classes. These results are in agreement with previous work, which showed that in macrophages the content of polyunsaturated FAs in the PL fraction was higher than in the neutral lipid fraction (Calder, et al. 1990). Our results are also in agreement with previous results obtained in isolated rat liver cells, which show that FAs were incorporated in the PL fraction to a very different extent, and in the order 18:0 > 16:0 (Woldseth, et al. 1993).

5.1.4. FA processing in A431 cells

In A431 cells, the incorporation of OA, LA, and LOA into nonpolar lipids occurred predominantly into DAG, while SA was more incorporated into TAG than DAG (Fig. 4.3C). DAG originates either from the hydrolysis of phosphatidic acid, from TAG, or from PL hydrolysis. Among the nonpolar lipids, FAs were more incorporated into diacylglycerols than into TAG in fibroblasts. In macrophages and HepG2 cells, in which more incorporation into nonpolar lipids occurred, FAs were mainly incorporated into TAGs.

To explain these results, Pulse-chase studies were carried out. Results indicate that DAGs are only transiently formed during the formation of TAGs. During short-time pulse periods (2h) we observed a major incorporation of radiolabeled oleate into diacylglycerol in fibroblasts, and A431 cells. In the other cell types, the major incorporation of labeled oleate was observed into TAG. In chase experiments, the level of labeled triacylglycerol was increased after 72h incubation in macrophages and fibroblasts. Similarly, the labeled triacylglycerol increased with time in HepG2 cells, and A431cells. Moreover, the incorporation of labeled PC was decreased during the chase periods in all cell types (Fig. 4.7). These observations indicated a role of the Lands' cycle to remodel phospholipids during the chase period.

5.1.5. FA processing in fibroblasts

Different FAs were added to cultured human fibroblasts. These cells are easy to prepare and are available in larger quantities. Human skin fibroblasts readily take up exogenous free FAs and incorporate them into cellular TAGs and PLs (Spector, et al. 1979: Rosenthal, et al. 1981). We found that within two hours, human skin fibroblasts incorporate all long chain FAs studied into diacylglycerol. SA was less incorporated into DAG than the other unsaturated FAs, and compared to unsaturated FAs, more SA was detected in free form. A previous study (Rosenthal, et al. 1981) has shown a progressive incorporation into diacylglycerols in

fibroblasts incubated in the presence of exogenous long chain saturated FAs. Incubation of fibroblasts with saturated FAs lead to increased levels of neutral lipids into lipid droplets.

Into phospholipids, OA was incorporated to a greater extent than other FAs. LOA is less incorporated into PLs, while LOA was found to a greater extent in nonpolar lipids than the other FAs. Our results show that between 82% (SA) and 76% (LOA) of the applied FAs were incorporated into PLs and much less into TAGs (7.9% for SA and 12% for LOA). These results are in agreement with previous work (Rosenthal, et al. 1981), which showed that when G10-fibroblasts were incubated with 2µM concentration of different FAs for 24h, about 89% (SA) and 66% (LOA) of the applied FAs were incorporated into PLs, and much less into TAGs (8.3% for SA and 26% for LOA). Furthermore, in G10-fibroblasts, more [^{14}C]OA, [^{14}C]LA, and [^{14}C]LOA was incorporated into TAGs than radiolabeled SA (Rosenthal, et al. 1981). These values are also in agreement with our cell culture experiments

Previous studies in hamster fibroblasts with 0.2µCi/mL of 1-[^{14}C]-labeled fatty acid (Maziere, et al. 1982) are in agreement with our results. The authors demonstrated an enhanced incorporation of saturated FA into SM and ceramide compared to unsaturated FAs. OA was more incorporated into polar lipids than the other FAs, and its incorporation into phosphatidylglycerol (PG) was higher than that observed for the other FAs. Previous work in normal fibroblasts (Daniel, et al. 1980) revealed a lower incorporation of polyunsaturated FAs in ethanolamine glycerophospholipids in normal cells. This is in agreement with our results, which show that the unsaturated FAs were less incorporated into PE compared with saturated fatty acid. When human skin fibroblasts were incubated in serum-free medium with up to 100nmol/mL OA bound to albumin in a 4.6:1 ratio, the rate of [^{14}C]OA incorporation into TAG was approximately 5-fold higher than that of radiolabeled eicosapentaenoic acid (EPA). The mass of TAG formed after incubation of fibroblasts with EPA was also significantly lower than that formed with OA (Miller, et al. 1993). Among the C_{18}-FAs, human skin fibroblasts incorporate more OA than LA into membrane lipids. The percentage of exogenous FA incorporated is dependent on cell density, length of incubation, and concentration of serum protein in the culture medium, but not on the concentration of OA (Rosenthal, 1980). Uptake of LA and OA bound in varying molar ratios to albumin (ranging between 0.25:1 and 2:1, and in 173µM concentration in the medium) by dermal fibroblasts was linear over an incubation period of 5min, with similar low uptake rates for both OA and LA over the initial phase (Schurer, et al. 1994). Thus, fibroblasts demonstrate no initial rapid influx of FA, and FA-uptake in fibroblasts was more consistent with uptake entirely by

passive diffusion. It was suggested that the ability to synthesize TAG plays a critical role in protection from lipotoxicity (Listenberger, et al, 2003).

In fibroblasts, the incorporation of 10µM FA into polar and nonpolar lipids has shown to be higher than macrophages and HepG2 cells as shown in table 4.2. Previous studies demonstrated that overexpression of the FA binding proteins FATP1 and FABPpm in 3T3 fibroblasts (Isola, et al. 1995) not only stimulated FA uptake, but also increased intracellular esterification of exogenously supplied FAs (Murphy, et al. 1996). Furthermore, expression of FAT/CD36 in fibroblasts (Ibrahimi, et al. 1996) and muscle cells (Bastie, et al. 2004) increased FA uptake rates. On the other hand, caveolin-1 expression is necessary for post-translational stabilization and membrane expression of CD36. Caveolin-1 ablation in fibroblasts reduced the plasma membrane content of CD36 in parallel with a reduction of cellular FA uptake (Ring, et al. 2006). Furthermore, dysfunctional NPC1 (the protein involved in the subcellular trafficking of cholesterol out of late endosomes and lysosomes) does not lead to aberrant fatty acid efflux from late endosomes and lysosomes in human fibroblasts (Passeggio, et al. 2005). In contrast, NPC1 mutation shows a decreased incorporation of LDL-derived fatty acid into phospholipids in macrophages (Leventhal, et al. 2004).

5.1.6. The effect of the cationic amphiphilic drug, desipramine, on FA uptake and processing

Desipramine as a prototypical CAD is able to induce phospholipidosis in humans (Hurwitz, et al. 1994; Kölzer, et al. 2004; Nioi, et al. 2008; Reasor, et al. 2006). Influences of desipramine on FA uptake and metabolic incorporation into cellular lipids have not been reported before. Since desipramine targets lipid processing in the endolysosomal system, an effect of desipramine on uptake and processing of FAs was not expected. Therefore, it was a surprise that profound changes are produced in FA metabolism by desipramine, when administered in different concentrations to various cell types.

Other CADs have been reported (Fig. 5.2), such as tianeptin (Frumenty, et al. 1989) and amineptin (Le Dinh, et al. 1988), to inhibit mitochondrial β-oxidation of short and medium-chain FAs.

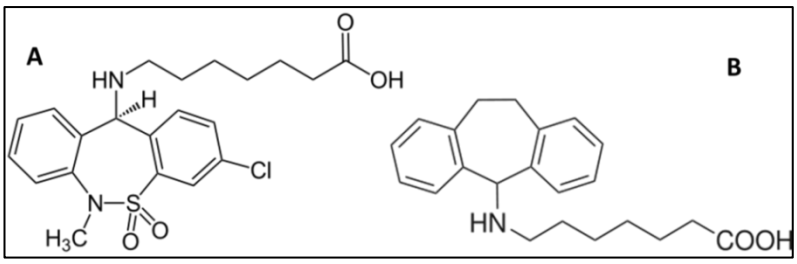

Figure 5.2: Chemical structure of A. tianeptin, B. amineptin.

CADs enter the cells and then lysosomes depending on the logP and pK_a-values within minutes to many hours (Kornhuber, et al. 2010). Desipramine has a fast lysosomal uptake kinetics (equilibrium within 30min), and a moderate lysosomal accumulation (accumulation ratio lysosome:extracellular < 100:1) (Kornhuber, et al. 2010). An effect of CADs on steps downstream of lysosomal depends on the applied CAD, on the identity of the FA, and on the cell type, as indicated by our experiments. In macrophages and A431 cells, treatment with desipramine leads to a drastically reduced incorporation of all FAs investigated into TAG and DAG, without reduction of FA uptake. In HepG2 cells, the effect of desipramine on saturated FA processing was less pronounced on the incorporation into TAG. 20µM desipramine decreases the incorporation of SA, OA, LA, and LOA into TAG by 10%, 14%, 35%, and 44%, but incorporation into polar lipids was significantly increased for unsaturated FAs 16%, 30%, 46%, and 54% when treated with 20µM desipramine, respectively (data not shown). In macrophages, pre-incubation with 20µM desipramine in the culture medium reduced the incorporation of radiolabeled SA, OA, LA, and LOA into TAG by 77%, 73%, 66%, and 45%, respectively. Also the level of intracellular free FA, and FA incorporation into DAG was reduced by 17%, 19%, 42%, and 44%, respectively, but to a lesser extent than into TAG (Fig. 4.9). In macrophages, when the concentration of desipramine increased, the incorporation of 1-[^{14}C]FA into neutral and acidic polar lipid increased in the presence of SA and OA, but is less affected with LA and LOA (data not shown). A possible explanation for the observed metabolic alterations is a reduced availability of DAG due to a desipramine-induced increase in endogenous sphingomyelin concentrations not only in the endolysosomal compartment, but also in the Golgi-apparatus (Fig. 5.4). This can drive the formation of PC from DAG, and deplete the DAG-pool available for TAG synthesis (Deevska, et al. 2009).

Another possible explanation is that phosphatidic acid phosphatase can be inhibited by desipramine in rat brain (Koul, et al. 1987) and by desipramine and other CADs in macrophages (Perry, et al. 1992). The resulting decreased availability of the DAG precursors might be the reason for the decreased incorporation of FAs into DAG, as shown in figure 5.3.

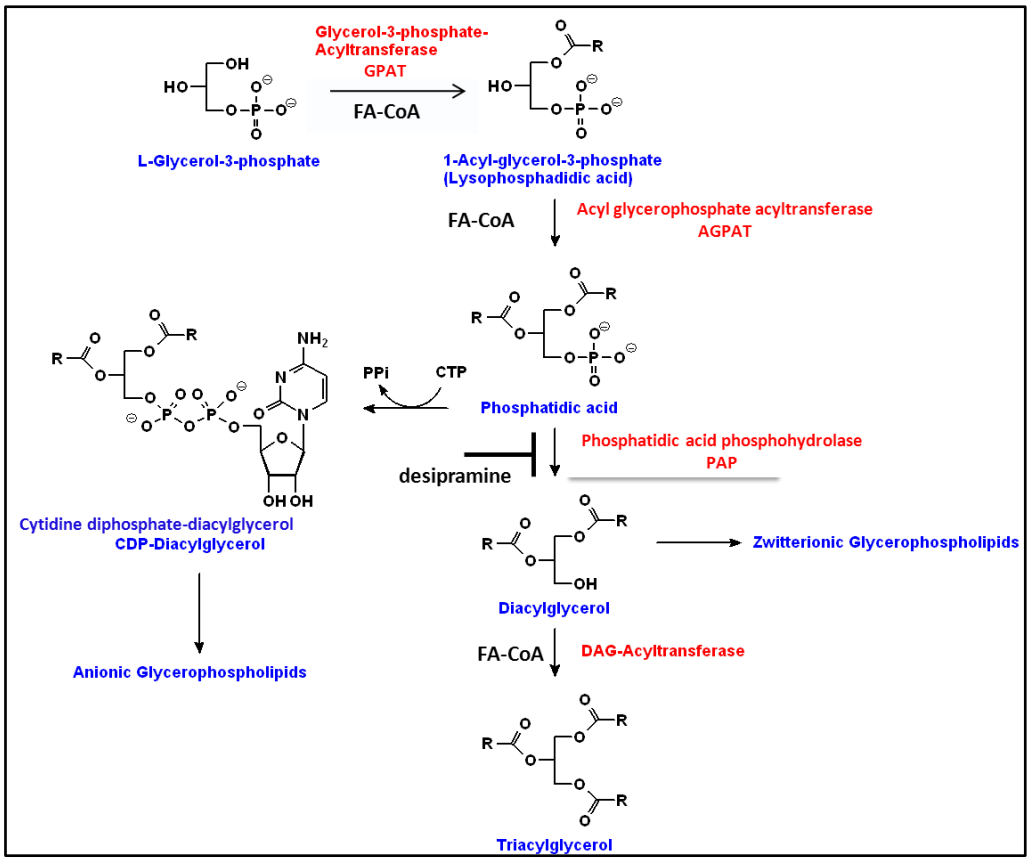

Figure 5.3: Desipramine-inhibits the activity of phosphatidic acid phosphohydrolase. This explains the decreased incorporation of FA into DAG, TAG, PC, and PE.

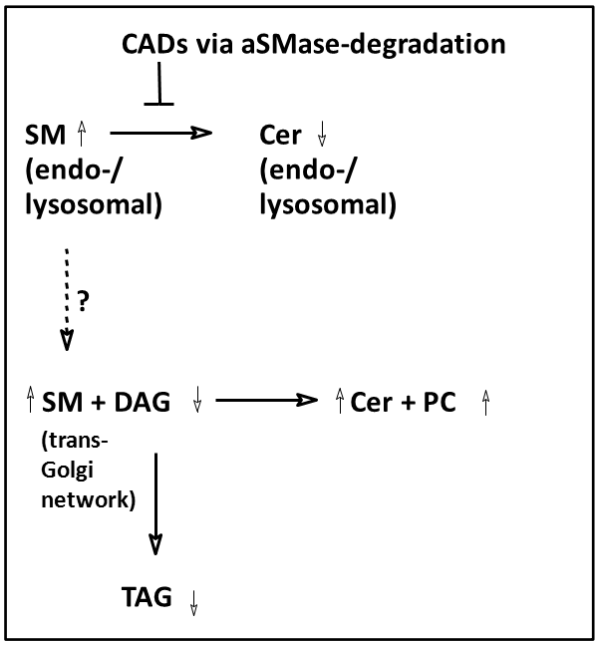

Figure 5.4: Model for the desipramine-induced reduction of incorporation of exogenous FAs into the neutral lipids DAG and TAG, and their increased incorporation into PC.

In A431 cells, 20μM desipramine reduced the incorporation of radiolabeled SA, OA, LA, and LOA into TAG by 97%, 83%, 94%, and 93%, respectively, while the levels of FAs incorporated into DAG were reduced by 74%, 29%, 49%, and 63 %, respectively (Fig. 4.8).
Unexpectedly, desipramine had a different effect on the uptakes of FAs in cultured fibroblasts. This was significantly increased in contrast to the other cell types (data not shown). The incorporation of the FAs into all analyzed lipids increased with increasing concentrations of desipramine. Treatment with 20μM desipramine increased the incorporation of SA, OA, LA, and LOA into TAG by 83%, 80%, 87%, and 88%, respectively, while the incorporation into DAG was increased by 65%, 77%, 73%, and 67%, respectively (Fig. 4.10). This is completely different from the uptake and processing by the other investigated cell types. The molecular reason for this phenomenon is unknown.
[^{3}H]-desipramine was used to measure desipramine uptake in the various cell types and to investigate if differential desipramine uptake accounts for the observed cell-type specific effects. Uptake of [^{3}H]desipramine by fibroblasts is much lower than by macrophages, A431 cells, and HepG2 cells (Fig. 4.13), so that this can only account for quantitative differences in its impact on FA processing. Antidepressants such as desipramine exhibit anticancer properties, although the mechanism for this is not clear (Ma, et al. 2011). Since some tumors cells depend on fatty acid uptake (Zhan, et al. 2008; Hess, et al. 2010), our new data on disturbance of fatty acid uptake in the presence of desipramine offer another possible mode of action of such antidepressants.

5.2. Analysis of two lipid delivery methods towards lipid uptake and processing

This work is the first comparative study of the uptake and metabolism of lipid probes delivered either as part of LDL-particles or in complex with BSA in four representative types of cultured cells. Despite the considerable attention devoted to studying the metabolism of LDL-lipids and apoB100, less is known about the uptake and metabolism of lipids associated with albumin. The ability of cargoes to bind to receptors at the cell surface is a fundamental cellular mechanism of specific transport into cells. LDL (from human) and delipidated BSA (from cattle) were loaded with [^{14}C]cholesteryloleate, [4-^{14}C]cholesterol, and [^{14}C]trioleoylglycerol. Incubation time of 6h or less led only to minor uptake and small values that were not high enough to allow reasonable experiments. 24h incubation time was suitable to demonstrate the significant differences in uptake and processing between the cell types. Therefore, this value was chosen for the experiments.

5.2.1. Cholesterol

Cholesterol is a major constituent of mammalian cell membranes. Hypercholesterolemia coupled with low HDL is a major risk factor for cardiovascular disease, CVD (Deng, et al. 2012). Accumulation of LDL-cholesterol in the blood is directly involved in the development of atherosclerosis. Most endocytic pathways like clathrin-dependent and clathrin-independent pathways are inhibited by the loss of cholesterol (Rodal, et al. 1999). Cholesterol itself shows only low solubility in blood and is transported in the circulatory system bound to lipoproteins. Therefore, in our study, we used low density lipoprotein as a carrier for cholesterol to address the receptor-mediated endocytic pathway, and compared it to albumin as an extracellular cholesterol carrier. Different types of cultured cells had an influence on the incorporation of the cholesterol into CE. This study asked to which extent the vehicle that delivers cholesterol to cells has an influence on its conversion to cholesteryl ester of lipid droplets.

It is known that LDL particles are internalized by cells via receptor-mediated endocytosis and are then sorted to endosomes and lysosomes. In our experiments, the uptake of [4-^{14}C]cholesterol delivered as LDL-complex is 3-fold higher in macrophages, 2.5-fold higher in fibroblasts, Hep-G2 cells, and A431 cells, when compared to [4-^{14}C]cholesterol delivered as BSA-complex. To explain these results, previous studies have reported that the LDL-receptor and the scavenger-receptor SR-BI have a specific role for the uptake of lipoprotein-associated cholesterol by various cell types (Urban, et al. 2000; Temel, et al. 1997; Goldstein, et al. 1987). In normal fibroblasts, the LDL receptor remained bound to LDL on the surface of cell membrane for less than 10min, within this time most of the surface bound LDL particles entered the cells, and the protein component of LDL was completely digested within another 60min (Goldstein, et al. 2009). SR-BI is participates in intracellular cholesterol trafficking (Silver, et al. 2001) and is also believed to facilitate the excretion of excess cholesterol into bile (Kozarsky, et al. 1997). Furthermore, the LDL particle is degraded in the endolysosomal compartment and free cholesterol is released by hydrolysis of cholesteryl esters in the core of LDL (Goldstein, et al. 2009). Cholesterol is sorted out of endosomes with the aid of the proteins NPC1 and NPC2 (Abdul-Hammed, et al. 2010), and then loaded on a cytoplasmic oxysterol-binding protein–related protein Orp-protein (Du, et al. 2011).

The incorporation of the 1µM [4-^{14}C]cholesterol delivered as LDL-complex into CE of macrophages is higher compared to other cell types. The incorporation of [4-^{14}C]cholesterol delivered as LDL-complex into CE of fibroblasts, macrophages, A431 cells, and HepG2 cells

were increased by 0.2%, 15%, 2%, and 4%, respectively (Fig. 4.18A). These results are in agreement with previous work (Wong, et al. 1987), which demonstrated that cholesterol is significantly esterified with endogenous FAs for the formation of cholesteryl ester droplet by macrophages. Furthermore, a previous study showed that in HeLa cells, BODIPY-labeled cholesterol analog (BODIPY-chol) moved from the plasma membrane (PM) to lipid droplets (LDs) with a half-time value of approximately 30min (Jansen, et al. 2011).

5.2.2. Cholesteryloleate

As shown in our results (Fig. 4.14), the uptake of [^{14}C]CE-LDL is 4-fold higher in fibroblasts and A431 cells, 3-fold higher in macrophages, and 2-fold higher in Hep-G2 cells during an 24h incubation at 37 °C, compared to [^{14}C]CE-BSA. Previous studies demonstrated that LDL-receptor and SR-BI are important for the delivery of LDL-CE to cultured cells (Minahk, et al. 2008). Minahk and co-workers demonstrated that LDL is internalized by cells via receptor-mediated endocytosis, in which CE-LDL complexes are taken up mainly by LDL receptor to 60-70% by primary hepatocytes. On the other hand, the uptake of the remaining 30-40% of CE-LDL complexes is mediated by the scavenger receptor class B, type I, which is considered an important receptor not only for LDL (Swarnakar, et al. 1999) but also for HDL (Rhainds, et al. 2003). Furthermore, various ligands, including native lipoproteins (LDL, VLDL, HDL, and chylomicrons) and modified lipoproteins (acetylated LDL, oxidized LDL, and oxidized HDL) are bound by specific amino acid residues of plasma membrane SRBI, which facilitates CE transport down its concentration gradient into the plasma membrane via a hydrophobic channel (Connelly, et al. 1999). SR-BI appears to have different binding sites for apolipoproteins and phospholipids (Urban, et al. 2000).

It has been observed that clathrin-dependent endocytosis is sensitive to low temperature (Wileman, et al. 1985; Baluška, et al. 2002). In our experiments, the effect of temperature on the lipid uptake was tested by adding lipid probe to cultured cells previously incubated at 4 ^{0}C for 30min and kept for a further 2h. The uptake of [^{14}C]CE, [^{14}C]Chol, and [^{14}C]TAG were significantly decreased by 83%, 78%, and 80%, when cells were incubated with the [^{14}C]CE-LDL, [^{14}C]Chol-LDL, and [^{14}C]TAG-LDL at 4 °C compared to control at 37 °C, respectively, (data not shown). These data demonstrate that the classical endocytic process mainly affected the uptake of [^{14}C]CE, [^{14}C]Chol, and [^{14}C]TAG and its transfer to early compartments, which is effectively inhibited by low temperature. These results support the

previous work (Minahk, et al. 2008) which demonstrated that LDL-receptor and SR-BI are important for the delivery of LDL-CE to cultured cells.

The metabolic processing of labeled oleic acid released from CE by lysosomal acid lipase in four types of cultured cells was followed. Liberated labeled oleate was incorporated significantly into glycerolipids, especially into phosphatidylcholine (Fig. 4.21B), and to a lesser extent into TAG. Similar results were previously reported in human fibroblasts (Groener, et al. 1996) and macrophages (Li, et al. 1995).

On the other hand, the incorporation of cholesteryl ester-derived [^{14}C]oleate delivered as BSA-complex into nonpolar and polar lipids is significantly lower in all cell types compared to cholesteryl ester-derived [^{14}C]oleate delivered as LDL-complex. Stromhaug *et al.* demonstrated that albumin is taken up by non-receptor fluid phase endocytosis (Stromhaug, et al. 1997) compared to the lipoprotein, which is taken up by receptor mediated endocytosis (Minahk, et al. 2008).

5.2.3. Triacylglycerol

A comparison of [1-^{14}C]trioleoylglycerol associated with LDL with that associated with BSA containing equimolar amounts by cultured cells has been investigated (Fig. 4.20). The experiments show that the incorporation of OA derived by the hydrolysis of TAG into other neutral and polar lipids was higher in macrophages compared to other cells. A possible explanation is that [1-^{14}C]trioleoylglycerol is hydrolysed by different enzyme capacities involved in different cell types. Previous studies have reported that J774 macrophages have very active triacylglycerol lipases that appear to be distinct from that of other cell types (Khoo, et al. 1984).

Our major findings are as follows: (i) lipid complexes with LDL show higher uptake compared to lipid complexes with albumin; (ii) LDL-lipids are metabolized differently than that delivered by BSA.

It is known that mammalian cells can take up macromolecules of a considerable size range. It has been suggested that within limits, an increase in molecular size may determine an increase in cellular uptake (Ryser, 1967). Since albumin and low density lipoprotein have not too different isoelectric points (4.7 and 5.4, respectively), but different molecular weight (66.5kDa and 3000kDa, respectively) (Chaiyasut, et al. 2001; Jachimska, et al. 2008), it is likely that the molecular size is a contributing factor in this case. Key factor, however, appears to be that a receptor-mediated pathway exists for LDL (Goldstein, 2009).

In contrast to FAs bound to BSA, many data suggest that there BSA-lipid complexes are taken up by the cell through a process independent of receptor-mediated endocytosis (Synnes, et al. 1999; Kobuna, et al. 2010; Berger, et al. 1994). Therefore, it is understandable that uptake and metabolism of the examined lipid probes depend on the mode of the delivery (LDL, BSA). These findings indicate that LDL-associated (CE, TAG, and Chol) are taken up into cells via a pathway that differs from that of BSA-associated (CE, TAG, and Chol). Essentially, lipid-BSA complexes were taken up at a lower rate compared to lipid-LDL complexes. With the exception of FA-BSA complexes, BSA-lipid complexes are largely taken up by fluid-phase endocytosis (Stromhaug, et.al 1997). Furthermore, we suspect that both the oleate derived from cholesteryl oleate and trioleoylglycerol delivered as LDL, are processed by the cell in a similar fashion. Only slightly different pattern were observed (Fig. 4.21) for metabolic products of cholesteryl oleate and trioleoylglycerol. We observed that there was a difference in the distribution of oleate among phospholipids even though oleate in cholesteryl oleate and trioleoylglycerol was delivered by the same carriers. A previous study (Teruya, et al. 1995) demonstrated different rates of lysosomal hydrolysis of oleate from cholesteryl oleate and from trioleoylglycerol in the lysosomes. Also in our experiments, free oleate delivered as albumin complexes (Fig. 4.3) shows pattern different from oleate derived from cholesteryl oleate and trioleoylglycerol delivered as LDL. Oleate delivered as trioleoylglycerol and cholesteryl oleate must first be hydrolyzed from the lipid probes, and can then be converted to fatty acyl-CoA for esterification. On the other hand, free oleate enters the cytoplasm of the cell without any metabolic step and is converted to fatty acyl-CoA for esterification into cellular complex lipids. The extra metabolic step in the lysosomes may account for differences in the rate of cellular metabolism of oleate. Moreover, the uptake of fatty acids from lipoproteins is receptor mediated, as opposed to the uptake of free oleate bound to albumin, which is predominantly non-receptor mediated uptake (Rosenthal, 1987). Furthermore, in both lipid probe delivered as LDL and free oleate delivered as BSA, the incorporation of oleate was predominantly into PC with all cell types. These results are also in agreement with previous results in human umbilical vein endothelial cells (Teruya, et al. 1995).

5.3. Analysis of lipid processing in Niemann-Pick A cells compared to normal fibroblasts.

Lysosomal storage diseases are a family of at least 40 inherited metabolic disorders that collectively have an incidence of 1 per 7700 live births (Meikle, et al. 1999). In most lysosomal storage diseases, multiple substrates accumulate. Some result from a distinct blockage in a metabolic pathway, whereas others appear to be secondary. Cholesterol accumulation represents a secondary metabolic defect that is observed in human patients with NPA disease, since sphingomyelin has a strong affinity for cholesterol (Leventhal, et al. 2001; Lönnfors, et al. 2011). Once sphingomyelin is degraded by lysosomal ASM, cholesterol can efflux from the lysosomal compartment (Leventhal, et al. 2001, Abdul-Hammed, et al. 2010).

Our results indicate that the processing of cholesterol and phosphatidylcholine through NPA is slightly reduced compared to normal cells. Nutrient uptake via endosomes and lysosomes is an important function of these organells. In mouse models of both GM1 and GM2 gangliosidoses, an impaired iron uptake via the endolysosomal system leads to progressive depletion of iron in brain tissue. Administration of iron prolonged survival in the diseased mice by up to 38% (Jeyakumar, et al. 2009). Our results indicate that also lipid uptake and processing is impaired in NPA, which might contribute to pathogenesis.

5.4. Effect of cationic amphiphilic drugs on uptake and processing of choline-containing phospholipids by cultured cells

5.4.1. Effect of CADs on SM uptake and processing

Several side-effects of clinically used CADs can be attributed to a drug-induced impairment of lysosomal function. It is known that the antidepressant desipramine leads to premature degradation of acid sphingomyelinase in the endolysosomal compartment and subsequently to SM accumulation (Kölzer, et al. 2004). Desipramine interferes with the binding of acid sphingomyelinase (ASMase) to intraendosomal membranes and lipid aggregates and thereby displaces the enzyme from the membranes where it is protected from degradation (Kölzer, et al. 2004). ASMase contributes to lysosomal SM turnover and is also secreted upon cellular treatment with inflammatory stimuli (Smith, et al. 2008; Gulbins, et al. 2003). Desipramine remains a useful medication for the treatment of several conditions, although many other

antidepressants have been developed since 1964. Displacement of ASMase from intraendosomal and intralysosomal membranes can be expected also for the antidepressant imipramine that shows a structure similar to desipramine, and has also been reported to lead to an apparent reduction of ASMase activity (Albouz, et al. 1981; Jensen, et al. 1999). The neuroleptic drug chlorpromazine interferes with clathrin-mediated endocytosis by inhibiting the relocation of clathrin (Wang, et al. 1993), so that it had to be expected that the amount of incorporated SM delivered as LDL-complex was reduced in the presence of this drug. Chlorpromazine is a cell-permeable CAD that inserts into lipid bilayers and binds to anionic lipids including polyphosphoinositides, and in this way influences a variety of cellular processes that depends on phosphoinositides (De Filippi, et al. 2007). Chloroquine has been reported to block LDLR recycling to the plasma membrane (Minahk, et al. 2008), and to an apparent inhibition of acid ceramidase (Elojeimy, et al. 2006). Chloroquine has been demonstrated to elevate the intracellular pH, thus altering lysosomal function and perturbing endosomal/recycling pathways (Anderson, et al. 2006). Moreover, chloroquine can affect the activity of lysosomal phospholipases (Matsuzawa, et al. 1980) that have an optimal pH in the acidic range. FTY720, a functional antagonist of one of the receptors of sphingosine-1-phosphate and currently used for the treatment of multiple sclerosis leads to an apparent inhibition of acid sphingomelinase by a mechanism similar to that exerted by tricyclic antidepressants (Dawson, et al. 2011). FTY720 and chlorpromazine also have been reported to inhibit ceramide synthases (Berdyshev, et al. 2009). It is noteworthy that we have only measured the affect of cationic amphiphilic drugs in hours, and the treatment of human patients with cationic amphiphilic drugs can be over many years. For example, most patients take desipramine for 6 months to a year.

For all cell types, we observed a dose-dependent (10, 20 and 40µM) and time-dependent reduction of the incorporation of SM-derived stearic acid and PC-derived palmitic acid into nonpolar lipids and polar lipids.

The impact on phospholipid processing of the investigated CADs was assessed at 20µM and 40µM concentration, because some changes in phospholipid turnover were clearly visible only at these concentrations. The effect of the drugs on PC and SM processing were dependent on its concentration: at 10µM, the drugs did not significantly alter lipid metabolism. High concentrations of more than 20µM chlorpromazine and FTY720 were found to be toxic. Concentrations of the CADs can reach millimolar levels in the lysosomal lamellar bodies isolated from the liver of rats treated with the drugs (Hostetler, et al. 1985). Prolonged incubation of the cells with PC-containing LDL or SM-containing LDL was

necessary to detect changes in the incorporation of PC-derived palmitic acid or SM-derived stearic acid into cellular lipids. Experiments with incubation time of 6h or less showed only effects too small to be observable, while 24h incubation time was suitable to demonstrate the impact of CADs on PC or SM uptake and metabolism.

In our experiments, all investigated drugs lead to an impaired SM processing in macrophages, but to a different extent. The incorporation of SM-derived 1-[^{14}C]stearic acid into neutral lipids (triacylglycerol and diacylglycerol) and into different glycerophospholipid classes was drastically reduced in the presence of the drugs.

Decreased degradation of exogenously added SM in the presence of FTY720, and also enhanced levels of SM-derived ceramide are in agreement with a premature degradation of acid ceramidase. Since increase of intracellular ceramide concentrations has been claimed to be a general measure to treat cancer (Radin, et al. 2004), the investigated CADs might be combined with radiation or chemotherapy to enhance ceramide levels and produce a more potent therapeutic effect.

A previous study demonstrated that FTY720 reduces cholesterol toxicity in primary human macrophages, e.g. by facilitating its release to extracellular physiological acceptors (Blom, et al. 2010). Our results show that the incorporation of SM-derived 1-[^{14}C]stearic acid or PC-derived [^{14}C]palmitic acid into cholesterylstearate or cholesteryloleate were drastically reduced in the presence of the 20µM FTY720. Our experiment shows that CE decrease 48% and 67% in the presence of 20µM FTY720, respectively, compared to control.

The different drugs used by us caused phospholipidosis in terms of labeled SM accumulation to a different extent. For example, macrophages showed a 54% and 21% increase in labeling of SM in the presence of 20µM chlorpromazine and FTY720, respectively, in the culture medium, and a 25% and 52% increase in the presence of 20µM imipramine and desipramine, respectively. When treated with 40µM of imipramine or desipramine, levels of labeled SM increased by 34% and 68%, respectively. These results indicate that our method is able to detect metabolic steps that are affected in the presence of CADs and to predict the potential of CADs to induce phospholipidosis.

The cells also showed increased levels of radiolabeled ceramide, when treated with 20µM desipramine, imipramine, and FTY720. This was not observed when the cells were treated with chlorpromazine and chloroquine. Increased levels of ceramide can result from reduced degradation of ceramide by acid ceramidase. FTY720 also causes a drastically reduced incorporation of SM-derived stearic acid into triacylglycerol. This was observed in all examined cell types supplemented with an SM-LDL complex at 1µM.

Macrophages treated with 20µM desipramine showed the similar effect on the incorporation into TAG, when [^{14}C]stearic acid or SM-derived 1-[^{14}C]stearic acid are used as a tracers. Previous experiments by us, in which macrophages were incubated with 1-[^{14}C]stearic acid, showed a substantial labeling of TAG (Fig. 4.3B). This labeling was reduced significantly by 62% and 54% in the presence of 20µM desipramine when cell treated with1-[^{14}C]stearic acid (Fig. 4.9) and SM-derived 1-[^{14}C]stearic acid (Fig. 4.26A), respectively. These data suggest that desipramine may affect the activity of phosphatidic acid-phosphatase (Koul, et al. 1987; Perry, et al. 1992) by decrease the incorporation of SM-derived 1-[^{14}C]stearic acid into TAG, as shown in figure 5.3.

Furthermore, in macrophages, CADs reduce the incorporation of radiolabeled stearate into TAG by 47%, and 20% with 40µM desipramine and imipramine, respectively, while further increasing that in SM by 68%, and 34%, respectively. A previous study reported that desipramine affects the *de novo* synthesis of sphingolipids by reducing the incorporation of radiolabeled fatty acid into TAG by 40% while further increasing that in ceramide and sphingomyelin (Deevska, et al. 2009) when HepG2 cells are supplemented with palmitic acid at different concentrations ranged between 0.1 - 1.0mM .

5.4.2. Effect of CADs on PC uptake and processing

PC is the most abundant phospholipid in numerous cell types and is generally thought to be essential for membrane structure and cellular function. Since there is only limited information on the fate of LDL-derived PC in cultured cells and the effect of CADs on their uptake and processing, we studied uptake and processing on LDL-derived PC in different cultured cells in the presence of CADs. Incorporation of PC-derived [^{14}C]palmitic acid into polar and nonpolar lipids of different cell types were studied in the presence of chlorpromazine (CPZ), chloroquine (CQ), desipramine (DMI), imipramine (IM), and fingolimod (FTY720).

Recent evidence had indicated that the consumption of diet rich in saturated fats, palmitate (but not other free fatty acids), can stimulate not only the rate of TAG accumulation but also stimulation of the *de novo* synthesis of ceramide, SM and generate excessive amounts of sphingolipid metabolites in a variety of cells (Deevska, et al. 2011). The flux through the sphingolipid synthetic pathway depends upon the availability of palmitate (Merrill, et al. 1988). Serine palmitoyl transferase (SPT) catalyzes the rate-limiting step in the synthesis of sphingolipids (Ikushiro, et al. 2011). Inhibition of SPT in animals by myriocin blocks the flux of palmitate through the *de novo* pathway (Holland, et al. 2008).

Previous studies demonstrated that dipalmitoyl PC is associated with phospholipidosis induced by amphiphilic drugs (Chatelain, et al. 1985; Miles, et al. 1986). Various amphiphilic drugs either bind to the hydrophobic moiety or hydrophilic head group of dipamitoyl PC with different potencies. The order of binding strength was chlorpromazine > imipramine > chloroquine (Joshi, et al. 1988). The drugs may interact with either or both of the hydrophilic and lipophilic sites of dipalmitoyl PC. Chlorpromazine and imipramine showed two binding affinities of these drugs to dipalmitoyl PC, whereas chloroquine displayed a single binding affinity site to dipalmitoyl PC (Joshi, et al. 1988).

In cultured macrophages, the incorporation of PC-derived palmitic acid into TAG was increased in cells pre-treated with 10μM desipramine (Fig. 4.27A). Minahk and co-worker (Minahk, et al. 2008) demonstrated that in cultured primary hepatocytes, 50% of LDL-[^{3}H]oleate-PC is converted to triacylglycerol via phospholipase C and DGAT2 rather than via lysosomal degradation. A recent study also demonstrated that PC plays a significant role to supply DAG for TAG synthesis via a PLC- mediated pathway (Robichaud, et al. 2009) , since desipramine has been demonstrated to stimulate PLC activity (Bouron, et al. 1999). Chinese hamster ovary cells can metabolize lipoprotein-associated PC to generate DAG by PLC that can be directly incorporated into TAG without prior formation of phosphatidic acid as the precursor (Igal, et al. 2001).

Our results show that after 24h incubation of cultured macrophages with 40μM DMI, the total phospholipid labeling of DMI-treated macrophages was increase compared to the untreated cells (Fig. 4.27B). More specifically, a marked accumulation of label in PC, phosphatidic acid (PA) and lysoPC (LPC) occurred in a concentration-dependent manner in the DMI-treated macrophages. We also observed a significantly decrease in the incorporation into TAG by 39%, and a 56% increase into PA with 40μM DMI compared to control. Several cationic amphiphilic drugs have been shown to inhibit soluble phosphatidate phosphohydrolase in C6 glioma cells (Leli, et al. 1987), rat liver, and rat brain (Koul, et al. 1987). These data suggest that desipramine at 40μM may affect the activity of PA phosphohydrolase with increased formation of phosphatidyl-CMP, the intermediate for the synthesis of acidic phospholipids (F.g. 5.3). This may explain the marked increase in PA and decrease in DAG labeling, which are translated into decreased labeling of PE and TAG when treated with 40μM DMI (Fig. 4.27B). The cell viability in the presence of 40μM desipramine was reduced 28% compared to untreated cells (Fig. 4.38); the amount of cellular protein was reduced by 8%. Therefore, the reduced incorporation of FAs into TAG and DAG in the presence of 40μM desipramine in macrophages cannot be attributed only to toxicity.

In cells pre-treated with CPZ or DMI for 24h, levels of labeled PC were increased 3- and 2-fold in the presence of 20µM CPZ or DMI, respectively. Furthermore, all drugs significantly reduced the amount of label in SM compared to the control cells and the experiments clearly show reduced processing of exogenously added PC with higher concentration of drugs. Some increase in FA was seen in macrophages treated with FTY720.

In macrophages, the recovery of [^{14}C]stearic acid derived from SM and [^{14}C]palmitic acid derived from PC as free FAs was significantly higher in cells treated with 20µM FTY720 than in cells treated with other CADs. One hypothetical explanation is that FTY720 might cause an elevated intralysosomal pH and lead to a lower degree of protonation of the fatty acid, which, in turn, will cause the fatty acid to diffuse more slowly across the lysosomal membrane since the pKa values of FTY720 is lower (7.8) than those of the other CADs, which have pKa values of 10.4, 9.4, 9.2, and 8.1 for desipramine, imipramine, chlorpromazine, and chloroquine, respectively. Other possible explanation assumed that this might be caused by the formation of complexes between positively charged FTY720 and the negatively charged fatty acid, which become trapped in the lysosomes.

Despite the difference in chain length of only two methylene groups, the saturated FAs present in our PC- and SM- probes should not necessarily undergo the same metabolic reaction. The metabolic fate of a saturated fatty acid derived from PC was significantly different from that derived from SM. For example, levels of palmitic acid derived from PC incorporated into TAG are increased in the presence of 10µM, and 20µM desipramine, while the opposite was observed for SM in the presence of desipramine. Furthermore, the incorporation of PC-derived [^{14}C]palmitic acid into TAG decreased slightly, but not significantly in fibroblasts and A431cells pre-treated with chlorpromazine compared to controls, while there were no significant differences in the incorporation of PC-derived [^{14}C]palmitic acid into TAG in HepG2 cells pre-treated with different concentrations of chlorpromazine and untreated control cells (data not shown).

In general, CADs are lysosomotropic agents that accumulate in lysosomes, can lead to pH elevation of the acidic compartment, and to the mis-localization of soluble lysosomal enzymes (Nanoyams, et al. 2008). Some CADs induce an inhibition of phospholipid catabolism in lysosomes and increase cellular phospholipids in human and other mammalian tissues. Effects of CADs on steps downstream of lysosomal release of the fatty acid from the lipid probe depend on the applied CAD, on the identity of the fatty acid and the cell type, as indicated by previous experiments. Independent studies in cultured primary hepatocytes demonstrated that PC present in LDL is taken up not only by LDL receptors (30%) and

scavenger receptors (class B, type I) (20-30%), but also by additional unknown mechanisms that are responsible for the remaining (40-50%) (Minahk, et al. 2008). Therefore, CADs might impact only processing of that fraction of LDL-derived PC, which enters the acidic compartments and not on that fraction for which the interaction between LDL and its receptor proteins are irrelevant (Truong, et al. 2000). Ishikawa *et al.* showed that in smooth muscle cells only a fraction of LDL-PC was degraded in lysosomes. They found 25% of LDL-derived PC in the lysosome-rich fraction and 25% in the cytosol-rich fraction. They concluded that LDL-derived PC was sorted equally between lysosomal and extralysosomal compartements (Ishikawa, et al. 1989) and also suggested that phospholipase A on plasma membranes, which can hydrolyze LDL-PC before LDL binds to LDL receptor, might affect the apoB epitope and the cellular uptake of LDL via the receptor. In addition, the authors found that phospholipases A1 and A2 are optimally active at neutral or alkaline pH ranges as well and might react with LDL-PC (Ishikawa, et al. 1988).

6. Material and Methods

6.1. Material

6.1.1. Analytical equipment

Autoclave	Systec V-150, Wettenberg, Germany
Blotter	Mini-TransBlot, BioRad, München, Germany
Centrifuges	Eppendorf, Hamburg, Germany
	L 8-80, mit SW-28-Ti-Rotor, Beckmann, Palo Alto, USA
Centrifuge tubes	12 mL: Costar, Cambridge, USA
	15 mL: Greiner, Nürtingen, Germany
	50 mL: Falcon/BectonDickinson, Bedford, USA
Glass tools	Schott-Duran, Jenaglas, Mainz, Germany
Incubator	Binder CO_2, Tuttlingen, Germany
Microscope	Helmut Hund GmbH, Typ h500, Germany
pH-meter	pH 537, WTW, Weinheim, Germany
Phosphoimager	Fujix BAS 1000 Bio Imaging Analyzer, Raytest, Straubenhardt, Germany
Phosphoimaging plates	^{14}C-screen BAS MS 2040, ^{3}H-Screen BAS Tr 2040, Raytest, Straubenhardt, Germany
Scintillation counter	Tri-Carb 2900TR, Packard Instruments Co., Inc, Downers Grove, USA
Shaker water bath	Gesellschaft für Labortechnik, Burgwedel, Germany
Sterile benches	LaminAirHA 2472 GS, Heraeus, Düsseldorf, Germany
TLC-tank	Desaga, Heidelberg, Germany
TLC-heater	TLC-Plate Heater III, Camag, Berlin, Germany
Ultrasound bath	Sonorex RK 100, Bandelin, Berlin, Germany
Heating-stirring module	Pierce, Therm III, Rockford

FLUO-STAR plate reader	BMG, Germany
Cell culture hood	Lamin Air, HB 2448, Heraeus, Hanau
Ultrasound	Cap horn, Sonifer B-12, Branson Ultrasonic Corp., Danbury, USA
Vortex	MS Minishaker, IKA-Werk, Staufen, Germany
Water purification	Millipore-Pelicon filtration device with polysulfone filter cassette, Millipore, Molsheim, France
Balance	Sartorius AG, Göttingen, Germany
Micropipette	Eppendorf
Pipette controllers	Brand, accu-jet, Wertheim, Germany
Oven	Memmert, Germany
Washer disinfectors for laboratory glassware	Miele G7783, Gütersloh, Germany
Multiskan photometer	Thermo scientific, Ascent, Vantaa, Finland
Water bath	GFI, Burgwedel, Germany
Refrigerator (4 ^{0}C, -20 ^{0}C)	Liebherr, Switzerland

6.1.2. Consumables and chemicals

Centrifuge Falcon tube (15 mL, 50 mL)	Greiner Bio-one, Solingen
Safe-lock tube (0.5mL, 1.5mL, 2mL)	Eppendorf, Germany
Glass fiber wadding, silanized	Macherey-Nagel, Düren, Germany
Micropipette tips (20µL, 200µL, 1000µL)	Brand, Wertheim, Germany
Cell culture petri-dish	BD Falcon, USA
Cell culture flask	BD Falcon, USA
Vivaspin, 3.000 MWCO	Sartorius, Germany
96-microplates	Greiner Bio-one, Solingen
Stripette, serological pipettes	Sigma-aldrich, Germany
Pasteur pipette	VWR, Germany
Bovine serum albumin	Sigma, Taufenkirchen, Germany
Low density lipoprotein	Invitrogen
Lipoprotein deficient serum	Sigma, Germany
DEAE sephadex A-25	GE Healthcare, Uppsala, Sweden
Sepharose CL-2B	GE Healthcare, Uppsala, Sweden

Copper (II) sulfate solution	Sigma-Aldrich, Germany
Bicinchoninic acid solution	Sigma-Aldrich, Germany
LiChroprep® RP18 (40-63 μM)	Merck, Darmstadt, Germany
Lipid standards	Sigma, Taufkirchen, Germany
Screw cap glass	Pyrex, BibbySterlin Ltd, Stone, Great Britain
Sterile filters	Sartorius, Göttingen, Germany
Scintillation polyethylene vials (6mL, 20mL)	Perkin Elmer, USA
TLC plates (glass coated with silica gel 60)	Merck, Darmstadt, Germany
Tris-(hydroxymethyl)-aminomethan Hydrochloride (Tris-HCl)	Applichem, Darmstadt, Germany
Acetic acid 99.8%	BDH Prolabo, Darmstadt, Germany
Chloroform	Fisher chemical
Ethanol 100%	Merck
Methanol	J.T.Baker
Diethyl ether	BDH Prolabo, Darmstadt, Germany
n-Hexane	Merck

6.1.3. Radiolabeled markers

[4-^{14}C]Cholesterol	Perkin Elmer, Boston, USA
Cholesteryl [^{14}C]-oleate	Perkin Elmer, Boston, USA
[^{14}C]Trioleoylglycerol	Perkin Elmer, Boston, USA
[1-^{14}C]-Stearic acid	GE Health Care, Amersham, UK
[1-^{14}C]-Oleic acid	GE Health Care, Amersham, UK
[1-^{14}C]-Linoleic acid	American Radio Labeled, USA
[1-^{14}C]-Linolenic acid	Perkin Elmer, Boston, USA
Phosphatidyl ethanol amine, [1-^{14}C-dioleoyl]	American Radio Labeled, USA
Phosphatidyl choline, [L-α-1-^{14}C-dipalmitoyl]	Perkin Elmer, Boston, USA

Phosphatidyl choline, [1-^{14}C-dioleoyl]	Perkin Elmer, Boston, USA
Sphingomyelin [Stearoyl-1-^{14}C]	American Radio Labeled, USA
Desmethyl imipramine hydrochloride, [benzene ring, 10, 11-^{3}H]	Perkin Elmer, Boston, USA

6.2. Biological materials

6.2.1. Cells and additives

Human skin fibroblasts	Kinderklinik St. Augustin, Germany
Mouse leukaemic monocyte macrophages, (Raw264.7)	DSMZ, Braunschweig, Germany
A431 cells	DSMZ,Braunschweig, Germany
Hep-G2 cells	DSMZ, Braunschweig, Germany
Niemann Pick A cells	Universitätsklinik, Münster, Germany
DMEM, RPMI	Gibco, Eggenstein, Germany
Foetal calf serum (FCS)	Cytogen, Berlin, Germany
Leupeptinhemi sulfate	Sigma, Taufkirchen, Germany
Streptomycin	Sigma, Deisenhofen, Germany
Trypsin	Cytogen, Berlin, Germany
N-Acetyl-L-Alanine-L-Glutamine	Seromed, Biochrom, Berlin, Germany

6.2.2. Kits

CellTiter Blue (CTB) assay	Promega, Germany

6.3. Methods

6.3.1. Cell culture

Fibroblasts, macrophages, and Hep-G2 cells were grown in DMEM, A431 cells were grown in RPMI-1640, supplemented with 2mM glutamine, 10% (v/v) heat-inactivated fetal bovine serum, and 100mg/L streptomycin. Cells were collected from liquid nitrogen storage (appropriate protective equipment). The frozen screw-cap vials were thawed immediately in a water bath at 37 ^{0}C and the vials were wiped with 70% ethanol before transferring to the cell culture hood to reduce bacterial contamination. The cell suspensions in the vial were

transferred to a 15mL Falcon tube with 2mL FCS. The cells were centrifuged at 300g for 5min at room temperature. After the supernatant was discarded, 1mL 10% DMEM was added and mixed with the cells by pipetting. The cell mixture was transferred to 25cm^2 flasks containing 1mL FCS and mixed. Growth medium (5 mL 10% DMEM) was added drop-wise and the flasks were incubated at 37 ^{0}C in a humidified atmosphere of 95% air and 5% CO_2. After reaching a confluent state, the cells were sub-cultured 1:2 in 75cm^2 culture flasks. For passaging of cells, the medium was removed and the cells were washed with PBS to remove the residual medium. To detach them from the flasks surface, the cells were incubated for 1-2min with 2mL trypsin/EDTA at 37 °C, upon gently shaking. 3mL DMEM containing 10% FBS was added to stop the digestion and pipetted to detach all cells from the flask wall. The cell suspension was transferred to a new Falcon tube and the cells were centrifuged at 300g for 5min, the supernatant was discarded, and the cells were re-suspended in growth medium. For our experiments, cells were grown until confluence in 35mm Petri-dishes or 25cm^2 flasks and were seeded to a density of 1 x l0^6 cells/mL (average count per square*dilution factor*10^4). The medium was replaced with fresh culture medium every 2-3 days. Cells were examined using an inverted microscope to check confluence and confirm the absence of contaminations. All procedures with cells were carried out under sterile conditions using aseptic techniques.

6.3.2. Protein determination

Cell protein was determined using the Bicinchoninic acid method with bovine serum albumin (BSA) as a standard (Smith et al., 1985). Prior to lipid extraction, cell pellets were homogenized 3 times (30sec) by ultrasonic treatment (120W) in 800µL of water, and aliquots were used for protein determination. 5µL of cell homogenate were diluted to 20µL with water in a multiwell plate (96-micro-plates). 200µL of freshly prepared reagent working solution (BicinChoninic Acid/copper (II) sulfate 50:1 by vol.) were added. After 30min incubation at 60 ^{0}C, the absorption of samples was measured at 562nm. Protein content was calculated on the basis of a standard curve, obtained by diluting BSA standard (0.25-5µg BSA/20µL).

6.3.3. Lipid extraction and analysis

Cell pellets were suspended in 0.86mL water by ultrasonic treatment (120W; 3 times, 30sec). 60µL aliquots for protein determination and radioactivity measurement were issued, and the remainder was extracted by adding chloroform/methanol/water (1:2:0.8, v/v/v) to the cell homogenate. The mixture was vortexed and incubated for 12h at 40 °C in a water bath.

Insoluble cellular components were separated by filtration through wadding and the extract was dried under a stream of nitrogen. 1mL chloroform/methanol (1:1, v/v) was added to re-dissolve the dried lipids in a glass tubes, vortexed and sonicated by ultrasound bath for 5min. 10µL of cell suspension in 1mL solvent mixture were mixed with 10mL liquid scintillation cocktails in plastic scintillation vials to count the radioactivity in the scintillation counter. Incorporation of radioactivity into the lipid extract was determined using a Packard 2900 TR scintillation counter (Packard, Frankfurt) with a counter efficiency of 90-92% for ^{14}C. Then, lipids were separated by thin layer chromatography (tlc).

6.3.4. Anion-exchange chromatography

Ion exchange chromatography was carried out to separate acidic and neutral lipids according to differences in their net charge. This was done with an anion exchanger, Diethylaminoethyl (DEAE) Sephadex A-25, in which negatively charged ions bind to a positively charged resin. First, the matrix was converted from the chloride to the acetate form to obtain maximum affinity of acidic lipids. 500g of ion exchanger was first slurred with 1L methanol for 12h to allow the resin to swell. Then, it was suspended in 0.5L water and allowed to stand for 24h. Then the resin was transferred into a glass column and suspended in 0.5L 1M sodium acetate solution. To eliminate chloride ions, the resin had to be washed several times with 1M sodium acetate solution, until no chloride was detectable by silver nitrate test. Then the acetate form was washed first with 1L water and then with 1L methanol and was stored as 1:1 suspension in methanol at 4 °C before use.

Small pieces of silanized glass fiber wadding were introduced into glass Pasteur pipettes and 1mL of DEAE-Sephadex A-25 (in acetate form)/methanol suspension (1:1) was added and equilibrated with 3mL of chloroform-methanol-water (3:7:1, v/v/v). Samples were dissolved in 2mL with the same solvent, sonicated for 5min and applied to the column. The column was rinsed with 6mL of the same solvent. The fractions that passed through the column contained the neutral sphingo and phospholipids. 8mL of chloroform-methanol-0.8M ammonium acetate (3:7:1, v/v/v) were applied to column to elute acidic lipids. The lipid fractions were evaporated to dryness under a nitrogen stream.

6.3.5. Alkaline hydrolysis

Alkaline hydrolysis is used to remove phospholipids from lipid mixtures. Lipid samples were first dissolved in 1mL of chloroform-methanol (1:1, v/v), vortexed, and then the glass of the reaction vessel were rinsed with 1.5mL of the same solvent. After 5min ultrasonic treatment,

62µL of 4N NaOH were added, and further 5min ultrasound, then the solution was incubated for 2h at 37 °C in a water bath. After the reaction mixture was cooled and neutralized with 10µL glacial acetic acid, the lipid fractions were evaporated to dryness under a stream of nitrogen.

6.3.6. Reversed-phase chromatography (RP18)

Desalting of lipid samples was performed according to the method described by (Williams and McCluer, 1980). In brief, small pieces of silanized glass fiber wadding were introduced into glass Pasteur pipettes and the Pasteur pipettes were clamped vertically. 1.5mL of LiChroprep RP-18 (40-63µm) (1:1) was added into the pipettes. The column was washed with solvents in the following order: 1mL methanol, 2mL water, 1mL methanol, 2mL of chloroform/methanol (1:1 (v/v)), 1mL methanol and 2mL of chloroform/methanol/0.1M KCl in water (3:48:47 (v/v/v)). After the washing process, lipid samples were dissolved in 1mL methanol, and sonicated for 5min. After further addition of 1mL ammonium acetate (300mM in water), the mixture was directly applied to the column. The glass vial was rinsed two times with 1mL ammonium acetate (200mM in methanol/water 1:1 (v/v)), and applied to the column. This was followed by washing with 6mL double distilled water to remove salt particles from the sample. 400µL methanol was added to the column. Glass tubes were placed under each column for lipid collection. Lipids were eluted with 600µL methanol followed by 8mL of chloroform/methanol (1:1 (v/v)). After the elution, the lipid fractions were evaporated to dryness under a stream of nitrogen.

6.3.7. Thin-layer chromatography

The dried lipids in the glass tubes were re-dissolved with 1mL chloroform/methanol (1:1, v/v), vortexed and sonicated by ultrasound bath for 5min. An aliquot of the re-dissolved lipid was then transferred to corresponding safe-lock tubes (Eppendorf), and evaporated to dryness under a stream of nitrogen. The dried lipids inside the safe-lock tubes were re-dissolved in 40µL chloroform-methanol (1:1, v/v) and applied to silica gel 60-precoated glass plates with glass capillaries. Each of the safe-lock tubes was rinsed with 25µL of the same solvent and again applied on the TLC plates. The TLC-plates were dried in an evacuated desiccator over sodium hydroxide. The chromatography tank was lined with filter paper and filled with 200mL of mobile phase using a one-dimensional system. For the separation of lipids, two developing systems were used:

To separate non-polar lipids, polar lipids and free FAs, the following solvent systems were used as the mobile phase:

Neutral lipids and free FAs:	n-hexane/diethyl ether/acetic acid 70:30:1 (v/v/v)
Phospholipids and sphingolipids:	chloroform/methanol/water 65:25:4 (v/v/v)

Lipid classes were assigned to visualized spots by co-chromatography with authentic standard substances (Schürer, et al. 1993). For monitoring and visualizing a radiolabeled lipid, the plate was exposed on a ^{14}C-Screen for 2-4 days. The radiolabeled spots were visualized using a phosphoimager (FUJIX BAS 1000, Raytest, Straubenhardt, Germany). Lipid spots were quantified using Tina software. Standard lipids were applied to the TLC plates along with the radiolabeled lipids, and the standards were visualized by immersing the area of the plate containing the standards spots in $CuSO_4$*$5H_2O$/H_3PO_4 (85%)/H_2O (15.6:9.4:75 (w/v/v)) (Yao and Rastetter, 1985) followed by heating on a heater plate.

6.3.8. Feeding with Lipid-BSA complexes

Stock solutions of 100nmol radiolabeled lipids in 300µL were complexed with FA-free bovine serum albumin (BSA; 7 mg) under vigorous stirring (Pütz and Schwarzmann, 1995). The lipid-BSA or FA-BSA complexes were diluted to a final concentration of 1µM for CE-BSA, Chol-BSA, and TAG-BSA, and to 10µM for FA-BSA complexes in the culture medium (DMEM in the case of fibroblasts, macrophages, and Hep-G2 cells; and RPMI medium in the case of A431 cells). The molar ratio of lipid to albumin was kept at 1:1. The cells were seeded in 25cm^2 plastic flasks with lipid-BSA complexes or 35mm Petri-dish with FA-BSA complexes. 1mL of radiolabeled lipid-BSA complexes were added to the cells for various incubation times as follows:

CE-BSA, TAG-BSA, and Chol-BSA	1h, 6h, and 24h.
SA-BSA, OA-BSA, LA-BSA, LOA-BSA	15min, 30min, and 120min.

The cells were rinsed two times with 1mL PBS, and harvested after trypsinization by a trypsin/EDTA solution. All procedures with cells were carried out under sterile conditions. All solutions were prepared freshly prior to the experiments. All experiments were conducted in duplicate and repeated two times.

6.3.9. Feeding with lipids-LDL complexes

Lipid-LDL complexes were prepared according to a modified procedure based on (Brown S., and Goldstein L. 1975). The probe (100nmol) was incorporated into LDL by a solvent

exchange technique (Howard, 1979). The dried lipid was dissolved in 30µL of MeOH, vortexed, sonicated by ultrasound bath for 5min, and added to 4mL of LDL (750µg) in Tris-Buffer. The resulting suspension was stirred for 4h at 37 ^{0}C in a water bath, and then filtered through a 3.000 MWCO Vivaspin filter (Howard, 1979). 30µL of the lipid-LDL suspension were taken before and after the filtration for scintillation counting and protein determination. The lipid-LDL complexes in suspension buffer were diluted to a final concentration with medium to yield 1µM for lipid-LDL complexes in medium containing 0.8% lipoprotein deficient serum (LPDS) in DMEM (0.8% LPDS in RPMI for A431cells). After adding 1mL of radiolabeled lipid-LDL complexes for various incubation times (1h, 6h, and 24h) to the medium, the cells were rinsed two times with 1mL PBS, and harvested after trypsinization by trypsin/EDTA solution. Although the lipid-LDL complex suspension appeared to be stable for more than a week, all lipid-LDL complexes were prepared freshly prior to the experiments.

6.3.10. Feeding with cationic amphiphilic drugs (CADs)

Fibroblasts, macrophages, and HepG2 cells were maintained in dulbecco´s modified eagle medium (DMEM), A431 cells were maintained in roswell park memorial institute (RPMI) medium, cultured in 25cm^2 culture flasks, and supplemented with 0.8% lipoprotein deficient serum. CADs (0, 10, 20, and 40µM), were added to the medium for 24h before the addition of the complexes. The lipid-LDL complexes were added to the medium and incubated for another 24h so that the drug was still present. Cells were maintained in an incubator containing 95% nitrogen and 5% CO_2. Control cells, cultured at the same time, were treated under the same conditions. After 24h the cells were harvested and the lipids were extracted using chloroform/methanol (1:2, v/v). 60µL aliquots of harvested cells were used for protein determination and counting. Radiolabeled polar and non-polar lipids were quantified after lipid extraction and separation by tlc using the following mobile phases: First, in n-hexane/diethylether/acetic acid (70:30:1, v/v/v) to separate non-polar lipids and free fatty acid, and chloroform/methanol/water (65:25:4, v/v/v) to separate polar lipids. Then, the separated lipids were visualized by phosphoimaging. Radioactivity of each lipid class was calculated from the distribution and the total radioactivity applied to each lane. Radioactivities in the homogenates were measured in a liquid scintillation counter. Lipid uptake was measured and the levels of the most prominent metabolites that did arise from incorporation of fatty acid derived from the exogenously added lipids into different lipid

classes were determined. A 24h incubation period was chosen for more detailed evaluation of the effects of the exogenously added lipids, because the changes during this period were more readily distinguishable.

6.3.11. Cell viability assay

In order to evaluate if the addition of inhibitors affected the cells viability or induced stress resulting in apoptosis of the cells, cell viability was measured using the CellTiter Blue (CTB) assay (Evans, et al. 2001, Brien, et al. 2000), following the manufacturer's instruction (Promega). The assay is based on the ability of living cells to convert the dye resazurin into the fluorescent product resorufin. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Therefore, the fluorescence produced is proportional to the number of viable cells. In brief, cells were cultured in 100µL DMEM medium per well in 96-well microplates (10,000 cells/well) supplemented with 5% fetal calf serum and 2mM L-glutamine. Cells were grown in the presence of 0µM (control), 10µM, 20µM, and 40µM CADs for 48h. Medium was removed, and 100µL CTB medium (85µL fresh medium and 15µL CTB reagent) were added directly to the cells of each well. After 90min incubation at 37 ^{0}C, the fluorescence was determined at excitation/emission wavelengths of 544/590nm using a FLUO-STAR plate reader (BMG, Germany). For each compound, the average value of the duplicate samples was calculated. Data were processed using an Excel spreadsheet. The cell viability ranged from 70 to 112 ± 20% in the presence of inhibitors.

6.4. Control experiments

The following experiments were used as a control.

6.4.1. Gel-filtration chromatography

Gel-filteration was carried out for separation non-bound lipids from lipid incorporate into lipoproteins by passing them through Sephacryl S-300. The column was packed with Sephacryl S-300 and equilibrated with 10mM Tris-HCl (pH 7.5) containing 0.3mM NaCl, and 0.3mM EDTA as a running buffer, and the column was eluted with the same buffer. The lipid-LDL complexes were loaded to the column, and the lipoproteins were eluted with Tris-buffer. Then, 2mL fractions were collected.

6.4.2. Pulse-chase experiment

Radiolabeled oleic acid was tracked in selected cells (fibroblasts, macrophages, A431 cells, and Hep-G2 cells) to determine the fate of incorporated radiolabeled fatty acids at various incubation times. Cells were incubated at different temperatures, either at 37 ^{0}C, or at 4 ^{0}C (which blocks the endocytosis pathways).

After 2h incubation time, the radiolabeled fatty acids were removed immediately from the culture medium. The cultured cells were rinsed three times with 1mL PBS to remove un-incorporated radiolabeled FA. Fresh 10% FCS in DMEM were added, and the cells were incubated again in chase medium at 37 °C. The medium was removed with five interval chase time points (24, 48, 72, 120, and 144h). At each time point, the cells were collected, washed with 1mL PBS, then trypsinized and transfered to a screw cup glass. After centrifuge for 5min, washed again with 1mL PBS. Centrifuge the cells again at 1000rpm for 5min, the pellet were store at –20°C until all time points have been completed.

6.4.3. Feeding with radiolabeled desipramine

[^{3}H]-Desipramine was used as a control to determine its uptake by different cell types. [^{3}H]-Desipramine (0.45nmol; 40µL in ethanol) was pipetted into Falcon tubes, dried to half contents under a stream of nitrogen, and then diluted with 12mL DMEM solution (containing different concentrations of unlabeled desipramine) to yield a 0.1µM [^{3}H]-desipramine in 1.5mL medium. After incubation of the cells with 1.5mL of various concentrations of [^{3}H]-desipramine (10, 20, and 40µM) for 48h incubation time, the cells were washed 3 times with 1mL PBS. After addition of 1mL trypsin/EDTA, the cells were incubated for 1-2min at 37 °C. Cells were carefully detached by gently tapping and the cell suspensions were transferred into a screw cap glass. The vials were washed with 1mL PBS, and the cell suspension was collected into a screw cap glass. The cells were centrifuged at 1000rpm for 5min, and washed again with 1mL PBS. After centrifugation of the cells at 1000rpm for 5min, the pellets were stored at –20 °C until use. Cell pellets were suspended in 0.86mL water and by ultrasonic treatment (120W; 3 times, 30sec). The amounts of protein and the radiolabeled [^{3}H]-desipramine taken up were determined.

7. References

Abdul-Hammed, M.; Breiden, B.; Adebayo, M.A.; Babalola, J.O.; Schwarzmann, G.; Sandhoff, K. (**2010**) The roles of endosomal membrane lipids and NPC2 in cholesterol transfer and membrane fusion. *J. Lipid Res., 51*: 1747-1760.

Aboulaich, N.; Vainonen, J.P.; Stralfors, P.; Vener, A.V. (**2004**) Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes. *Biochem. J., 383*: 237–248.

Aden, D.P.; Fogel, A.; Plotkin, S.; Damjanov I.; Knowles, B.B. (**1979**) Controlled synthesis of HBsAG in a differentiated human liver carcinomca-derived cell line. *Nature, 282*: 615-616.

Aderem, A.; Underhill, D.M. (**1999**) Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol., 17*: 593–623.

Albouz, S.; Hauw, J.J.; Berwaldnetter, Y.; Boutry, J.M.; Bourdon, R.; Baumann, N. (**1981**) Tricyclic anti-depressants induce sphingomyelinase deficiency in fibroblast and neuro-blastoma cell-cultures. *Biomed. Express., 35*: 218-220.

Anderson, N.; Borlak, J. (**2006**) Drug-induced phospholipidosis. *FEBS Letter, 580*: 5533-5540.

Anderson, R.G. (**1998**) The caveolae membrane system. *Annu. Rev. Biochem.,67*: 199–225.

Arikketh, D.; Nelson, R.; Vance, J.E. (**2008**) Defining the important of phosphatidylserine synthase-1 (PSS1). *J. Biol. Chem., 283*: 12888-12897.

Aviram, M.; Bierman, E.L.; Chait, A. (**1988**) Modification of low density lipoprotein by lipoprotein lipase or hepatic lipase induces enhanced uptake and cholesterol accumulation in cells. *J. Biol. Chem., 263*: 15416-15422.

Baillie, A.G.; Coburn, C.T.; Abumrad, N.A. (**1996**) Reversible binding of long-chain fatty acids to purified FAT, the adipose CD36 homolog. *J. Mem. Biol., 153*: 75-81.

Bajjalieh, S.; Batchelor, R. (**2000**) Ceramide kinase. *Methods Enzymol., 311*: 207-215.

Baluška, F.; Hlavacka, A.; Samaj, J.; Palme, K.; Robinson, D.G.; Matoh, T.; McCurdy, D.; Menzel, D.; Volkmann, D. (**2002**) F-actindependent endocytosis of cell wall pectins in meristematic root cells. insights from brefeldin A-induced compartments. *Plant Physiol., 130*: 422–431.

Barlow, C.A.; Laishram, R.S.; Anderson, R.A. (**2010**) Nuclear phosphoinositides: a signaling enigma wrapped in a compartmental conundrum. *Trends Cell Biol., 20*: 25-35.

Bastie, C.C.; Hajri, T.; Drover, V.A.; Grimaldi, P.A.; Abumrad, N.A. (**2004**) CD36 in myocytes channels fatty acids to a lipase-accessible triglyceride pool that is related to cell lipid and insulin responsiveness. *Diabetes, 53*: 2209-2216.

Beck, M. (**2010**) Therapy for lysosomal storage disorders. *IUBMB Life, 62*: 33–40.

Bendedouch, D.; Chen, S.H. (**1983**) Structure and interparticle interactions of bovine serum albumin in solution studied by small angle neutron scattering. *J. Phys. Chem., 87*: 1473-1477.

Berdyshev, E.V.; Gorshkova, I.; Skobeleva, A.; Bittman, R.; Lu, X.; Dudek, S.M.; Mirzapoiazova, T.; Garcia, J.G.; Natarajan, V. (**2009**) FTY720 inhibits ceramide synthases and up-regulates

dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J. Biol. Chem., 284*: 5467-5477.

Berger, M.; Wetzler, E.; August, T.; Tartakoff, A.M. (**1994**) Internalization of type I complement receptors and de novo multivesicular body formation during chemoattractant-induced endocytosis in human neutrophils. *J. Clin. Invest., 94*: 1113-1125.

Berg, J.M.; Tymoczko, J.L.; Strayer, L. (**2003**) Biochemistry. 5th ed.; Spektrum, Akad. Verl.: Berlin, Germany.

Berg, T.; Tolleshaug, H. (**1980**) The effects of ammonium ions and chloroquine on uptake and degradation of 125I-labeled asialo-fetuin in isolated rat hepatocytes. *Biochem. Pharmacol., 29*: 917-925.

Bernlohr, D.A.; Jenkins, A.E.; Bennaars, A.A. (**2002**) Adipose tissue and lipid metabolism. In: Biochemistry of Lipids, Lipoproteins and Membranes, Eds. D.E. Vance and J.E. Vance, 4th ed., Elsevier, Amsterdam, pp. 263-289.

Bhattacharya, A.A.; Grüne, T.; Curry, S. (**2000**) Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *J. Mol. Biol., 303*: 721–732.

Bishop-Bailey, D. (**2011**) PPARs and angiogenesis. *Biochem. Soc. Trans., 39*: 1601-1605.

Blom, T.; Back, N.; Mutka, A.L.; Bittman, R.; Li, Z.; De Lera, A.; Kovanen, P.T.; Diczfalusy, U.; Ikonen, E. (**2010**) FTY720 stimulates 27-hydroxycholesterol production and confers atheroprotective effects in human primary macrophages. *Circ. Res., 106*: 720-729.

Bojesen, I.N.; Bojesen, E. (**1994**) Binding of arachidonate and oleate to bovine serum albumin. *J. Lipid Res., 35*: 770-778.

Botham, K.M.; Moore, E.H.; De Pascale, C.; Bejta, F. (**2007**) The induction of macrophage foam cell formation by chylomicron remnants. *Biochem. Soc. Trans., 35*: 454–458.

Bouron, A., Chatton, J.Y. (**1999**) Acute application of the tricyclic antidepressant desipramine presynaptically stimulates the exocytosis of glutamate in the hippocampus. *Neuroscience., 90*: 729-36.

Brien, J.O.; Wilson, I.; Orton, T.; Pognan, F. (**2000**) Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem., 267*: 5421-5426.

Brindley, D.N. (**1991**) Metabolism of triacylglycerols. In: Biochemistry of Lipids, Lipoproteins and Membranes, Eds. D.E. Vance and J.E. Vance, Elsevier, New York, pp. 171–203.

Briscoe, C.P.; Tadayyon, M.; Andrews, J.L.; Benson, W.G.; Chambers, J.K.; Eilert, M.M.; Ellis, C.; Elshourbagy, N.A.; Goetz, A.S.; Minnick, D.T.; Murdock, P.R.; Sauls, H.R.; Shabon, U.; Spinage, L.D.; Strum, J.C.; Szekeres, P.G.; Tan, K.B.; Way, J.M.; Ignar, D.M.; Wilson, S.; Muir, A.I. (**2003**) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J. Biol. Chem., 278*: 11303-11311.

Brousseau, M.; Schaefer, E.; Wolfe, M.; Bloedon, L.; Digenio, A.; Clark, R.W.; Mancusu, J.P.; Rader, D.J. (**2004**) Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. *N Engl. J. Med., 350*: 1505–1515.

Brown, M.S.; Faust, J.; Goldstein, J.L. (**1975**) Role of the low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest., 55*: 783-793

Brown, M.S.; Goldstein, J.L. (**1999**) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci., 96*: 11041–11048.

Brown, M.S.; Goldstein, J.L. (**1986**) A receptor-mediated pathway for cholesterol homeostasis. *Science, 232*: 34–47.

Brown, M.S.; Goldstein, J.L. (**2004**) A tribute to Akira Endo, discoverer of a "Penicillin" for cholesterol. *Atherosclerosis Suppl., 5*: 13–16.

Brown, M.S.; Goldstein, J.L.; Krieger, M.; Ho, Y.K.; Anderson, R.G. (**1979**) Cholesteryl ester in macrophages incubated with acetylated lipoproteins. *J. Cell. Biol., 82*: 597-613.

Brown, M.S.; Goldstein, J.L. (**1975**) Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell, 6*: 307–3l6.

Brown, S.; Dana, E.; Goldstein, L. (**1975**) Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Nat. Acad. Sci., 72*: 2925-2929.

Brunzell, J.D.; Davidson, M.; Furberg, C.D.; Goldberg, R.B.; Howard, B.V.; Stein, J.H.; Witztum, J.L. (**2008**) Lipoprotein management in patients with cardiometabolic risk. *J. Am. Coll. Cardiol., 51*: 1512-1524.

Buckland, A.G.; Wilton, D.C. (**2000**) Anionic phospholipids, interfacial binding and the regulation of cell functions. *Biochim. Biophys. Acta, 1483*: 199-216.

Burack, W.R.; Biltonen, R.L. (**1994**) Lipid bilayer heterogeneities and modulation of phospholipase A2 activity. *Chem. Phys. lipids, 73*: 209-222.

Calder, P.C.; Bond, J.A.; Harvey, D.J.; Gordon, S.; Newsholme, E.A. (**1990**) Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J., 269*: 807-814.

Calder, P.C. (**2011**) Fatty acids and inflammation: the cutting edge between food and pharma. *Eur. J. pharmacol., 668*: 50-58.

Calder, P.C. (**2010**) Omega-3 fatty acids and inflammatory processes. *Nutrients, 2*: 355-374.

Cantor, R.S. (**1999**) lipid composition and the lateral pressure profile in bilayers. *Biophys. J., 76*: 2625-2639.

Carter, D.C.; He, X.; Munson, S.H.; Twigg, P.D.; Gernert, K.M.; Broom, M.B.; Miller, T.Y. (**1989**) Three-dimensional structure of human serum albumin. *Science, 244*: 1195–1198.

Carter, D.C.; Ho, J.X. (**1994**) Structure of serum albumin. *Adv. Protein Chem., 45*: 153–203.

Cevikbas, F.; Seeliger, S.; Fastrich, M.; Hinte, H.; Metze, D.; Kempkes, C.; Homey, B.; Steinhoff, M. (**2011**) Role of protease-activated receptors in human skin fibrosis and scleroderma. *Exp. Dermatol., 20*: 69-71.

Chaiyasut, C.; Tsuda, T. (**2001**) Isoelectric points estimation of proteins by electroosmotic flow: PH relationship using physically adsorbed proteins on silica gel. *Chromatography, 22*: 91-95.

Charbonneau, D.M.; Tajmir-Riahi, H.A. (**2010**) Study on the interaction of cationic lipids with bovine serum albumin. *J. Phys. Chem. B., 114*: 1148–1155.

Chatelain, P.; brotelle, R. (**1985**) Phospholipid composition of rat lung after amiodarone treatment. *Res. Commun. Chem. Pathol. Pharmacol., 50*: 407-418.

Chaurio, R.A.; Janko, C.; Muñoz, L.E.; Frey, B.; Herrmann, M.; Gaipl, U.S. (**2009**) Phospholipids: key players in apoptosis and immune regulation. *Molecules, 14*: 4892-4914.

Cheng, D.; Iqbal, J.; Devenny, J.; Chu, C.H.; Chen, L.; Dong, J.; Seethala, R.; Kelm, W.J.; Azzara, A.V.; Lawrence, R.M.; Pelleymounter, M.A.; Hussain, M.M. (**2008**) Acylation of acylglycerols by acyl coenzyme A: Diacylglycerol acyltransferase 1 (DGAT1). *J. Biol. Chem., 283*: 29802-29811.

Chen, S.; Gjerde, A.U.; Holmsen, H.; Nerdal, W. (**2005**) Importance of polyunsaturated acyl chains in chlorpromazine interaction with phosphatidylserines: a 13C and 31P solid-state NMR study. *Biophys.chem., 117*: 101-109.

Choi, J.K.; Ho, J.; Curry, S.; Qin, D.; Bittman, R.; Hamilton, J.A. (**2002**) Interactions of very long-chain saturated fatty acids with serum albumin. *J. Lipid Res., 43*: 1000–1010.

Chun, J.; Hartung, H.P. (**2010**) Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. *Clin. Neuropharm., 33*: 91-101.

Connelly, M.A.; Klein, S.M.; Azhar, S.; Abumrad, N.A.; Williams, D.L. (**1999**) Comparison of class B scavenger receptors, CD36 and scavenger receptor BI (SR-BI), shows that both receptors mediate high density lipoprotein-cholesteryl ester selective uptake but SR-BI exhibits a unique enhancement of cholesteryl ester uptake. *J. Biol. Chem., 274*: 41-47.

Cox, C.; Cao, S.; Lu, Y. (**2009**) Enhanced detection and study of murine noroviruse-I using a more efficient microglial cell line. *Virol. J., 6*: 196-202.

Damyanova, R.; Markovska, T.; Mitev, V.; Staneva, G.; Petkova, D. (**2010**) Uptake of fatty acids by fibroblasts cultured in three-dimensional matrix. *Comp. Rend. Acad. Bulg. Sci., 63*: 999-1002.

Daniel, L.W.; Kucera, L.S.; Waite, M. (**1980**) Metabolism of fatty acids by cultured tumor cells and their diploid precursor fibroblasts. *J. Biol. Chem., 255*: 5697-5702.

Dawson, G.; Qin, J.; (**2011**) Gilenya (FTY720) inhibits acid sphingomyelinase by a mechanism similar to tricyclic antidepressants. *Biochem. Biophys. Res. Commun., 404*: 321-323.

Deevska, G.M.; Nikolova-Karakashian, M.N. (**2011**) The twists and turns of sphingolipid pathway in glucose regulation. *Biochim., 93*: 32-38.

Deevska, G.M.; Rozenova, K.A.; Giltiay, N.V.; Chambers, M.A.; White, J.; Boyanovsky, B.B.; Wei, J.; Daugherty, A.; Smart, E.J.; Reid, M.B.; Merrill, A.H.; Nikolova-Karakashian, M. (**2009**) Acid sphingomyelinase deficiency prevents diet-induced hepatic triacylglycerol accumulation and hyperglycemia in mice. *J. Biol. Chem., 284*: 8359-8368.

De Filippi, L.; Fournier, M.; Cameroni, E.; Linder, P.; De Virgilio, C.; Foti, M.; Deloche, O. (**2007**) Membrane stress is coupled to a rapid translational control of gene expression in chlorpromazine-treated cells. *Curr. Genet., 52*: 171-185.

Demant, E.J.F.; Richieri, G.V.; Kleinfeld, A.M. (**2002**) Stopped-floe kinetic analysis of long-chain fatty acid dissociation from bovine serum albumin. *Biochem. J., 363*: 809-815.

Deng, W.; Bukiya, A.N.; Rodríguez-Menchaca, A.A.; Zhang, Z.; Baumgarten, C.M.; Logothetis, D.E.; Levitan, I.; Rosenhouse-Dantsker, A. (**2012**) Hypercholesterolemia induces up-regulation of KACh cardiac currents via a mechanism independent of phosphatidylinositol 4,5-bisphosphate and Gβγ. *J. Biol. Chem., 287*: 4925-4935.

Derksen, J.T.; Morselt, H.W.; Scherphof, G.L. (**1987**) Processing of different liposome markers after in vitro uptake of immunoglobulin-coated liposomes by rat liver macrophages. *Biochim. Biophys. Acta., 931*: 33-40.

Diegelmann, R.F.; Evans, M.C. (**2004**) Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci., 9*: 283–289.

Dixon, J.L.; Ginsberg, H.N. (**1993**) Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J. Lipid Res., 34*: 167-79.

Dubois, B.W.; Cherian, S.F.; Evers, A.S. (**1993**) Volatile anesthetics compete for common binding sites on bovine serum albumin: A ^{19}F-NMR study. *Pharmacol., 90*: 6478-6482.

Doherty, G.J.; McMahon, H.T. (**2009**) Mechanisms of Endocytosis. *Annu. Rev. Biochem., 78*: 857–902.

Doherty, P.; Benedek, G.B. (**1974**) The effect of electric charge on the diffusion of macromolecules. *J. Chem. Phys., 61*: 5426-5435.

Dokko, R.C.; Cho, B.H.; Chung, B.H. (**1998**) Cellular uptake of stearic, oleic, linoleic, and linolenic acid and their effects on synthesis and secretion of lipids in Hep-G2 cells. *Inter. J. Biochem. Cell Biol., 30*: 65-76.

Downes, C.E.; MacPhee, C.H. (**1990**) Myo-inositol metabolites as cellular signals. *Eur. J. Biochem., 193*: 1-18.

Dixon, J.L.; Furukawa, S.; Ginsberg, H.N. (**1991**) Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep-G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem., 266*: 5080-5086.

Duttaroy, A.K. (**2009**) Transport of fatty acids across the human placenta: a review. *Prog. lipid Res., 48*: 52-61.

Du, X.; Kumar, J.; Ferguson, C.; Schulz, T.A.; Ong, Y.S.; Hong, W.; Prinz, W.A.; Parton, R.G.; Brown, A.J.; Yang, H. (**2011**) A role for oxysterol-binding protein–related protein 5 in endosomal cholesterol trafficking. *J. Cell biol., 192*: 121-135.

Edidin, M. (**2003**) Lipids on the frontier: a century of cell-membrane bilayers. *Nat. Rev. Mol. Cell Biol., 4*: 414-418.

ElBawab, S.; Mao, C.; Obeid, L.M.; Hannun, Y.A. (**2002**) Ceramidases in the regulation of ceramide levels and function. *Subcell. Biochem., 36*: 187–205.

Elojeimy, S.; Holman, D.H.; Liu, X.; El-Zawahry, A.; Villani, M.; Cheng, J.C.; Mahdy, A.; Zeidan, Y.; Bielwaska, A.; Hannun, Y.A.; Norris, J.S. (**2006**) New insights on the use of desipramine as an inhibitor for acid ceramidase. *FEBS letters, 580*: 4751-4756.

Evans, S.M.; Casartelli, A.; Herreros, E.; Minnick, D.T.; Day, C.; George, E.; Westmorelan, C. (**2001**) Development of a high throughput in vitro toxicity screen predictive of high acute in vivo toxic potential. *Toxicol. In Vitro., 15*: 579–584.

Fahy, E.; Subramaniam, S.; Brown, H.A.; Glass, C.K.; Merrill, A.H.; Murphy, R.C.; Raetz, C.R.H.; Russell, D.W.; Seyama, Y.; Shaw, W.; Shimizu, T.; Spener, F.; van Meer, G.; VanNieuwenhze, M.S.; White, S.H.; Witztum, J.L.; Dennis, E.A. (**2005**) A comprehensive classification system for lipids. *J. Lipid Res., 46*: 839-862.

Falcone, S.; Cocucci, E.; Podini, P.; Kirchhausen, T.; Clementi, E.; Meldolesi, J. (**2006**) Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events. *J. Cell Sci., 119*: 4758–69.

Ferlinz, K.; Hurwitz, R.; Sandhoff, K. (**1991**) Molecular basis of acid sphingomyelinase deficiency in a patient with Niemann-Pick disease type A. *Biochem. Biophys. Res. Commun., 179*: 1187–1191.

Fielding, P.E.; Fielding, C.E. (**2002**) Dynamics of lipoprotein transport in the human circulatory system. In: Biochemistry of Lipids, Lipoproteins and Membranes, Eds. D.E. Vance and J.E. Vance, 4th ed., Elsevier, Amsterdam, pp. 527–552.

Frank, P.; Lisanti, M. (**2004**) Caveolin-1 and caveolae in atherosclerosis: differential roles in fatty streak formation and neointimal hyperplasia. *Curr. Opin. Lipidol.,15*: 523–9.

Fritsche, K. (**2006**) Fatty acids as modulators of the immune response. *Annu. Rev. Nut., 26*: 45-73.

Fromenty, B.; Freneaux, E.; Labbe, G.; Deschamps, D.; Larrey, D.; Letteron, P.; Pessayre, D. (**1989**) Tianeptine, a new tricyclic antidepressant metabolized by beta-oxidation of its heptanoic side chain, inhibits the mitochondrial oxidation of medium and short chain fatty acids in mice. *Biochem. Pharmacol., 38*: 3743-3751.

Fujimoto, T.; Ohsaki, Y.; Cheng, J.; Suzuki, M.; Shinohara, Y. (**2008**) Lipid droplets: a classic organelle with new outfits. *Histochem. Cell Biol., 130*: 263-279.

Fujimoto, Y.; Onoduka, J.; Homma, K.J.; Yamaguchi, S.; Mori, M.; Higashi, Y.; Makita, M.; Kinoshita, T.; Noda, J.; Itabe, H.; Takanoa, T. (**2006**) Long-chain fatty acids induce lipid droplet formation in a cultured human hepatocyte in a manner dependent of Acyl-CoA synthetase. *Biol. Pharm. Bulletin., 29*: 2174-2180.

Futerman, A.H.; van Meer, G. (**2004**) The cell biology of lysosomal storage disorders. *Nat. Rev. Mol. Cell Biol., 5*: 554-65.

Garbarino, J.; Padamsee, M.; Wilcox, L.; Oelkers, P.M.; D'Ambrosio, D.; Ruggles, K.V.; Ramsey, N.; Jabado, O.; Turkish, A.; Sturley, S.L. (**2009**) Sterol and diacylglycerol acyltransferase deficiency triggers fatty acidmediated cell death. *J. Biol. Chem., 284*: 30994–31005.

Giacomelli, C.E.; Norde, W. (**2001**) The adsorption-desorption cycle. Reversibility of the BSA-silica system. *J. Colloid Interface Sci., 233*: 234-240.

Giard, D.J.; Aaronson, S.A.; Todaro, G.J.; Arnstein, P.; Kersey, J.H.; Dosik, H.; Parks, W.P. (**1973**) Invitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst., 51*: 1417-1423.

Gibellini, F.; Smith, T.K. (**2010**) The kenndy pathway-De novo synthesis of phosphatidylethanolamine and phophatidylcholine. *IUBMB, 62*: 414-428.

Gilmore, R.; Mandon, E.C. (**2012**) Understanding integration of α-helical membrane proteins: the next steps. *Trends Biochem. Sci., 37*: 303-308.

Gimeno, R.E.; Ortegon, A.M.; Patel, S.; Punreddy, S.; Ge, P.; Sun, Y.; Lodish, H.F.; Stahl, A. (**2003**) Characterization of a heart-specific fatty acid transport protein. *J. Biol. Chem., 278*: 16039–16044.

Gjerde, A.U.; Holmsen, H.; Nerdal, W. (**2004**) Chlorpromazine interaction with phosphatidylserines: a (13)C and (31)P solid-state NMR study. *Biochim. Biophys. Acta, 1682*: 28-37.

Glatz, J.F.; Luiken, J.J.; Bonen, A. (**2010**) Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol. Rev., 90*: 367–417.

Goldstein, J.L.; Brown, M.S. (**1987**) Regulation of low-density lipoprotein receptors: implication for pathogenesis and therapy of hypercholesterolemia and atherosclerosis. *Circulation., 76*: 504-507.

Goldstein, J.L.; Brown, M.S. (**2009**) The LDL receptor. *Arterioscler. Thromb. Vasc. Biol., 29*: 431-438.

Goldstein, J.L.; Brunschede, G.Y.; Brown, M.S. (**1975**) Inhibition of proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A, and Triton WR 1339. *J. Biol. Chem., 250*: 7854-7862.

Goni, F.M.; Alonso, A. (**2002**) Sphingomyelinases: enzymology and membrane activity. *FEBS Letters, 531*: 38-46.

Goodman, J.M. (**2008**) The gregarious lipid droplet. *J. Biol. Chem., 283*: 28005-28009.

Granot, E.; Tabas, I.; Tall, A.R. (**1987**) Human plasma cholesteryl ester transfer protein enhances the transfer of cholesteryl ester from high density lipoproteins into cultured HepG2 cells. *J. Biol. Chem., 262*: 3482-3487.

Groener, J.E.M.; Bax, W.; Poorthuis, B.J. (**1996**) Metabolic fate of oleic acid derived from lysosomal degradation of cholesteryl oleate in human fibroblasts. *J. Lipid Res., 37*: 2271-2279.

Gulbins, E. (**2003**) Regulation of death receptor signaling and apoptosis by ceramide. *Pharmacol. Res., 47*: 393-399.

Haberland, M.E.; Fogelman, A.M. (**1985**) Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. *Proc. Natl. Acad. Sci. USA., 82*: 2693-2697.

Hagberg, C.E.; Falkevall, A.; Wang, X.; Larsson, E.; Huusko, J.; Nilsson, I.; van Meeteren, L.A.; Samen, E.; Lu, L.; Vanwildemeersch, M.; Klar, J.; Genove, G.; Pietras, K.; Stone-Elander, S.; Claesson-Welsh, L.; Yla-Herttuala, S.; Lindahl, P.; Eriksson, U. (**2010**) Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature, 464*: 917-921.

Hanada, K. (**2006**) Discovery of the molecular machinery CERT for endoplasmic reticulum-to-Golgi trafficking of ceramide. *Mol. Cell Biochem., 286*: 23-31.

Hanada, K. (**2003**) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta., 1682*:16-30.

Hamilton, J.A. (**2007**) New insights into the roles of proteins and lipids in membrane transport of fatty acids. *Prostaglandins Leukot. Essent. Fatty Acids, 77*: 355–361.

Hartley, J.W.; Evans, L.H.; Green K.Y.; Naghashfar, Z.; Macias, A.R.; Zerfas, P.M.; Ward, J.M. (**2008**) Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirol., 5*: 1-6.

Hatch, G.M. (**1994**) Cardiolipin biosynthesis in the isolated heart. *Biochem. J., 297*: 201–208.

Haunerland, N.H.; Spener, F. (**2004**) Fatty acid-binding proteins--insights from genetic manipulations. *Prog. lipid Res., 43*: 328-349.

Heimburg, T. (**2009**) Physical properties of biological membranes. In: Handbook of Molecular Biophysics. Ed. H.G. Bohr. Wiley-VCH, pp. 593-616.

Heravi, J.; Waite, M. (**1999**) Transacylase formation of bis(monoacylglycerol)phosphate. *Biochim. Biophys. Acta, 1437*: 277-286.

Hess, D.; Chisholm, J.W.; Igal, R.A. (**2010**) Inhibition of stearoylCoA desaturase activity blocks cell cycle progression and induces programmed cell death in lung cancer cells. *PloS one, 5*: e11394.

Hevonoja, T.; Pentikäinen, M.O.; Hyvönen, M.T.; Kovanen, P.T.; Ala-Korpela, M. (**2000**) Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. *Biochim. Biophys. Acta, 1488*: 189-210.

Holland, W.L.; Summers, S.A. (**2008**) Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. *Endocrine Rev., 29*: 381-402.

Holthuis, J.C.; Pomorski, T.; Raggers, R.J.; Sprong, H.; Van Meer, G. (**2001**) The organizing potential of sphingolipids in intracellular membrane transport. *Physiol. Rev., 81*: 1689–1723.

Horinouchi, K.; Erlich, S.; Perl, D.P.; Ferlinz, K.; Bisgaier, C.L.; Sandhoff, K. (**1995**) Acid sphingomyelinase deficient mice: a model of type A and b Niemann-Pick disease. *Nat. Genet., 10*: 288-293.

Horton, J.D.; Goldstein, J.L.; Brown, M.S. (**2002**) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest., 109*: 1125–31.

Hostetler, K.Y.; Reasor, M.; Yazaki, P.J. (**1985**) Chloroquine-induced phospholipid fatty liver. Measurement of drug and lipid concentrations in rat liver lysosomes. *J. Biol. Chem., 260*: 215-219.

Howard, B.V. (**1979**) Uptake of very low density lipoprotein triglyceride by bovine aortic endothelial cells in culture. *J. Lipid Res., 18*: 561-571.

Huang, C.J.; Cheng, H.H.; Chou, C.T.; Kuo, C.C.; Lu, Y.C.; Tseng, L.L.; Chu, S.T.; Hsu, S.S.; Wang, J.L.; Lin, K.L.; Chen, I.S.; Liu, S.I.; Jan, C.R. (**2007**) Desipramine-induced Ca^{2+} movement and cytotoxicity in PC3 human prostate cancer cells. *Toxicol. In Vitro, 21*: 449-456.

Huang, W.C.; Chen, C.L.; Lin, Y.S.; Lin, C.F. (**2011**) Apoptosis sphingolipid ceramide in cancer therapy. *J. Lipids, 2011*: 1-15.

Hunter, J.E. (**2006**) Dietary trans fatty acids: review of recent human studies and food industry responses. *Lipids, 41*: 967-992.

Hurwitz, R.; Ferlinz, K.; Sandhoff, K. (**1994**) The tricyclic antidepressant desipramine causes proteolytic degradation of lysosomal sphingomyelinase in human fibroblasts. *Biol. Chem. Hoppe-Seyler, 375*: 447-450.

Ibrahimi, A.; Sfeir, Z.; Magharaie, H.; Amri, E.Z.; Grimaldi, P.; Abumrad, N.A. (**1996**) Expression of the CD36 homolog (FAT) in fibroblast cells: effects on fatty acid transport, *Proc. Nat. Acad. Sci. U.S.A., 93*: 2646-2651.

Ichimura, A.; Hirasawa, A. Poulain-Godefroy, O.; Bonnefond, A.; Hara, T.; Yengo, L.; Kimura, I.; Leloire, A.; Liu, N.; Iida, K.; Choquet, H.; Besnard, P.; Lecoeur, C.; Vivequin, S.; Ayukawa, K.; Takeuchi, M.; Ozawa, K.; Tauber, M.; Maffeis, C.; Morandi, A.; Buzzetti, R.; Elliott, P.; Pouta, A.; Jarvelin, M.R.; Korner, A.; Kiess, W.; Pigeyre, M.; Caiazzo, R.; Van Hul, W.; Van Gaal, L.; Horber, F.; Balkau, B.; Levy-Marchal, C.; Rouskas, K.; Kouvatsi, A.; Hebebrand, J.; Hinney, A.; Scherag, A.; Pattou, F.; Meyre, D.; Koshimizu, T.A.; Wolowczuk, I.; Tsujimoto, G.; Froguel, P. (**2012**) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature, 483*: 350-354.

Ikushiro, H.; Hayashi, H. (**2011**) Mechanistic enzymology of serine palmitoyltransferase. *Biochim. Biophys. Acta., 1814*: 1474-1480.

Imokawa, G.; Yada, Y.; Higuchi, K.; Okuda, M.; Ohashi, Y.; Kawamata, A. (**1994**) Pseudo-acylceramide with linoleic acid produces selective recovery of diminished cutaneous barrier function in essential fatty acid-deficient rats and has an inhibitory effect on epidermal hyperplasia. *J. Clin. Invest., 94*: 89-96.

Ishikawa, Y.; Nishide, T.; Sasaki, N.; Shirai, K.; Saito, Y.; Yoshida, S. (**1989**) Effects of chloroquine on the metabolism of phosphatidylcholine associated with low density lipoprotein in arterial smooth muscle cells. *Atherosclerosis, 80*: 1-7.

Ishikawa, Y.; Nishide, T.; Sasaki, N.; Shirai, K.; Saito, Y.; Yoshida, S. (**1988**) Hydrolysis of low-density lipoprotein phosphohpids in arterial smooth muscle cells. *Biochim. Biophys. Acta., 961*: 170-176.

Isola, L.M.; Zhou, S.L.; Kiang, C.L.; Stump, D.D.; Bradbury, M.W.; Berk, P.D. (**1995**) 3T3 fibroblasts transfected with a cDNA for mitochondrial aspartate aminotransferase express plasma membrane fatty acid-binding protein and saturable fatty acid uptake, *Proc. Nat. Acad. Sci. U.S.A., 92*: 9866-9870.

Jachimska, B.; Wasilewska, M.; Adamczyk, Z. (**2008**) Characterization of globular protein solutions by dynamic light scattering, electrophoretic mobility, and viscosity measurements. *Am. Chem. Soc., 24*: 6866-6872.

Jacobson, T.A.; Miller, M.; Schaefer, E.J. (**2007**) Hypertriglyceridemia and cardiovascular risk reduction. *Clin. Therap., 29*: 763-777.

Jaffrezou, J.P.; Laurent, G., Levade, T. (**2002**) Ceramide in regulation of apoptosis. Implication in multitoxicant resistance. *Subcell. Biochem., 36*: 269–284.

Jan, C.R.; Lu, Y.C.; Tseng, L.L.; Jiann, B.P.; Chang, H.T.; Wang, J.L.; Chen, W.C.; Huang, J.K. (**2003**) Effect of the antidepressant desipramine on cytosolic Ca(2+) movement and proliferation in human osteosarcoma cells. *Pharmacol., 69*: 190-196.

Jansen, M.; Ohsaki, Y.; Rega, L.R.; Bittman, R.; Olkkonen, V.M.; Ikonen, E. (**2011**) Role of ORPs in sterol transport from plasma membrane to ER and lipid droplets in mammalian cells. *Traffic, 12*: 218–231.

Jauhiainen, M.; Metso, J.; Pahiman, R.; Blomqvist, S.; Tol, A.V.; Ehnhom, C. (**1993**) Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem., 269*: 4032-4036.

Javitt, N.B. (**1990**) Hep-G2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J., 4*: 161-8.

Jensen, J.M.; Schutze, S.; Forl, M.; Kronke, M.; Proksch, E. (**1999**) Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J. Clin. Inves., 104*: 1761-1770.

Jeyakumar, M.; Williams, I.; Smith, D.; Cox, T.M.; Platt, F.M. (**2009**) Critical role of iron in the pathogenesis of the murine gangliosidoses. *Neurobiol Dis., 34*: 406–416.

Johnsen, G.M.; Weedon-Fekjaer, M.S.; Tobin, K.A.; Staff, A.C.; Duttaroy, A.K. (**2009**) Long-chain polyunsaturated fatty acids stimulate cellular fatty acid uptake in human placental choriocarcinoma (BeWo) cells. *Placenta, 30*: 1037-1044.

Jones, A.T. (**2007**) Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J. Cell Mol. Med., 11*: 670-84.

Joshi, U.M.; Kodavanti, P.R.; Coudert, B.; Dwyer, T.M.; Mehendale, H.M. (**1988**) Types of interaction of amphiphilic drugs with phospholipid vesicles. *J. Pharmacol. Exp. Therap., 246*: 150-157.

Jump, D.B. (**2004**) Fatty acid regulation of gene transcription. *Crit. Rev. Clin. Lab. Sci., 41*: 41-78.

Kamata, N.; Chida, K.; Rikimaru, K.; Horikoshi, M.; Enomoto, S.; Duroki, R. (**1986**) Growth-inhibitory effects of epidermal growth factor and overexpression of its receptors on human squamous cell carcinomas in culture. *Cancer Res., 46*: 1648-1653.

Kasahara, T.; Tomita, K.; Murano, H.; Harada, T.; Tsubakimoto, K.; Ogihara, T.; Ohnishi, S.; Kakinuma, C. (**2006**) Establishment of an in vitro high-throughput screening assay for detecting phospholipidosis-inducing potential, *Toxicol. Sci., 90*: 133-141.

Kawamoto, T.; Sate, J.D.; Le. A.; Polikoff, J.; Sato, G.H.; Mendelsohn, J. (**1983**) Growth stimulation of A431 cells by epidermal growth factor: Identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci., 80*: 1337-1341.

Khoo, J.C.; Vance, J.E.; Mahoney, E.M.; Jensen, D.; Wancewicz, E.; Steinberg. D. (**1984**) Neutral triglyceride lipase in macrophages. *Arteriosclerosis, 4*: 34-40.

Kleinman, Y.; Krul E.S.; Burnes, M.; Aronson, W.; Pfleger, B.; Schonfeld, G. (**1988**) Lipolysis of LDL with phospholipase A2 alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells. *J. lipid Res., 29*: 729-743.

Kliewer, S.A.; Sundeth, S.S.; Jones, S.A. (**1997**) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and β. *Proc. Natl. Acad. Sci. USA., 94*: 4318–4323.

Kobayashi, T.; Beuchat, M.; Chevallier, J.; Makino, A.; Mayran, N.; Escola, J.M.; Lebrand, C.; Cosson, P.; Kobayashi, T.; Gruenberg, J. (**2002**) Separation and characterization of late endosomal membrane domains. *J. Biol. Chem., 277*: 32157–32164.

Kobayashi, T.; Startchev, K.; Whitney, A.J.; Gruenberg, J. (**2001**) Localization of lysobisphosphatidic acid-rich membrane domains in late endosomes. *Biol. Chem., 382*: 483–485.

Kobuna, H.; Inoue, T.; Shibata, M.; Gengyo-Ando, K.; Yamamoto, A.; Mitani, S.; Arai, H. (**2010**) Multivesicular body formation requires OSBP–related proteins and cholesterol. *PLoS Genet., 6*: e1001055.

Kodavanti, U.P.; Mehendale, H.M. (**1990**) Cationic amphiphilic drugs and phospholipid storage disorder. *Pharmacol. Rev., 42*: 327-354.

Kölzer, M.; Werth, N.; Sandhoff, K. (**2004**) Interactions of acid sphingomyelinase and lipid bilayers in the presence of the tricyclic antidepressant desipramine. *FEBS letters, 559*: 96-98.

Kolter, T.; Sandhoff, K. (**2010**) Lysosomal degradation of membrane lipids. *FEBS Letter, 584*: 1700-1712.

Kolter, T.; Sandhoff, K. (**2005**) Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids. *Annu. Rev. Cell Dev. Biol., 21*: 81–103.

Kolter, T.; Sandhoff, K. (**1999**) Sphingolipids - their metabolic pathways and the pathobiochemistry of neurodegenerative diseases. *Angew. Chem. Int. Ed. 38*: 1532-1568.

Kondo, A.; Oku, S.; Higashitani, K. (**1991**) Structural changes in protein molecules adsorbed on ultrafine silica particles. *J. Colloid Interface Sci., 143*: 214-221.

Kornhuber, J.; Tripal, P.; Reichel, M.; Muhle, C.; Rhein, C.; Muehlbacher, M.; Groemer, T.W.; Gulbins, E. (**2010**) Functional Inhibitors of Acid Sphingomyelinase (FIASMAs): a novel pharmacological group of drugs with broad clinical applications. *Cell. Physiol. Biochem., 26*: 9-20.

Koul, O.; Hauser, G. (**1987**) Modulation of rat brain cytosolic phosphatidate phosphohydrolase: effect of cationic amphiphilic drugs and divalent cations. *Arch. Biochem. Biophys., 253*: 453-461.

Knowles, B.B.; Howe, C.; Aden, P. (**1980**) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science, 209*: 497-499.

Kobilka, B.K.; Lefkowitz, R.J. (**2012**) Studies of G-protein-coupled receptors. The Royal Swedish Academy of Sciences, Nobel Prize in Chemistry. pp.1-15.

Kozarsky, K.F.; Donahee, M.H.; Rigotti, A.; Iqbal, S.N.; Edelman E.R., Krieger M. (**1997**) Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature, 387*: 414–417.

Kuehnel, M.P.; Goethe, R.; Habermann, A.; Mueller, E.; Rohde, M.; Griffiths, G.; Valentin-Weigand, P. (**2001**) Characterization of the intracellular survival of mycobacterium avium ssp. Paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cell. Microbiol., 3*: 551-566.

Kuehnel, W. (**2003**) Color Atlas of Cytology, Histology, and Microscopic Anatomy (4th ed.). Thieme. pp. 34. ISBN 1-58890-175-0.

Kwak, S.M.; Myung, S.K.; Lee, Y.J.; Seo, H.G. (**2012**) Efficacy of omega-3 fatty acid supplements (eicosapentaenoic acid and docosahexaenoic acid) in the secondary prevention of cardiovascular disease. *Arch. Intern. Med., 172*: 686-694.

Ladha, S. (**1998**) Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *J. Membrane Biol., 165*: 1-10.

LaLonde, J.M.; Bernlohr, D.A.; Banaszak, L.J. (**1994**) The up-and-down beta-barrel proteins. *FASEB J., 8*: 1240-1247.

Lands, W.E. (**1958**) Metabolism of glycerolipides: A comparison of lecithin and triglyceride synthesis. *J. Biol. Chem., 231*: 883–888.

Lansmann, S.; Schuette, C.G.; Bartelsen, O.; Hoernschemeyer, J.; Linke, T.; Weisgerber, J.; Sandhoff, K. (**2003**) Human acid sphingomyelinase, assignment of the disulfide bond pattern. *Eur. J. Biochem., 270*: 1076-1088.

Le Dinh, T.; Freneaux, E.; Labbe, G.; Letteron, P.; Degott, C.; Geneve, J.; Berson, A.; Larrey, D.; Pessayre, D. (**1988**) Amineptine, a tricyclic antidepressant, inhibits the mitochondrial oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. *J. Pharmacol. Exp. Ther., 247*: 745-750.

Lee, A.G. (**2003**) Lipid-protein interactions in biological membranes: a structural perspective. *Biochim. Biophys. Acta., 1612*: 1-40.

LeLay, S.; Dugail, I. (**2009**) Connecting lipid droplet biology and the metabolic syndrome. *Prog. Lipid Res., 48*: 191-195.

Leli, U.; Hauser, G. (**1987**) Modifications of phospholipid-metabolism induced by chlorpromazine, desmethylimipramine and propranolol in C6 glioma-cells. *Biochem. Pharmacol., 36*: 31-37.

Lemaitre, G.; Gonnet, F.; Vaigot, P.; Gidrol, X.; Martin, M.T.; Tortajada, J.; Waksman, G. (**2005**) CD98, a novel marker of transient amplifying human keratinocytes. *Proteomics, 5*: 3637–3645.

Leventhal, A.R.; Chen, W.; Tall, A.R.; Tabas, I. (**2001**) Acid sphingomyelinase deficient macrophages have defective cholesterol trafficking and efflux. *J. Biol. Chem., 276*: 44976 – 44983.

Leventhal, A.R.; Leslie, C.C.; Tabas, I. (**2004**) Suppression of macrophage eicosanoid synthesis by atherogenic lipoproteins is profoundly affected by cholesterol-fatty acyl esterification and the Niemann-Pick C pathway of lipid trafficking. *J. Biol. Chem. 279*: 8084-8092.

Leventis, P.A.; Grinstein, S. (**2010**) The distribution and function of phosphatidylserine in cellular membranes. *Annu. Rev. Biophys., 39*: 407–427.

Lim, J.P.; Gleeson, P.A. (**2011**) Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunol. Cell Biol., 89*: 836-843.

Lin, Y.; Smit, M.J.; Havinga, R.; Verkade, H.J.; Vonk, R.J.; Kuipers, F. (**1995**) Differential effects of eicosapentaenoic acid on glycerolipid and apolipoprotein B metabolism in primary human hepatocytes compared to HepG2 cells and primary rat hepatocytes. *Biochim. Biophys. Acta., 1256*: 88-96.

Li, Q.M.; Fuhrman, B.; Aviram. M. (**1995**) Low density lipoprotein-cholesteryl ester-derived linoleic acid is mainly incorporated into the phospholipid component in macrophages. *Isr. J. Med. Sci., 31*: 474-478.

Listenberger, L.L.; Han, X.; Lewis, S.E.; Cases, S.; Farese, R.V.; Ory, D.S.; Schaffer, J.E. (**2003**) Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc. Nat. Acad. Sci. U.S.A., 100*: 3077-3082.

Lönnfors, M.; Doux, J.P.F.; Killian, J.A.; Nyholm, T.K.M.; Slotte, J.P. (**2011**) Sterols have higher affinity for sphingomyelin than for phosphatidylcholine bilayers even at equal acyl-chain order. *Biophys. J., 100*: 2633–2641.

Lopez-Montero, I.; Rodriquez, N.; Cribier, S.; Pohl, A.; Velez, M.; Devaux, P.F. (**2005**) Rapid transbilayer movement of ceramides in phospholipid vesicales and in human erythrocytes. *J. Biol. Chem., 280*: 25811-25819.

Lucas, A.D.; Greaves, D.R. (**2001**) Atherosclerosis: role of chemokines and macrophages. *Expert. Rev. Mol. Med., 3*: 1–18.

Lucas, L.H.; Price K.E.; Larive, C.K. (**2004**) Epitope mapping and competitive binding of HSA drug site II ligands by NMR diffusion measurements. *J. Am. Chem. Soc., 126*: 14258–14266.

Lüllmann-Rauch, R.; Scheid, D. (**1975**) Intra alveolar foam cells associated with lipidosis-like alterations in lung and liver of rats treated with tricyclic psychotropic drugs. *Virchows Arch. B Cell path., 19*: 255-268.

Lüllmann, H.; Lüllmann-Rauch, R.; Wassermann, O. (**1978**) Lipidosis induced by amphiphilic cationic drugs. *Biochem. Pharmacol., 27*: 1103-1108.

Lysy, P.A.; Smets, F.; Sibille, C.; Najimi, M.; Sokal, E.M. (**2007**) Human skin fibroblasts: from mesodermal to hepatocyte-like differentiation. *Hepatol., 46*: 1574-1585.

Macierzanka, A.; Szelag, H.; Moschakis, t.; Murray, B.S. (**2006**) Phase transitions and microstructure of emulsion system prepared with acylglycerols/zinc stearate emulsifier. *Langmuir, 22*: 2487-2497.

Ma, J.; Qiu, Y.; Yang, L.; Peng, L.; Xia, Z.; Hou, L.N.; Fang, C.; Qi, H.; Chen, H.Z. (**2011**) Desipramine induces apoptosis in rat glioma cells via endoplasmic reticulum stress-dependent CHOP pathway. *J. Neurooncol., 101*: 41-48.

Mandon, E. C.; Ehses, I.; Rother, J.; Van Echten, G.; Sandhoff, K. (**1992**) Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver. *J. Biol. Chem., 267*: 11144-11148.

Matsuo, H.; Chevallier, J.; Mayran, N.; Le Blanc, I.; Ferguson, C.; Faure´, J.; Blanc, N.S.; Matil, S.; Dubochet, J.; Sadoul, R.; Parton, R.G.; Vilbois, F.; Grunenberg, J. (**2004**) Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science, 303*: 531–34.

Matsuzawa, Y.; Hostetler, K.Y. (**1980**) Inhibition of lysosomal phospholipase A and phospholipase C by chloroquine and 4,4'-bis(diethylaminoethoxy) alpha, beta-diethyldiphenylethane. *J. Biol. Chem., 255*: 5190-5194.

Mayes, P.A.; Botham, K.M. (**2003**) Cholesterol synthesis, transport, and extraction. In: Murray, R.K.; Granner, D.K.; Mayes, P.A.; Rodwell, V.W. Harper's Illustratted biochemistry. 26th ed., Lange Medical Books/McGraw-Hill, U.S.A.

Mayes, P.A.; Botham, K.M. (**2003**) Metabolism of acylglycerols and sphingolipids. In: Murray, R.K.; Granner, D.K.; Mayes, P.A.; Rodwell, V.W. Harper's Illustratted biochemistry. 26th edition, Lange Medical Books/McGraw-Hill, U.S.A.

Mayor, S.; Pagano, R.E. (**2007**) Pathways of clathrin-independent endocytosis. *Mol. Cell Biol., 8*: 603-612.

Maziere, C.; Maziere, J.C.; Mora, L.; Polonovski, J. (**1982**) Incorporation of exogenous fatty-acids into phospholipids by cultured hamster fibroblasts - effect of Sv40 transformation. *Biochim. Biophys. Acta., 712*: 712-715.

McArthur, M.J.; Atshaves, B.P.; Frolov, A.; Foxworth, W.D.; Kier, A.B.; Schroeder, F. (**1999**) Cellular uptake and intracellular trafficking of long chain fatty acids. *J. Lipid Res., 40*: 1371-1383.

McNutt, M.C.; Lagace, T.A.; Horton, J.D. (**2007**) Catalytic activity is not required for secreted PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. *J. Biol. Chem., 282*: 20799-20803.

Meikle, P.J.; Hopwood, J.J.; Clague, A.E.; Carey, W.F. (**1999**) Prevalence of lysosomal storage disorders. *JAMA; 20*: 249 – 254.

Mellman, I. (**1996**) Membranes and sorting. *Curr. Opin. Cell Biol., 8*: 497-498.

Merlino, G.T.; Xu, Y.H.; Ishii, S.; Clark, A.J.L.; Semba, K.; Toyoshima, K.; Yamamoto, T.; Pastan, I. (**1984**) Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science, 224*: 417-419.

Merrill, A.H.; Wang, E.; Mullins, R.E. (**1988**) Kinetics of long-chain (sphingoid) base biosynthesis in intact LM cells: effects of varying the extracellular concentrations of serine and fatty acid precursors of this pathway. *Biochem., 27*: 340-345.

Miles, P.R.; Bowman, L.; Tucker, J.; Reasor, M.J.; Wright, J.R. (**1986**) Alteration in rat alveolar surfactant phospholipids and proteins induced by adminstration of chlorphentermine. *Biochim, Biophys. Acta, 877*: 167-178.

Miller, M.; Motevalli, M.; Westphal, D.; Kwiterovich, P.O. (**1993**) Incorporation of oleic acid and eicosapentaenoic acid into glycerolipids of cultured normal human fibroblasts. *Lipids, 28*: 1-5.

Minahk, C.; Kim, K.W.; Nelson, R.; Trigatti, B.; Lehner, R.; Vance, D.E. (**2008**) Conversion of low density lipoprotein-associated phosphatidylcholine to triacylglycerol by primary hepatocytes. *J. Biol. Chem., 283*: 6449-6458.

Möbius, W.; van Donselaar, E.; Ohno-Iwashita, Y.; Shimada, Y.; Heijnen, H.F.; Slot, J.W.; Geuze, H.J. (**2003**) Recycling compartments and the internalvesicles of multivesicular bodies harbor most of the cholesterol found in theendocytic pathway. *Traffic, 4*: 222–231.

Montagner, A.; Rando, G.; Degueurce, G.; Leuenberger, N.; Michalik, L.; Wahli, W. (**2011**) New insights into the role of PPARs. *Prostaglandins Leukot. Essent. Fatty Acids*, 85: 235–243.

Morishita, S.; Watanabe, S. (**1997**) Effect of the tricyclic antidepressant desipramine on protein kinase C in rat brain and rabbit platelets in vitro. *Psychiatry Clin. Neurosci., 51*: 249-252.

Munro, S. (**2003**) Lipid rafts: elusive or illusive?. *Cell, 115*: 377-388.

Murphy, E.J.; Prows, D.R.; Jefferson, J.R.; Schroeder, F. (**1996**) Liver fatty acid-binding protein expression in transfected fibroblasts stimulates fatty acid uptake and metabolism. *Biochim. Biophys. Acta., 1301*: 191-198.

Murphy, E.J.; Schapiro, M.B.; Schapiro, M.B.; Shetty, H.U. (**2000**) Phospholipid composition and levels are altered in down syndrome brain. *Brain Res., 867*: 9–18.

Nawa, M.; Takasaki, T.; Yamada, K.; Kurane, I.; Akatsuka, T. (**2003**) Interference in Japanese encephalitis virus infection of Vero cells by a cationic amphiphilic drug, chlorpromazine. *J. Gen. Virol., 84*: 1737-1741.

Nelson, D.L.; Cox, M.M. (**2005**) Lehninger's principles of biochemistry, 4th ed., W. H. Freeman and Company, New York.

Nioi, P.; Pardo, I.D.; Snyder, R.D. (**2008**) Monitoring the accumulation of fluorescently labeled phospholipids in cell cultures provides an accurate screen for drugs that induce phospholipidosis, *Drug Chem. Toxicol., 31*: 515-528.

Nonoyama, T.; Fukuda, R. (**2008**) Drug-induced phospholipidosis-pathological aspects and its prediction. *J. Toxical. Pathol., 21*: 9-24.

Norde, W.; Favier, J.P. (**1992**) Structure of adsorbed and desorbed proteins, *Colloids Surf., 64*: 87-93.

Oh, D.Y.; Talukdar, S.; Bae, E.J.; Imamura, T.; Morinaga, H.; Fan, W.; Li, P.; Lu, W.J.; Watkins, S.M.; Olefsky, J.M. (**2010**) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell, 142*: 687-698.

Okwu, A.K.; Xu, X.X.; Shiratori, Y.; Tabas, I. (**1994**) Regulation of the threshold for lipoprotein-induced acyl-CoA:cholesterol 0-acyltransferase stimulation in macrophages by cellular sphingomyelin content. *J. Lipid Res.. 35*: 644-655.

Orso, E.; Grandl, M.; Schmitz, G. (**2011**) Oxidized LDL-induced endolysosomal phospholipidosis and enzymatically modified LDL-induced foam cell formation determine specific lipid species modulation in human macrophages. *Chem. Phys. Lipids, 164*: 479-487.

Papp, A.C.; Pinsonneault, J.K., Wang, D.; Newman, L.C.; Gong, Y.; Johnson, J.A.; Pepine, C.J. Kumari, M.; Hingorani, A.D.; Talmud, P.J.; Shah, S.; Humphries, S.E.; Sadee, W. (**2012**) Cholesteryl ester transfer protein (CETP) polymorphisms affect mRNA splicing, HDL levels, and sex-dependent cardiovascular risk. *PLoS ONE, 7*: e31930.

Pappu, A.; Hostetler, K.Y. (**1984**) Effect of cationic amphiphilic drugs on the hydrolysis of acidic and neutral phospholipids by liver lysosomal phospholipase A. *Biochem. Pharmacol., 33*: 1639-1644.

Pastores, G.M. (**2006**) Miglustat: substrate reduction therapy for lysosomal storage disorders associated with primary central nervous system involvement. *Recent Pat. CNS Drug Discov., 1*: 77-82.

Passeggio, J.; Liscum, L. (**2005**) Flux of Fatty Acids through NPC1 Lysosomes. *J. Biol. Chem., 280*: 10333-10339.

Paugh, S.W.; Cassidy, M.P.; He, H.; Milstien, S.; Sim-Selley, L.J.; Spiegel, S.; Selley, D.E. (**2006**) Sphingosine and its analog, the immunosuppressant 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol, interact with the CB1 cannabinoid receptor. *Mol. Pharmacol., 70*: 41-50.

Payne, S.G.; Oskeritzian, C.A.; Griffiths, R.; Subramanian, P.; Barbour, S.E.; Chalfant, C.E.; Milstien, S.; Spiegel, S. (**2007**) The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. *Blood, 109*: 1077-1085.

Pelkmans, L. (**2005**) Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses. *Biochim. Biophys. Acta, 1746*: 295–304.

Pelech, S.L.; Vance, D.E. (**1984**) Trifluoperazine and chlorpromazine inhibit phosphatidylcholine biosynthesis and CTP:phosphocholine cytidylyltransferase in HeLa cells. *Biochim. Biophys. Acta, 795*: 441–446

Perry, D.K.; Hand, W.L.; Edmondson, D.E.; Lambeth, J.D. (**1992**) Role of phospholipase D-derived diradylglycerol in the activation of the human neutrophil respiratory burst oxidase. Inhibition by phosphatidic acid phosphohydrolase inhibitors. *J. Immunol., 149*: 2749-2758.

Peters, T. (**1985**) Serum albumin. *Adv. Protein Chem., 37*: 161-245.

Pewzner-Jung, Y.; Ben-Dor, S.; Futerman, A.H. (**2006**) When do lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis. *J Biol Chem., 281*: 25001-25005.

Pittman, R.C.; Carew, T.E.; Attie, A.D.; Witztum, J.L.; Watanabe, Y.; Steinberg, D. (**1982**) Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. *J. Biol. Chem., 257*: 7994-8000.

Pohl, J.; Fitscher, B.A.; Ring, A.; Ihl-Vahl; R.; Strasser, R.H.; Stremmel; W. (**2000**) Fatty acid transporters in plasma membranes of cardiomyocytes in patients with dilated cardiomyopathy. *Eur. J. Med. Res., 5*: 438–442.

Pohl, J.; Ring, A.; Stremmel, W. (**2002**) Uptake of long-chain fatty acids in HepG2 cells involves caveolae: analysis of a novel pathway. *J. Lipid Res., 43*: 1390-1399.

Pomorski, T.; Hrafnsdottir, S.; Devaux, P.F.; Van Meer, G. (**2001**) Lipid distribution and transport across cellular membranes. *Semin Cell Dev. Biol., 12*: 139-148.

Prinz, W. (**2002**) Cholesterol trafficking in the secretory and endocytic systems. *Semin Cell Dev. Biol., 13*: 197-203.

Pütz, U.; Schwarzmann, G. (**1995**) Golgi staining by two fluorescent ceramide analogues in cultured fibroblasts requires metabolism. *Eur. J. Cell Biol., 68*: 113-121.

Pussinen, P.J.; Jauhinainen, M.; Metso, J.; Pyle, L.E.; Marcel, Y.L.; Fidge, N.H.; Ehnholm, C. (**1998**) Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. *J. Lipid Res., 39*: 152-161.

Puzio-Kuter, A.M. (**2011**) The role of p53 in metabolic regulation. *Gen. Can., 4*: 385-391.

Quinn, P.J. (**2002**) Plasma membrane phospholipid asymmetry. *Subcell. Biochem., 36*: 39–60.

Radin, N.S. (**2001**) Killing cancer cells by poly-drug elevation of ceramide levels: a hypothesis whose time has come?. *Eur. J. Biochem., 268*: 193-204.

Reasor, M.J.; Hastings, K.L.; Ulrich, R.G. (**2006**) Drug-induced phospholipidosis: issues and future directions. *Exp. Opin. Drug Saf., 5*: 567-583.

Rehm, M.; Zahler, S.; Lötsch, M.; Welsch, U.; Conzen P.; Jacob M.; Becker B.F. (**2004**) Endothelial glycocalyx as an additional barrier determining extravasation of 6% hydroxyethylstarch or 5% albumin solutions in the coronary vascular bed. *Anesthesiology, 100*: 1211–1223.

Renooij, W.; Snyder, F. (**1981**) biosynthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor and a hypotension lipid) by cholinephosphotransferase in various rat tissues. *Biochim. Biophys. Acta, 663*: 545-556.

Reynaert, H.; Rombouts, K.; Vandermonde, A.; Urbain, D.; Kumar, U.; Bioulac-Sage, P.; Pinzani, M.; Rosenbaum, J.; Geerts, A. (**2004**) Expression of somatostatin receptors in normal and cirrhotic human liver and in hepatocellular carcinoma. *Gut, 53*: 1180-1189.

Rhainds, D.; Brodeur, M.; Lapointe, J.; Charpentier, D.; Falstrault, L.; Brissette, L. (**2003**) The role of human and mouse hepatic scavenger receptor class B type I (SR-BI) in the selective uptake of low-density lipoprotein-cholesteryl esters. *Biochem., 42*: 7527-7538.

Richieri, G.V.; Anel, A.; Kleinfeld, A.M. (**1993**) Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochem., 32*: 7574–7580.

Richieri, G.V.; Ogata, R.T.; Kleinfeld, A.M. (**1994**) Equilibrium constants for the binding of fatty acids with fatty acid-binding proteins from adipocyte, intestine, heart, and liver measured with the fluorescent probe ADIFAB. *J. Biol. Chem., 269*: 23918–23930.

Richmond, G.S.; Smith, T.K. (**2011**) Phospholipases A1. *Int. J. Mol. Sci., 12*: 588-612.

Ring, A.; Le Lay, S.; Pohl, J.; Verkade, P.; Stremmel, W. (**2006**) Caveolin-1 is required for fatty acid translocase (FAT/CD36) localization and function at the plasma membrane of mouse embryonic fibroblasts. *Biochim. Biophys. Acta., 1761*: 416-423.

Roda, S.K.; Skretting, G.; Garred, Q.; Vilhardt, F.; Deurs, B.V.; Sandvig, K. (**1999**) Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin coated endocytosis vesicles. *Mol. Biol. Cell., 10*: 961-974.

Roeber, B.M.; Pical, C. (**2002**) Inositol phospholipid metabolism in arabidopsis. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C1. *Plant Physiol., 130*: 22–46.

Rock, C.O. (**2008**) Fatty acid and phospholipid metabolism in prokaryotes. In: Biochemistry of lipids, lipoproteins and membrane, Eds. Vance, D.E; Vance, J.E, 5th ed. Elsevier, pp. 59-95.

Rohwedder, A.; Zhang, Q.; Rudge, S.; Wakelam, M. (**2011**) LipidomicNet Steering Committee Annual Meeting 27-28 June, Kraków, Poland, Receptor Mediated Generation of Lipid Droplets in Huh-7 Cells.

Rosenson, R.S. (**2004**) Statins in atherosclerosis: lipid-lowering agents with antioxidant capabilities. *Atherosclerosis, 173*: 1–12.

Rosenthal, M.D. (**1981**) Accumulation of neutral lipids by human skin fibroblasts: differential effects of saturated and unsaturated fatty acids. *Lipids, 16*: 173-182.

Rosenthal, M.D. (**1987**) Fatty acid metabolism of isolated mammalian cells. *Prog. Lipid Res., 26*: 87-124.

Rosenthal, M.D. (**1980**) Selectivity in incorporation, utilization and retention of oleic and linoleic acids by human skin fibroblasts. *Lipids, 15*: 838-848.

Ross, J.A.; Auger, M.J.; Burke, B.; Lewis, C.E. (**2002**) The biology of the macrophage. In: The Macrophage, Eds. Burke, B.; Lewis, C.E., 2nd ed., Oxford Medical Publication, pp. 1-72.

Rother, J.; Van Echten, G.; Schwarzmann, G.; Sandhoff, K. (**1992**) Biosynthesis of sphingolipids: dihydroceramide and not sphinganine is desaturated by cultured cells. *Biochem. Biophys. Res. Commun., 189*, 14-20.

Rudenko, G.; Henry, L.; Henderson, K.; Ichtchenko, K.; Brown, M.S.; Goldstein, J.L.; Deisenhofer, J. (**2002**) Structure of the LDL receptor extracellular domain at endosomal pH. *Science, 298*: 2353–2358.

Ryser, H.J. (**1967**) A membrane effect of basic polymers dependent on molecular size. *Nature, 215*: 934-936.

Saito, H.; Lund-Katz, S.; Phillips, M.C. (**2004**) Contributions of domain structure and lipid interaction to the functionality of exchangeable human apolipoproteins. *Prog. Lipid Res., 43*: 350–380.

Samaj, J.; Read, N.D.; Volkmann, D.; Menzel, D.; Baluska, F. (**2005**) The endocytic network in plants. *Trends Cell Biol.,15*: 425–33.

Sandhoff, K.; Linke, T.; Wilkening, G.; Sadeghlar, F.; Moczall, H.; Bernardo, K.; Schuchman, E. (**2001**) Interfacial regulation of acid ceramidase activity: stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator proteins. *J. Biol. Chem., 276*: 5760-5768.

Saqr, H.E.; Pearl, D.K.; Yates, A.J. (**1993**) A review and predictive of ganglioside uptake by biological membrane. *J. Neurochem., 61*: 395-411.

Schaap, F.G.; Van der Vusse, G.J.; Glatz, J.F.C. (**1998**) Fatty acid-binding proteins in the heart. *Mol. Cell. Biochem., 180*: 43-51.

Scherer, M.; Schmitz, G.; Liebisch, G. (**2010**) Simultaneous quantification of cardiolipin, bis(monoacylglycero)phosphate and their precursors by hydrophilic interaction LC-MS/MS including correction of isotopic overlap. *Anal. Chem., 82*: 8794-8799.

Schuchman, E.H. (**2010**) Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. *FEBS letters, 584*: 1895-1900.

Schürer, N.Y.; Köhne, A.; Schliep, V.; Barlag, K.; Goerz, G. (**1993**) Lipid composition and synthesis of HacaT cells, an immortalized human keratinocyte line, in comparison to normal human adult keratinocytes. *Exp. Dermatol., 2*: 179-185.

Schürer, N.Y.; Monger, D.J.; Hincenbergs, M.; Williams, M.L. (**198**8) Fatty acid metabolism in human keratinocytes cultured at an air-medium interface. *J. Invest. Derma., 92*: 196-202.

Schürer, N.Y.; Stremmel, W.; Grundmann, J.U.; Schliep, V.; Kleinert, H.; Bass, N.M.; Williams, M.L. (**1994**) Evidence for a novel keratinocyte fatty acid uptake mechanism with preference for linoleic acid: comparison of oleic and linoleic acid uptake by cultured human keratinocytes, fibroblasts and a human hepatoma cell line. *Biochim. Biophys. Acta., 1211*: 51-60.

Schwendener, R.A. (**1988**) Incorporation of chlorpromazine into bilayer liposomes for protection against microsomal metabolism and liver absorption. *Eur. J. Drug Metab. Pharmacokinet., 13*: 135-141.

Schwenk, R.W.; Holloway, G.P.; Luiken, J.J.; Bonen, A.; Glatz, J.F. (**2010**) Fatty acid transport across the cell membrane: regulation by fatty acid transporters. *Prostaglandins Leukot. Essent. Fatty Acids, 82:* 149-154.

Scott, J. (**2004**) Pathophysiology and biochemistry of cardiovascular disease. *Curr. Opin. Genet. Dev., 14*: 271–279.

Shcharbin, D.; Janicka, M.; Wasiak, M.; Palecz, B.; Przybyszewska, M.; Zaborski, M.; Bryszewska, M. (**2007**) Serum albumins have five sites for binding of cationic dendrimers. *Biochim. Biophys. Acta, 1774*: 946–951.

Shcharbin, D.; Klajnert, B.; Mazhul, V.; Bryszewska, M. (**2005**) Dendrimer interactions with hydrophobic fluorescent probes and human serum albumin. *J. Fluoresc., 15*: 21–28.

Shen, M.M.S.; Krauss, R.M.; Lindgren, F.T.; Forte, T.M. (**1981**) Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res., 22*: 236-244.

Shields, D.J.; Altarejos, J.Y.; Wang, X.; Agellon, L.B.; Vance, D.E. (**2003**) Molecular dissection of the S-Adenosylmethionine-binding site of phosphatidylethanoamine N-Methyltransferase. *J. Biol. Chem., 278*: 35826-35836.

Shkulipa, S.A. (**2006**) Computer simulations of lipid bilayer dynamics. Thesis. University of Twente, Enschede, pp. 1-139. ISBN: 90.365.2408.3.

Silver, D.L.; Wang, N.; Xiao, X.; Tall, A.R. (**2001**) High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. *J. Biol. Chem., 276*: 25287–25293.

Simonsen, A.; Wurmser, A.E.; Emr, S.D.; Stenmark, H. (**2001**) The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol., 13*: 485-492.

Simons, K.; Ikonen, E. (**1997**) Functional rafts in cell membranes. *Nature, 387*: 569–572.

Singer, S.J.; Nicolson, G.L. (**1972**) The fluid mosaic model of the structure of cell membranes. *Science, 175*: 720-731.

Simard, J.R.; Zunszain, P.A.; Ha, C.E.; Yang, J.S.; Bhagavan, N.V.; Petitpas, I.; Curry, S.; Hamilton, J.A. (**2005**) Locating high-affinity fatty acid-binding sites on albumin by X-ray crystallography and NMR spectroscopy. *Proc. Natl. Acad. Sci., 102*: 17958–17963.

Smith, E.L.; Schuchman, E.H. (**2008**) The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *FASEB J., 22*: 3419-3431.

Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. (**1985**) Measurement of protein using bicinchoninic acid. *Anal. Biochem., 150*: 76-85.

Sorkin, A. (**2004**) Cargo recognition during clathrin-mediated endocytosis: a team effort. *Curr. Opin. Cell Biol., 16*: 392-399.

Sorrentino, D.; Robinson, R.B.; Kiang, C.L.; Berk, P.D. (**1989**) At physiologic albumin/oleate concentrations oleate uptake by isolated hepatocytes, cardiac myocytes, and adipocytes is a saturable function of the unbound oleate concentration. Uptake kinetics are consistent with the conventional theory. *J. Clin. Invest., 84*: 1325-1333.

Sovic, A.; Balazs, Z.; Hrzenjak, A.; Reicher, H.; Panzenboeck, U.; Malle, E.; Sattler, W. (**2004**) Scavenger receptor class B, type I mediates uptake of lipoprotein-associated phosphatidylcholine by primary porcine cerebrovascular endothelial cells. *Neurosci. letters, 368*: 11-14.

Spady, D.K.; Bilheimer, D.W.; Dietschy, J.M. (**1983**) Rates of receptor-dependent and -independent low density lipoprotein uptake in the hamster. *Proc. Natl. Acad. Sci. USA., 80*: 3499-3503.

Spector, A.A.; Kiser, R.E.; Denning, G.M.; Koh, S.W.; DeBault, L.E. (**1979**) Modification of the fatty acid composition of cultured human fibroblasts. *J. Lipid Res., 20*: 536-547.

Spector, A.A. (**1986**) Structure and lipid binding properties of serum albumin. *Methods Enzymol., 128*: 320-339.

Sprenger, R.R.; Speijer, D.; Back, J.W.; De Koster, C.G.; Pannekoek, H.; Horrevoets, A.J. (**2004**) Comparative proteomics of human endothelial cell caveolae and rafts using two dimensional gel electrophoresis and mass spectrometry. *Electrophoresis, 25*: 156–172.

Sprong, H.; Van der Sluijs, P.; Van Meer, G. (**2001**) How proteins move lipids and lipids move proteins. *Nat. Rev. Mol. Cell Biol., 2*: 504-513.

Stathopoulos, V.M.; Coco-Maroney, A.; Wei, C.W.; Goth, M.; Zaricznyj, C.; Macara, I.G. (**1990**) Identification of two cytosolic diacylglycerol kinase isoforms in rat brain, and in NIH-3T3 and ras-transformed fibroblasts. *Biochem. J., 272*: 569-575.

Stiban, J.; Caputo, L.; Colombini, M. (**2008**) Ceramide synthesis in the endoplasmic reticulum can permeabilize mitochondria to proapoptotic protein. *J. lipid Res., 49*: 625-634.

Storch, J.; Thumser, A.E. (**2000**) The fatty acid transport function of fatty acid-binding proteins. *Biochim. Biophys. Acta, 1486*: 28-44.

Strelow, A.; Bernardo, K.; Adam-Klages, S.; Linke, T.; Sandhoff, K.; Krönke, M. (**2000**) Overexpression of acid ceramidase protects from tumor necrosis factor-induced cell death. *J. Exp. Med., 192*: 601-611.

Stremmel, W.; Diede, H.E. (**1989**) Fatty acid uptake by human hepatoma cell lines represents a carrier-mediated uptake process. *Biochim. Biophys. Acta., 1013*: 218-222.

Stremmel, W.; Pohl, L.; Ring, A.; Herrmann, T. (**2001**) A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. *Lipids, 36*: 981–989.

Stremmel, W.; Potter, B.J.; Berk, P.D. (**1983**) Studies of albumin binding to rat liver plasma membranes. Implications for the albumin receptor hypothesis. *Biochim. Biophys. Acta., 756*: 20-27.

Stromhaug, P.E.; Berg, T.O.; Gjoen, T.; Seglen P.O. (**1997**) Differences between fluid-phase endocytosis (pinocytosis) and receptor-mediated endocytosis in isolated rat hepatocytes. *Eur. J. Cell Biol., 73*: 28-39.

Stubhaug, I.; Tocher, D.R.; Bell, J.G.; Dick, J.R.; Torstensen, B.E. (**2005**) Fatty acid metabolism in Atlantic salmon (Salmo salar L.) hepatocytes and influence of dietary vegetable oil. *Biochim. Biophys. Acta., 1734*: 277-288.

Stuhne-Sekalec, L.; Chudzik, J.; Stanacev, N.Z. (**1987**) Effect of chlorpromazine on the synthesis, hydrolysis, and transfer of microsomal cytidine liponucleotides and mitochondrial polyglycerophosphatides. Can. *J. Physiol. Pharmacol., 65*: 377-384.

Swarnakar, S.; Temel, R.E.; Connelly, M.A.; Azhar, S.; Williams, D.L. (**1999**) Scavenger receptor class B, type I, mediates selective uptake of low density lipoprotein cholesteryl ester. *J. Biol. Chem., 274*: 29733–29739.

Synnes, M.; Prydz, K.; Lovdal, T.; Brech, A.; Berg, T. (**1999**) Fluid phase endocytosis and galactosyl receptor-mediated endocytosis employ different early endosomes. *Biochim. Biophys. Acta, 1421*: 317-328.

Tafesse, F.G.; Huitema, K.; Hermansson, M.; Van der Poel, S.; Van den Dikkenberg, J.; Uphoff, A.; Somerharju, P.; Holthuis, J.C. (**2007**) Both sphingomyelin synthases SMS1 and SMS2 are required for sphingomyelin homostasis and growth in human HeLa cells. *J. Biol. Chem., 282*: 17537-17547.

Takahashi, E.; Inanami, O.; Asanuma, T.; Kuwabara, M. (**2006**) Effect of ceramide inhibition on radiation-induced apoptosis in human leukemia MOLT-4 cells. *J. Radiat. Res., 47*: 19-25.

Tanaka, A. (**1995**) Acid lipase deficiency: Wolman disease and cholesteryl ester storage disease. *Nihon. Rinsho., 53*: 3004-3008.

Tanford, C. (**1980**) The hydrophobic effect: formation of micelles and biological membranes. 2nd ed. John Wiley and Sons, New York.

Tayeh, N.; Rungassamy, T.; Albani, J.R. (**2009**) Fluorescence spectral resolution of tryptophan residues in bovine and human serum albumins. *J. Pharm. Biomed. Anal., 50*: 107–116.

Temel, R.E.; Trigatti, B.; De Mattos, R.B.; Azhar, S.; Krieger, M.; Williams, D.L. (**1997**) Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc. Natl. Acad. Sci. U.S.A. 94*: 13600–13605.

Teruya, J.; Cluette-Brown, J.; Szczepiorkowski, Z.M.; Laposata, M. (**1995**) Mode of transport of fatty acid to endothelial cells influences intracellular fatty acid metabolism. *J. Lipid Res., 36*: 266-276.

Teufel, A.; Maass, T.; Galle, P.R.; Malik, N. (**2009**) The longevity assurance homologue of yeast lag1 (Lass) gene family (review). *Int. J. Mol. Med., 23*: 135-140.

Thiele, C.; Spandl, J. (**2008**) Cell biology of lipid droplets. *Curr. Opin. Cell Biol., 20*: 378-385.

Tollefson, J.H.; Ravnik, S.; Albers, J.J. (**1988**) Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. *J. Lipid Res., 29*:1593–1602.

Tolleshaug, H.; Berg, T. (**1979**) Chloroquine reduces the number of asialo-glycoprotein receptors in the hepatocyte plasma membrane. *Biochem. Pharmacol., 28*: 2919-2922.

Trigatti, B.L.; Krieger, M.; Rigotti, A. (**2003**) Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol., 23*: 1732-1738.

Truong, T.Q.; Auger, A.; Denizeau, F.; Brissette, L. (**2000**) Analysis of low-density lipoprotein catabolism by primary cultures of hepatic cells from normal and low-density lipoprotein receptor knockout mice. *Biochim. Biophys. Acta., 1484*: 307-315.

Ueda, Y.; Royer, L.; Gong, E.; Zhang, J.; Cooper, P.N.; Francone, O.; Rubin, E.M. (**1999**) Lower plasma levels and accelerated clearance of high density lipoprotein (HDL) and non-HDL cholesterol in scavenger receptor class B type I transgenic mice. *J. Biol. Chem., 274*: 7165-7171.

Urban; S.; Zieseniss, S.; Werder, M.; Hauser, H.; Budzinski, R.; Engelmann, B. (**2000**) Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. *J. Biol. Chem. 275*: 33409–33415.

Vance, D.E. (**2002**) Phospholipid biosynthesis in eukaryotes. In: biochemistry of lipids, lipoprotein and membrane, Eds. Vance, D.E.; Vance, J.E., 4th ed. Elsevier Science.

Vance, J.E. (**2008**) Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. *J. Lipid. Res., 49*: 1377–1387.

Van Meer, G. (**2000**), Cellular organelles: How lipids get there, and back. *Trends Cell. Biol., 10*: 550–552.

Van Meer G.; Voelker, D.R.; Feigenson, G.W. (**2008**) Membrane lipids: where they are and how they behave. *Nature, 9*: 112-124.

Varga, T.; Czimmerer, Z.; Nagy, L. (**2011**) PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim. Biophy. Acta, 1812*: 1007-1022.

Veldman, R.J.; Koning, G.A.; Hell, A.V.; Zerp, S.; Vink, S.R.; Storm, G.; Verheij, M., Van Blitterswijik, W.J. (**2005**) Conformulated N-octanoyl-glucosylceramide improves cellular delivery and cytotoxicity of liposomal doxorubicin. *J. Pharmacol. Exp. The., 315*: 704-710.

Verkleij, A.J.; Zwaal, R.F.; Roelofsen, B.; Comfurius, P.; Kastelijn, D.; Van Deenen, L.L. (**1973**) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta, 323*: 178–193.

Verspohl, E.J.; Roth, R.A.; Vigneri, R.; Goldfine, I.D. (**I984**) Dual regulation of glycogene metabolism by insulin and insulin-like growth factors in human hepatoma cells (HEP-G2). *J. Clin. Invest., 74*: 1436-1443.

Vigh, L.; Maresca, B.; Harwood, J.L. (**1998**) Does the membrane's physical state control the expression of heat shock and other genes?. *Trends Biochem. Sci., 23*: 369-374.

Voet, D.; Voet, J.G. (**2011**) Biochemistry, 4th ed., United States of America. John Wiley and Sons. ISBN 978-0470570951.

Vogel, S.; Piantedosi, R.; Frank, J.; Lalazar, A.; Rockey, D.C.; Friedman, S.L.; Blaner, W.S. (**2000**) An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J. Lipid Res., 41*: 882–893.

Vork, M.M.; Glatz, J.F.; Van Der Vusse, G.J. (**1993**) On the mechanism of long chain fatty acid transport in cardiomyocytes as facilitated by cytoplasmic fatty acid- binding protein. *J. Theor. Biol., 160*: 207–222.

Vorum, H.; Brodersen, R.; Kragh-Hansen, U.; Pedersen, A.O. (**1992**) Solubility of long- chain fatty acids in phosphate buffer at pH 7.4. *Biochim. Biophys. Acta, 1126*: 135–142.

Wakelam, M.; Rohwedder, A; Zhang, Q.; Rudge, S. (**2012**) LipidomicNet 5 October, Regulation of lipid droplet formation by oleate in Huh7 cells.

Wang, L.H.; Rothberg, K.G.; Anderson, R.G. (**1993**) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol., 123*: 1107-1117.

Wang, X.; Devaiaha, S.P.; Zhang, W.; Welti, R. (**2006**) Signaling functions of phosphatidic acid. *Prog. Lipid Res., 45*: 250–278.

Wang, Y.; Oram, J.F. (**2007**) Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C delta pathway. *J. lipid Res., 48*: 1062-1068.

Weisiger, R.A.; Ma, W.L. (**1987**) Uptake of oleate from albumine solution by rat liver. *J. Clin. Invest., 79*: 1070-1077.

Wijk, N.V.; Watkins, C.L.; Hageman, R.J.J.; Sijben, J.W.C.; Kamphuis, P.G.; Wurtman, R.J.; Broersen, L.M. (**2012**) Combined dietary folate, vitamin B-12, and docosahexaenoic acid concentration in rats. *Nut. Metab., 9*: 1-8.

Wileman, T.; Harding, C.; Stahl, P. (**1985**) Receptor mediated endocytosis. *Biochem. J., 262*: 19404–19413.

Williams, M.A.; Mc Cluer, R.H. (**1980**) The use of Sep-Pak C18 cartridges during the isolation of gangliosides. *J. Neurochem., 35*: 266-269.

Wirtz, K.A.W. (**2006**) Phospholipid transfer proteins in perspective. *FEBS Letters, 580*: 5436-5441.

Woldseth, B.; Christensen, E.; Christophersen, B.O. (**1993**) Incorporation of stearic acid (18:0) and palmitic acid (16:0) in phospholipid molecular species studied in isolated rat liver cells. *Biochim. Biophys. Acta., 1167:* 296-302.

Wolfrum, C.; Buhlmann, C.; Rolf, B.; Borchers, T.; Spener, F. (**1999**) Variation of liver-type fatty acid binding protein content in the human hepatoma cell line HepG2 by peroxisome proliferators and antisense RNA affects the rate of fatty acid uptake. *Biochim. Biophys. Acta., 1437*: 194-201.

Wong, H.; Hashimoto, S. (**1987**) Accumulation of cholesteryl ester and lipid droplets in macrophages after uptake of cholesterol-rich necrotic products. *Arterioscler. Thromb. Vasc. Biol., 7*:185-190

Wüstner, D.; Mondal, M.; Tabas, I.; Maxfield, F.R. (**2005**) Direct observation of rapid internalization and intracellular transport of sterol by macrophage foam cells. *Traffic, 6*: 396–412.

Yao, D.; Ehrlich, M.; Henis, Y.I.; Leof, E.B. (**2002**) Transforming growth factor-beta receptors interact with AP2 by direct binding to beta 2 subunit. *Mol. Biol. Cell, 13*: 4001-4012.

Yao, J.K.; Rastetter, G.M. (**1985**) Microanalysis of complex tissue lipids by high-performance thin-layer chromatography. *Anal. Biochem., 150*: 111-116.

Yoshida, Y.; Arimoto, K.; Sato, M.; Sakuragawa, N.; Arima, M.; Satoyoshi, E. (**1985**) Reduction of acid sphingomyelinase activity in human fibroblasts induced by AY-9944 and other cationic amphiphilic drugs. *J. Biochem., 98*: 1669-1679.

Zeidan, Y.H.; Pettus, B.J.; Elojeimy, S.; Taha, T.; Obeid, L.M.; Kawamori, T.; Norris, J.S.; Hannun, Y.A. (**2006**) Acid ceramidase but not acid sphingomyelinase is required for tumor necrosis factor-{alpha}-induced PGE2 production. *J. Biol. Chem., 281*: 24695-24703.

Zhan, Y.; Ginanni, N.; Tota, M.R.; Wu, M.; Bays, N.W.; Richon, V.M.; Kohl, N.E.; Bachman, E.S.; Strack, P.R.; Krauss, S. (**2008**) Control of cell growth and survival by enzymes of the fatty acid synthesis pathway in HCT-116 colon cancer cells. *Clin. Can. Res., 14*: 5735-5742.

Zhou, J.; Stubhaug, I.; Torstensen, B.E. (**2010**) Trans-membrane uptake and intracellular metabolism of fatty acids in Atlantic salmon (Salmo salar L.) hepatocytes. *Lipids, 45*: 301-311.

Zinser, E.; Sperka-Gottlieb, C.D.; Fasch, E.V.; Kohlwein, S.D.; Paltauf, f.; Daum, G. (**1991**) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote saccharomyces cerevisiae. *J. Bacteriol., 173*: 2026-2034.

8. ABBREVIATIONS

ACSL	acyl-CoA synthetase
BSA	bovine serum albumin
PBS	phosphate buffered saline
DMEM	dulbecco's modified eagle's medium
RPMI	roswell park memorial institute
TLC	thin layer chromatography
LDL	low density lipoprotein
LPDS	lipoprotein deficient serum
CAD	cationic amphiphilic drug
CE	cholesteryl ester
DAG	diacylglycerol
TAG	triacylglycerol
Chol	cholesterol
LD	lipid droplet
FA	fatty acid
FABP	fatty acid binding protein
LCPUFA	long chain polyunsaturated fatty acid
PL	polar lipid
SA	stearic acid
OA	oleic acid
LA	linoleic acid
LOA	linolenic acid
PC	phosphatidylcholine
PG	phosphatidylglycerol
PA	phosphatidic acid
PE	phosphatidylethanolamine
BMP	bis(monoacylglycero)phosphate
PI	phosphatidylinositol
SM	sphingomyelin
Cer	ceramide
GlcCer	glucosylceramide
LacCer	lactosylceramide