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TITOLO TESI

MEMBRANE LIPIDOMICS: THE REORGANIZATION OF FATTY ACIDS AS A BIOMARKER OF CELL CONDITION

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"...the mighty whales which swim in a sea of water,
and have a sea of oil swimming in them..."

MOBY DICK
Herman Melville

ABBREVIATIONS

AA Arachidonic Acid
ALA α-Linolenic Acid
CL Cardiolipin
DGLA di-homo-γ-Linolenic Acid
DHA Docosaesanoic Acid
EPA Eicosapentaenoic Acid
FA Fatty Acids
GLA γ-Linolenic Acid
LA Linoleic Acid
MUFA Monounsaturated Fatty Acids
OA Oleic Acid
PA Palmitic Acid
PA Phosphatidic Acid
PC Phosphatidylcholine
PE Phosphatidylethanolamine
PI Phosphatidylserine
POA Palmitoleic Acid
PS Phosphatidylserine
PUFA Polyunsaturated Fatty Acids
SA Stearic Acid
SFA Saturated Fatty Acids
UFA Unsaturated Fatty Acids
VA Vaccenic Acid

INTRODUCTION

CHAPTER 1
THE PLASMA MEMBRANE

1.1 An istorical perspective

Given their biological importance, membranes have been surprisingly neglected until recently. Perhaps this is understandable in view of the technical hurdles that working with them presents. Most methods require purification and observation in aqueous environments alien to the molecular design of a membrane, and so the field had to rely on oversimplified views that still dominate the texts and teaching in this area. But now we have a rising number of high-resolution structures, an abundance of functional data and an evolving conceptual basis for framing more pointed questions. This is leading to a great expansion of interest in the area, after the very tough path that traces the way in which the lipid-bilayer model developed over the past one-hundred years.

At the beginning, cells had only an 'end layer' — an outer layer of protoplasm of unknown composition and properties, which was often described in nineteenth century literature as a precipitate (Overton E, 1899-1968). This end layer was explored by physiologists, chemists and morphologists. Physiologists characterized the cell surface in terms of its functions; they measured the ease or difficulty with which migrant molecules and ions crossed the frontier. These physiological measurements showed that fat-soluble molecules generally crossed the frontier more easily than water-soluble molecules and ions. The cell-surface barrier was therefore inferred to be a lipid of some sort — in the words of the pioneering study, a "fatty oil" — rich in cholesterol and phospholipids (Overton E, 1899-1968). Later, physiological and biophysical experiments developed this initial model into a combined chemical and morphological model that was a layer, just a few lipids thick, which was coated with proteins. In the 1920s and 1930s measurements of cell-membrane capacitance by Fricke H (1923) indicated that the plasma membrane was only 4-nm thick, and measurements of the surface tension of many kinds of cells (Cole KS, 1932) indicated that the surface was covered with proteins rather than being naked lipid. The model was elaborated in a 1935 review by Danielli JN and Davson H.

Membrane chemistry and physics as we know them today began with observations of the spreading of oils and fats on water. In 1917, Langmuir I developed a method for measuring the pressure that is exerted by molecular films as they spread on water; he showed that lipids that spread in this way form a monomolecular layer on the surface of water. Simple arithmetic gave the area per lipid molecule and also showed that the hydrocarbon chains of the lipids were flexible; they did not extend straight out from the surface of the water, but were bent (Langmuir I, 1917).

This work paved the way for the resolution of the bilayer structure of the plasma membrane. The first step in this resolution came when Langmuir's methods for measuring the area per lipid molecule were applied to lipid extracts of erythrocyte membranes by Gorter E and Grendel F(1925). Using Langmuir's method, they measured the area occupied by lipids that were extracted from a known number of erythrocytes. Then, they measured the surface area of whole erythrocytes and calculated that the lipids of a single erythrocyte could be accommodated by a lipid bilayer. After summarizing their measurements and calculations for the erythrocytes of six different mammalian species, they concluded that,"It is clear that all our results fit in well with the supposition that the erythrocytes are covered by a layer of fatty substances that is two molecules thick". So, the lipid-bilayer membrane was born.

Optical imaging of membrane morphology had to wait for the advent of electron microscopy and the resolution that it can obtain. However, once a structure that corresponded to a bilayer had been imaged, it became clear that it was not only the plasma membrane that had this 75-Å-thick structure and, by 1959, it was being argued by Robertson that all cell-organelle membranes had a common structure (Robertson JD, 1959). Even ten years later, though, the bilayer was not accepted as the basic structure of cell membranes, and an important review by Stoeckenius W and Engelman DM (1969) was devoted to weighing up the evidence for the bilayer structure against the possibility that cell membranes were made of discrete, globular subunits. However, within a few more years, the reinterpretation of older work on the X-ray diffraction patterns of membranes (Fernandez-Morán H and Finean JB, 1957) and the accumulation of new evidence on the physical state of membrane lipids (Blasie JK and Worthington CR, 1969) consolidated the bilayer model for membranes. Rapidly evolving magnetic resonance methods — NMR and electron spin resonance — showed that bilayer lipids were in motion over numerous scales of time and distance, flexing and diffusing in the plane of the membrane. In short, the bilayer was more like a fluid than a solid. This work, which was mainly from the laboratories of McConnell and Chapman, is summarized in a contemporary review (Chapman D, 1975). The review also mentions the possibility that bilayer lipids are asymmetrically distributed — that is, that the two membrane leaflets have a different lipid composition and fluidity . Studies of erythrocyte membrane proteins (Bretscher MS, 1973; Fairbanks G *et al*., 1971) and surveys of proteins that were extracted from various other membranes led Singer SJ and Nicolson GL (1972) to make a crucial distinction between integral and peripheral membrane proteins in 1972. This took us to the model that is still the way most of us see membranes — the fluid mosaic model. The mosaic is made of proteins that are inserted into the fluid, which is the lipid bilayer. The model is more of a cartoon than a predictive model, but it

successfully managed to capture and integrate diverse experiments on membrane physics and chemistry.

In the bilayer membrane model of the 1980s, cell membranes were based on a largely fluid lipid bilayer in which proteins were embedded. The bilayer was highly dynamic; lipids (Smith RL and Oldfield E, 1984) and proteins (Edidin M, 1974) could flex, rotate and diffuse laterally in a two-dimensional fluid. The fluid was isotropic, that is, the diffusion of proteins and lipids was random unless it was constrained by the cytoskeleton or by the high concentration of membrane proteins. The lipids immediately surrounding a membrane protein could affect the function of the protein, which might be one explanation for the large number of lipid species (some 500–1,000 different kinds of lipids) that are present in a single membrane. There were numerous ideas about the coupling of reactions by diffusion (Gupte S. *et al*.,1984; Jans DA, 1977), but often the diffusion measurements were made on a μm scale, when the relevant reactions occurred on a scale of 10s of Å. The 1980s model captures the complexity of the fluid bilayer and the possibilities for molecular interactions in it by diffusion and collision. Although there had been a brief interest in detecting lipid-phase transitions in cell membranes, by 1980 the model largely neglected the possibility that lipids might not be randomly distributed in the bilayer and also understated the degree of local order that might be possible in membranes.

As the fluid mosaic picture was being assimilated by cell biologists, another picture was being sketched in which membranes contained patches of lipids, the composition and physical state of which differed from the average for the bilayer. This sketch by Jain and White started with model membranes (Jain MK and White HB, 1977), and was followed by a lot of work on the formation of lipid patches in model membranes. The lipids were said to form ‘domains’, which implies that the patches are not at equilibrium and so are not as stable or as long-lived as separated phases, which are at equilibrium. Some measurements on whole cells and intact membranes also detected lipid domains. Lipid domains were proposed to solve the problem of sorting and trafficking lipids and lipid-anchored proteins in polarized epithelial cells (Simons K and van Meer G, 1988). These molecules are differentially presented on the apical surface of morphologically polarized cells, which indicates that the cytoplasmic cell-sorting machinery can recognize them, even though they are on the inner surface of trafficking vesicles (Rodriguez-Boulan E and Nelson WJ, 1989). The ‘lipid-raft’model proposed that lipids that are to be sorted segregate into a raft, which is rich in cholesterol and sphingolipids. The entire raft is then recognized for trafficking either because it also contains transmembrane proteins or because the state of the raft lipids is somehow detected by cytoplasmic proteins. In 1992, the first, careful test of the raft hypothesis by Brown and Rose showed that a lipid-anchored protein could indeed enter a cholesterol- and sphingolipid-rich lipid

domain, which could be isolated in cold detergent (Brown DA and Rose JK, 1992). Later work found that many other molecules, such as signalling kinases, could be isolated in this detergent insoluble complex and attention therefore shifted from lipid rafts as trafficking units to lipid rafts as signalling platforms (Simons K and Ikonnen E, 1997).

1.2 Where we stand today

1.2.1 Lipid repertoire

From the ongoing cataloguing of lipid structures (lipidomics), it is clear that eukaryotic cells invest substantial resources in generating thousands of different lipids. Why do cells use ~5% of their genes to synthesize all of these lipids? The fundamental biological maxim that 'structure subserves function' implies that there must be evolutionary advantages that are dependent on a complex lipid repertoire. Although we now understand the specific functions of numerous lipids, the full definition of the utility of the eukaryotic lipid repertoire remains elusive.

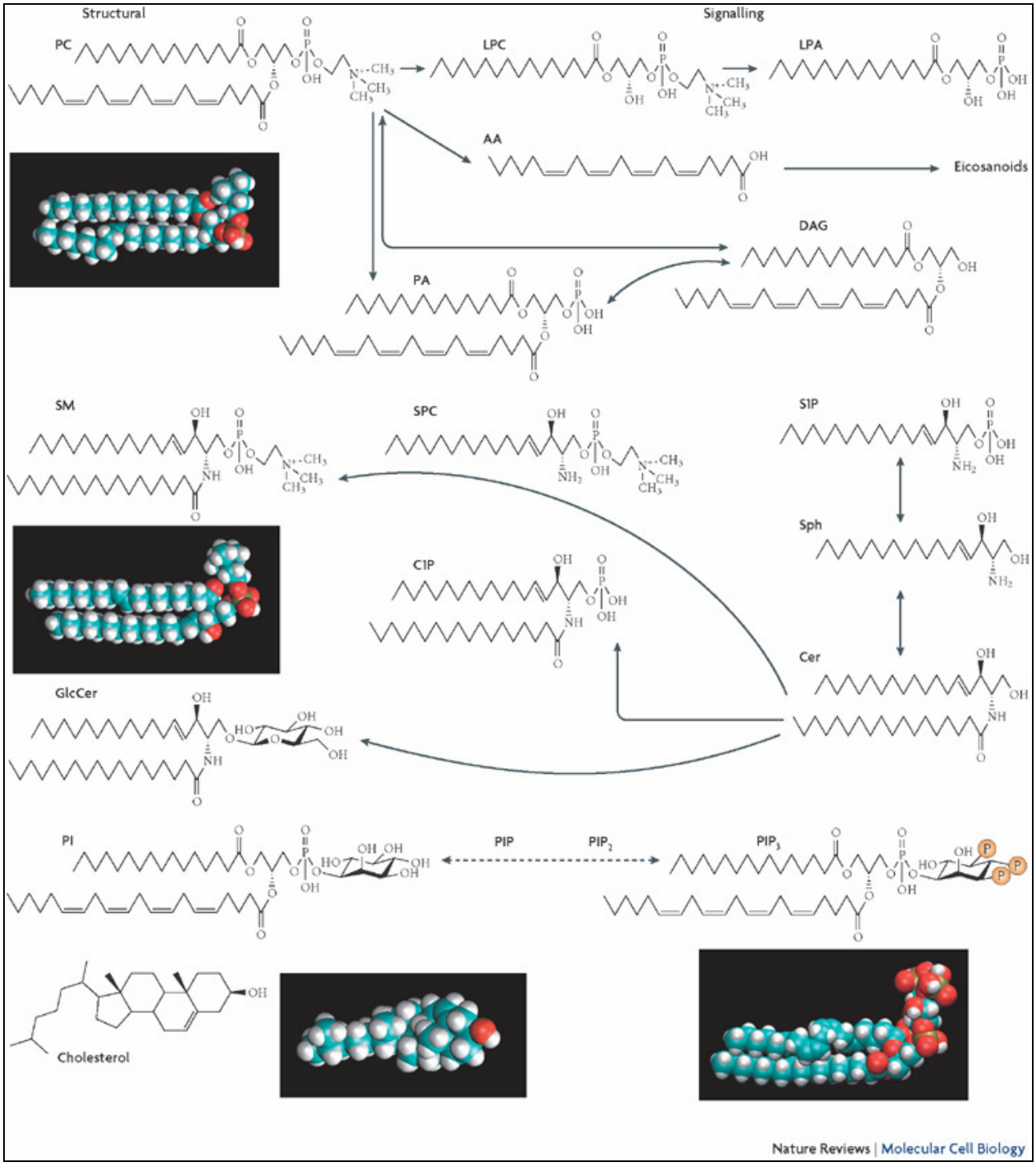
The major structural lipids in eukaryotic membranes are the glycerophospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA). Their hydrophobic portion is a diacylglycerol (DAG), which contains saturated or *cis*-unsaturated fatty acyl chains of varying lengths (Figure 2). PC accounts for >50% of the phospholipids in most eukaryotic membranes. It self-organizes spontaneously as a planar bilayer in which each PC has a nearly cylindrical molecular geometry, with the lipidic tails facing each other and the polar headgroups interfacing with the aqueous phase. Most PC molecules have one *cis*-unsaturated fatty acyl chain, which renders them fluid at room temperature. PE assumes a conical molecular geometry because of the relatively small size of its polar headgroup. The inclusion of PE in PC bilayers imposes a curvature stress onto the membrane, which is used for budding, fission and fusion (Marsh D, 2007). Non-bilayer lipids like PE and cardiolipin (CL) may also be used to accommodate membrane proteins and modulate their activities (Marsh D, 2007; Dowhan W and Bogdanov M, 2002). An additional factor that contributes to curvature stress in biomembranes is the asymmetric distribution of various lipids between the two bilayer leaflets.

The sphingolipids constitute another class of structural lipids. Their hydrophobic backbone is ceramide (Cer). The major sphingolipids in mammalian cells are sphingomyelin (SM) and the glycosphingolipids (GSLs), which contain mono-, di- oligosaccharides based on glucosylceramide (GlcCer) and sometimes galactosylceramide (GalCer)(Van Meer G and Lisman Q, 2002). Gangliosides are GSLs with terminal sialic acids. Sphingolipids have saturated (or *trans*-unsaturated) tails so they are able to form taller, narrower cylinders than PtdCho lipids of the same chain length and they pack more tightly, adopting the solid 'gel' or so phase; they are fluidized by sterols. Sterols are the major non-polar lipids of cell membranes: cholesterol predominates in mammals whereas ergosterol predominates in yeast. According to the umbrella model (Huang J and Feigenson GW, 1999; Ali MR *et al*., 2006), the preferential mixing of sterols with sphingolipids is

caused by shielding of the non-polar sterol by the sphingolipid headgroup rather than being caused by preferential intermolecular interactions.

Signalling-induced hydrolysis of glycerolipids and sphingolipids produces parallel series of messenger lipids: lysoPC (LPC), lysoPA (LPA), PA and DAG, versus sphingosylphosphorylcholine (SPC), sphingosine (Sph), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) and Cer (Figure 2). LPC, LPA, SPC, Sph and S1P carry only one aliphatic chain and readily leave membranes; they signal through related membrane receptors (Meyer zu Heringdorf D and Jakobs KH, 2007). By contrast, PA, DAG, C1P and Cer remain in the membrane and can recruit cytosolic proteins (Fernandis AZ and Wenk MR, 2007). Interestingly, when signalling lipids such as Cer are generated in large quantities (Kolesnick R and Hannun YA, 1999; Tepper AD *et al*., 2000), they can also affect membrane physical properties such as lipid phase behaviour. Cer can displace cholesterol from lipid umbrellas (Megha Sawatzki P, 2007) and drive its esterification. Phosphorylated derivatives of PtdIns (Figure 1) participate in signalling and recognition. These phosphoinositides are important in defining organelle identity and in recruiting both soluble and membrane proteins to specific membranes.

Figure 1. The main eukaryotic membrane lipids are the glycerophospholipids such as phosphatidylcholine (PC). Their diacylglycerol (DAG) backbone carries a phosphate (phosphatidic acid; PA) esterified to either a choline (forming PC), ethanolamine (forming phosphatidylethanolamine (PE); not shown), serine (forming phosphatidylserine (PS); not shown), or inositol (forming phosphatidylinositol (PI)). The prototypical phospholipid, dipalmitoyl-PC, exhibits nearly cylindrical molecular geometry with a cross-sectional surface area of 64 $Å^2$ and a head-to-tail length of 19 Å. The phosphosphingolipid sphingomyelin (SM) and the glycosphingolipid glucosylceramide (GlcCer) have a ceramide (Cer) backbone, consisting of a sphingoid base (such as sphingosine; Sph), which is amide-linked to a fatty acid. Yeast sphingolipids carry a C26 fatty acid and have phosphoinositol-X substituents that contain additional mannoses and phosphates. Breakdown products of membrane lipids serve as lipid second messengers. The glycerolipid-derived signalling molecules include lysoPC (LPC), lysoPA (LPA), PA and DAG. The sphingolipid-derived signalling molecules include sphingosylphosphorylcholine (SPC), Sph, sphingosine-1-phosphate (S1P), Cer-1-phosphate (C1P) and Cer. Arachidonic acid (AA) yields the signalling eicosanoids and endocannabinoids (not shown). The various phosphorylated PtdIns molecules (PIPs; also known as the phosphoinositides) mark cellular membranes and recruit cytosolic proteins. They are interconverted by the actions of kinases and phosphatases. (van Meer G *et al*., 2008).


Structural
Signalling
PC
LPC
LPA
AA
Eicosanoids
DAG
PA
SM
SPC
S1P
Sph
C1P
Cer
GlcCer
PI
PIP
PIP2
PIP3
Cholesterol
Nature Reviews | Molecular Cell Biology

1.2.2 Strucrure

An influential step in the study of membranes was taken with the development of the "fluid mosaic model" (Singer SJ and Nicolson GL, 1972), which pulled together findings and ideas from the preceding decade. The model has become the standard conceptualization of membrane architecture and is shown redrawn in Figure 2 as it appears in virtually all biochemistry texts. As important and insightful as this model has been, the emergence of new findings during the passage of 36 years has weakened the generalizations it contains. The model includes the ideas that the proteins of a membrane are dispersed, are at low concentration and that they match the hydrophobic dimension of an unperturbed lipid bilayer with peripheral belts of exposed hydrophobic side chains. The lipid is seen as a sea in which mainly monomeric proteins float unencumbered, and the bilayer surface is exposed directly to the aqueous environment. Each of these ideas is misleading. Most of the authors of the following reviews write of the preferential associations of molecules in the membrane plane; such associations are expected and membranes are typically crowded with their bilayers varying considerably in thickness. Is a membrane a random two-dimensional liquid? In the Singer–Nicholson model, molecules are distributed randomly in two dimensions. But we know from first principles and from experimental observation that non-randomness is the rule. Considering a mixture of n lipid and protein components in a membrane, the planar distribution can be random only if all pairwise interaction energies of the n different molecular species are within thermal energies (about 0.6 kcal mol^{-1} at room temperature) of each other. In a plasma membrane there are many species of lipids and proteins. The *E. coli* genome, for example, codes for more than a thousand putative helical transmembrane proteins (Liu Y *et al*., 2002), giving more than half a million kinds of pairwise combinations. A narrow range of interaction energies is a highly improbable condition given the range of known intermolecular interactions from hydrogen bonds, packing, electrostatics and the hydrophobic effect. Indeed, simply rotating a pair of identical helices against each other or changing a single interfacial side chain can result in interaction variations of several times kT (Doura AK *et al*., 2004; Adams PD *et al*., 1996). Thus, it should have been expected that regions of biased composition would exist and that the environments of proteins should vary, because it is highly improbable that interaction energies will match each other across all protein and lipid species in a membrane. Time-invariant complexes, transient associations and biased distributions should be the norm. Evolution, ever seeking to exploit the natural tendencies of molecules, has seized the opportunity to craft functional associations, and it is clear that there are functional protein complexes, separated lipid compositional areas and regions of functional specialization, although we do not yet know their extent. Views of the participation of membrane

proteins in organizing large functional complexes are beginning to emerge (Wong W and Scott JD., 2004). In common with proteins, lipids also tend to group together, forming lipid–lipid and lipid–protein interactions. Many lipids are seen in crystal structures to form specific complexes with proteins, most famously in the only structure of an entire membrane that we know — the purple membrane from Halobacterium salinarium (Grigorieff N *et al*., 1996). A large body of literature shows that lipids on their own form regions of separated composition in the plane of pure lipid vesicles (Reviewed by Maxfield FR and Tabas I, 2005). So, it would seem that patchiness is the order of the day.

The fluid mosaic model posits that "the structures of the lipid in the membrane and of the lipid in isolated aqueous dispersion are closely similar" and that "hydrophobic and hydrophilic interactions are to be maximized and the lowest free energy state is to be attained for the intact membrane in an aqueous environment" (Singer SJ and Nicolson GL, 1972). It follows that membrane proteins should have evolved their hydrophobic regions to fit the lipid dimension perpendicular to the membrane plane, since the exposure of hydrophobic surface area to water is unfavourable by about 25 cal $Å^{-2}$. Inspection of the known structures shows that they vary in hydrophobic dimension around their peripheries and also from one to another. Something must give — either the protein distorts to match the bilayer or the lipid distorts to match the protein, or both. The fluidity of the lipid and the relative rigidity of the proteins (Zaccai G, 2000) suggest that it is the main lipid that distorts to match the protein, and both modelling and experiment support this view (Mitra K *et al*., 2004), although protein distortion may occur in extreme cases of protein–lipid mismatch (Williamson IM et al., 2002). If the lipid distorts to cover the hydrophobic area of a membrane protein, the transmembrane dimension of a bilayer in membranes with high protein-to-lipid ratios must be variable. Further, if the distortion is asymmetrical across a bilayer, curvature can result (Reviewed by McMahon HT and Gallop JL, 2005). Local distortion of the bilayer is likely to influence protein interactions; for example, the peripheral energy of distorting the bilayer may enhance interactions that reduce the peripheral contour length. What effect the energy of distorting the bilayer might have on the protein itself is not known, but might have functional relevance for cases where the protein varies its transmembrane conformation in the course of activity (Perozo E *et al*., 2002).

In general it is not known how much of the membrane bilayer area is occupied by protein the answer to this question; yet the answer strongly influences concepts of membrane organization. Further, proteins may occupy small areas at the bilayer level but have large ectodomains covering lipid and creating steric restrictions. Most drawings of the fluid mosaic model greatly exaggerate the lipid area in both senses — the area occupied by protein and the area covered by protein are

shown as small and zero, respectively. Membrane protein shapes vary greatly. Some are largely contained within the bilayer (Abramson J *et al*., 2003; Fu D *et al*., 2000; Luecke H *et al*., 1999; Zheng L *et al*., 2004; Abramson J *et al*., 2000), while many protein complexes, such as the ATP synthase, have large structures outside the lipidic region that will create steric contacts and other interactions outside the bilayer (Iverson TM *et al*., 1999; Long SB *et al*., 2005; Kurisu G *et al*., 2003; Miyazawa A *et al*., 2003; Stock D *et al*., 1999; Stroebel D *et al*., 2003; Xia D *et al*., 1997; Zouni A *et al*., 2001). These may occupy larger areas in projection onto the membrane than do the transmembrane regions. Proteins anchored by single helices or by lipidic anchors such as fatty acids can cover large regions of a membrane with protein surfaces (Binda C *et al*., 2002; Bracey MH *et al*., 2002). Interactions of the ectodomain structures are known to be functionally important in many cases, such as the tyrosine kinase receptors (Ferguson KM *et al*., 2003). The exposure of membrane lipid surface may be rather small, for example when a plasma membrane is viewed from either the cytoplasm or the extracellular milieu. However, some proteins associate and dissociate with lipid as part of their function (Newton AC, 2003), so some lipid exposure must be maintained. Whether lipid exposure to the cytoplasm might be used to control or focus such interactions is at present unexplored.

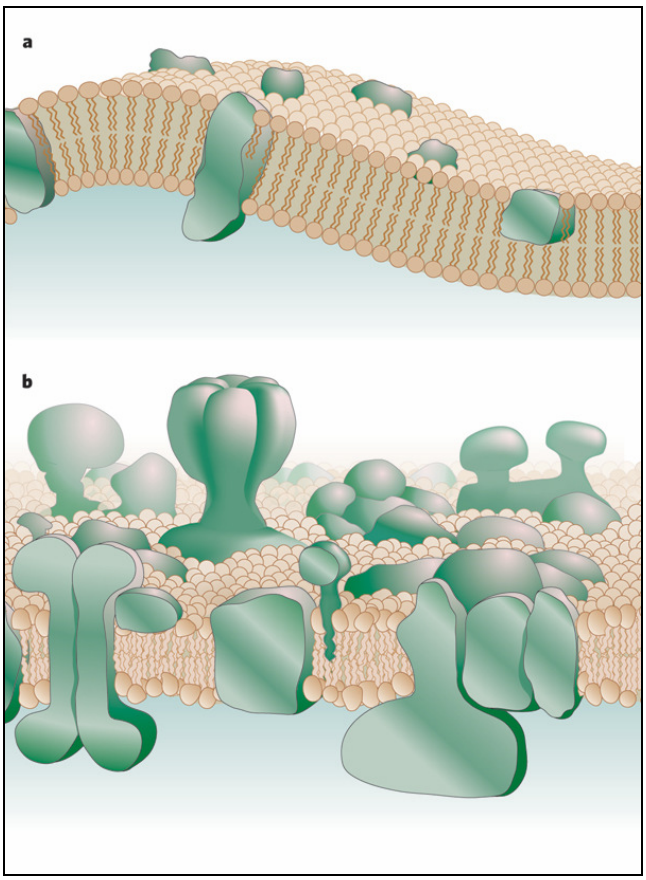


Figure 2. **a**, The Singer–Nicholson "fluid mosaic model". **b**, An amended and updated version. (Engelman DM, 2005).

Although most membranes exhibit fluidity, with rapid diffusion observed for some lipid and protein species in the plane of a membrane, recent measurements using single-particle tracking reveal a complex set of restrictions on protein lateral mobility. These include directed motion, confined motion and anomalous diffusion (Kusumi A *et al*., 2005). Although the observation that some proteins can move relatively freely suggests that a subset may not be in larger assemblies, crowding, ectodomain collisions, transbilayer interactions, adhesion sites and cytoskeletal structure produce a variety of restrictions on the motion of most proteins and lipids (Saxton MJ and Jacobson K, 1997). Not yet considered are the additional constraints imposed by lipid–protein interactions through complex formation and thickness perturbation. Fluidity must be reconciled with order. It follows that the patchiness of many membranes must be local enough for there to be channels of lipid separating regions of protein assemblies, but this constraint would still allow large segregated regions. The sizes and variability of segregated regions are still to be established.

The concepts developed above lead to a view of a membrane that has variable patchiness, variable thickness and higher protein occupancy than has generally been considered. But, while we await improved measurements and models, the modified view sketched in figure 2b is suggested as a guide to thinking.

1.2.3 Functions

All membranes of eukaryotic cells separate functional compartments, but the plasma membrane is an extreme. It is the frontier between the cell and its environment. The composition of the plasma membrane regulates frontier crossings by molecules between cell's surroundings and its interior, and the properties of the bilayer are different from those of any of its components alone. The reductionist view of biology, to which many adhere, rests in part on the structure–function hypothesis: that the structures we find are there for specific functional reasons selected by evolution. In the case of plasma membrane, it is meaningful starting with the origin of life. The matrix of cellular membranes is formed by amphipathic lipids, which consist of a hydrophobic and a hydrophilic portion. The propensity of the hydrophobic moieties to self-associate entropically driven by water, and the tendency of the hydrophilic moieties to interact with aqueous environments and with each other is the physical basis of the spontaneous formation of membranes. This fundamental principle of amphipathic lipids is a chemical property that enabled the first cells to segregate their internal constituents from the external environment. This same principle is recapitulated within the cell to produce discrete organelles. The cell membrane surrounds the cytoplasm of a cell and, in animal cells, physically separates the intracellular components from the extracellular environment. In fungi, some bacteria, and plants, an additional cell wall forms the outermost boundary; however, the cell wall plays mostly a mechanical support role rather than a role as a selective boundary. The compartmentalization enables segregation of specific chemical reactions for the purposes of increased biochemical efficiency and restricted dissemination of reaction products. In addition to the barrier function, lipids provide membranes with the potential for budding, tubulation, fission and fusion, characteristics that are essential for cell division, biological reproduction and intracellular membrane trafficking. Compartmentalization is essential for an organism, and that with compartmentalization must come specific ways to surmount the barrier defining the boundary of the compartment — the membrane. Thus, the lipid bilayer, which spontaneously forms permeability barriers surrounding aqueous interiors, must be modified by macromolecules for the uptake of nutrients and the disposal of waste. Further refinements led to the use of the barrier for its energy-storage properties and to the creation of ways to pass information between a cell and its environment. The cell membrane also plays a role in anchoring the cytoskeleton to provide shape to the cell, and in attaching to the extracellular matrix to help group cells together in the formation of tissues. The barrier is selectively permeable and able to regulate what enters and exits the cell, thus facilitating the transport of materials needed for survival. The movement of substances across the membrane can be either passive, occurring without the input of

cellular energy, or active, requiring the cell to expend energy in moving it. Moreover, membranes play a big role in tissues organization, with some receptors on the external surface of the cell membrane that participate in the grouping of cells to form tissues (cellular adhesion).

The processing of informations is one of the best goals obtained by cells through evolution, and enables them to communicate with the sorrounding envinronment, exploiting the very powerful tools that membranes developed during the history of life. Specific proteins embedded in the cell membrane can act as molecular signals that allow cells to communicate and protein receptors are found ubiquitously and function to receive signals from both the environment and other cells. These signals are transduced and passed in a different form into the cell. The movement of bacteria toward food and the response of target cells to hormones such as insulin are two examples of processes that hinge on the detection of a signal by a specific receptor in a cell membrane. Cell membranes can also serve as an assembly that organizes the specific enzymes involved in a given metabolic pathway. Binding the enzymes to the membrane in sequential order enables the series of chemical reactions in the pathway to be carried out efficiently. Some membranes have the capacity to generate chemical or electrical signals; for example cell membranes of nerve cells, muscle cells, and some eggs are excitable electrically. Other proteins on the surface of the cell membrane can serve as "markers" that identify a cell to other cells. The interaction of these markers with their respective receptors forms the basis of cell-cell interaction in the immune system. Lipids can also act as first and second messengers in signal transduction and molecular recognition processes. The degradation of amphipathic lipids allows for bipartite signalling phenomena, which can be transmitted within a membrane by hydrophobic portions of the molecule and also propagated through the cytosol by the soluble polar portion of the molecule. In addition, some lipids function to define membrane domains, which recruit proteins from the cytosol that subsequently organize secondary signalling or effector complexes.

In the last decades, great discoveries regarding lipid rafts, caveole, phospholipids asimmetry in leaflets and protein recruitment through membrane charge have contribute to open a very huge field of research in this area and to deeply modify our understanding of membrane dinamics.

Lipid rafts are defined as liquid ordered (*lo*) lipid domains enriched with sphingolipids and cholesterol segregated from bulk liquid disordered (*ld*) membranes (reviewed by London E, 2005). In the *lo* phase, the lateral mobility of the lipid molecules is similar to that in the *ld* phase, whereas the conformational order of the lipid hydrocarbon chains in the *lo* phase is similar to that in the solid ordered (*so*) phase. Cholesterol plays a crucial role in *lo* phase formation via interaction with the hydrocarbon chains of phospholipids or sphingolipids. Rafts have been implicated in a number of processes and systems both physiological and pathological. These include cell signalling, molecular

trafficking, the function of the immune, vascular, digestive and reproductive systems. The pathogenesis of diseases such as HIV (viral), Salmonella (bacterial) and malaria (eukaryotic) has been linked to the role of rafts. Typically this involves the exploitation of the host cell raft function by the pathogen for its own purposes, that is to gain access to the interior of a host cell. Certain proteins associated with cellular signaling processes have been shown to associate with lipid rafts (Brown D and Rose JK, 1992). Proteins that have shown association to the lipid rafts include glycosylphosphatidylinositol (GPI)-anchored proteins, doubly-acylated tyrosine kinases of the Src family and transmembrane proteins. This association can at least be partially contributed to the acylated, saturated tails of both the tyrosine kinases and the GPI-anchored proteins, which matches the properties of sphingolipids more than the rest of the membrane (Simons K and Ikonen E, 1997). While these proteins tend to continuously be present in lipid rafts, there are others that associate with lipid rafts only when the protein is activated. Some examples of these include, but are not limited to, B cell receptors (BCRs), T cell receptors (TCRs), thyrotropin receptor (TSHR), PAG, and an enzyme called CD39 (Horejsí V *et al*., 1999; Matkó J and Szöllõsi J, 2002; Latif R *et al*., 2003; Papanikolaou A *et al*., 2005; Petrie RJ *et al*., 2000). Other proteins are excluded from rafts, such as transferrin-receptor and a member of the Ras family. Sphingolipid and cholesterol-rich liquid ordered lipid domains have been studied in both eukaryotic cells and model membranes. However, while the coexistence of ordered and disordered liquid phases can now be easily demonstrated in model membranes, the situation in cell membranes remains ambiguous. Unlike the usual situation in model membranes, under most conditions, cell membranes rich in sphingolipid and cholesterol may have a "granular" organization in which the size of ordered and/or disordered domains is extremely small and domains may be of borderline stability. After 20 years since the proposal of the original concept though, structure and functions of lipid rafts are still obscure.

The cell biology of *caveolae* is a rapidly growing area of biomedical research. *Caveolae* are small (50–100 nanometer) invaginations of the plasma membrane in many vertebrate cell types, especially in endothelial cells and adipocytes. These flask-shaped structures are rich in proteins as well as lipids such as cholesterol and sphingolipids and have several functions in signal transduction (Anderson RG, 1998). They are known primarily for their ability to transport molecules across endothelial cells, but modern cellular techniques have dramatically extended our view of *caveolae*. They form a unique endocytic and exocytic compartment at the surface of most cells and are capable of importing molecules and delivering them to specific locations within the cell, exporting molecules to the extracellular space, and compartmentalizing a variety of signaling activities. They are not simply an endocytic device with a peculiar membrane shape but constitute an entire membrane system with multiple functions essential for the cell. They are also believed to

play a role in oncogenesis and in the uptake of pathogenic bacteria and certain viruses (Frank P and Lisanti M, 2004; Li X *et al*., 2005; Pelkmans L, 2005). *Caveolae* are one source of clathrin-independent endocytosis involved in turnover of adhesive complexes. Formation and maintenance of *caveolae* is primarily due to the protein caveolin. This protein has both a cytoplasmic C-terminus and a cytoplasmic N-terminus, linked together by a hydrophobic hairpin that is inserted into the membrane. The presence of caveolin leads to the local change in morphology of the membrane. Because of their specific lipid content, *caveolae* are sometimes considered as a caveolin-positive subset of lipid rafts.

The plasma membrane of mammalian cells contains about 20 mol % of anionic lipids on the inner leaflet (Figure 3). The preferential accumulation of negative charges creates an electric field, estimated at 105 V/cm. The negative surface charge of the inner leaflet determines the targeting of proteins containing polycationic motifs, including peripheral membrane proteins (Olivotto M *et al*., 1996). This electrostatic interaction has been best documented for the myristoylated alanine-rich C kinase substrate (MARCKS), which interacts with the plasmalemma through a polycationic domain, in conjunction with a myristoyl anchor (McLaughlin S and Aderem A, 1995). Hydrolysis of phosphoinositides and displacement of phosphatidylserine accounted for the change in surface potential at the phagosomal cup (Young *et al*., 2005); signaling molecules such as K-Ras, Rac1, and c-Src that are targeted to the membrane by electrostatic interactions were rapidly released from membrane subdomains where the surface charge was altered by lipid remodeling during phagocytosis. The realization of this charge-dependent anchorage led to the postulation of an "electro-static switch" model (McLaughlin S and Aderem A, 1995), which predicts that the formation and stability of electrostatic associations can be regulated by changes in the charge of either the cationic protein complex or the anionic lipid layer. The unique negativity of the plasmalemmal inner leaflet has been attributed, in part, to its high polyphosphoinositide content. Phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] are required to target and retain polycationic proteins to the plasma membrane (Heo WD *et al*., 2006). Although they are polyvalent, polyphosphoinositides represent only a minor fraction of the phospholipids of the plasma membrane and are less abundant than phosphatidylserine (PS), the predominant anionic species, which represents 10 to 20% of all surface lipid (Vance JE and Steenbergen R, 2005). The accumulation of this anionic lipid produces the accretion of negative surface charge. As a result, polycationic proteins, particularly those bearing a hydrophobic anchorage site, associate with PS-enriched compartments, including endosomes and/or

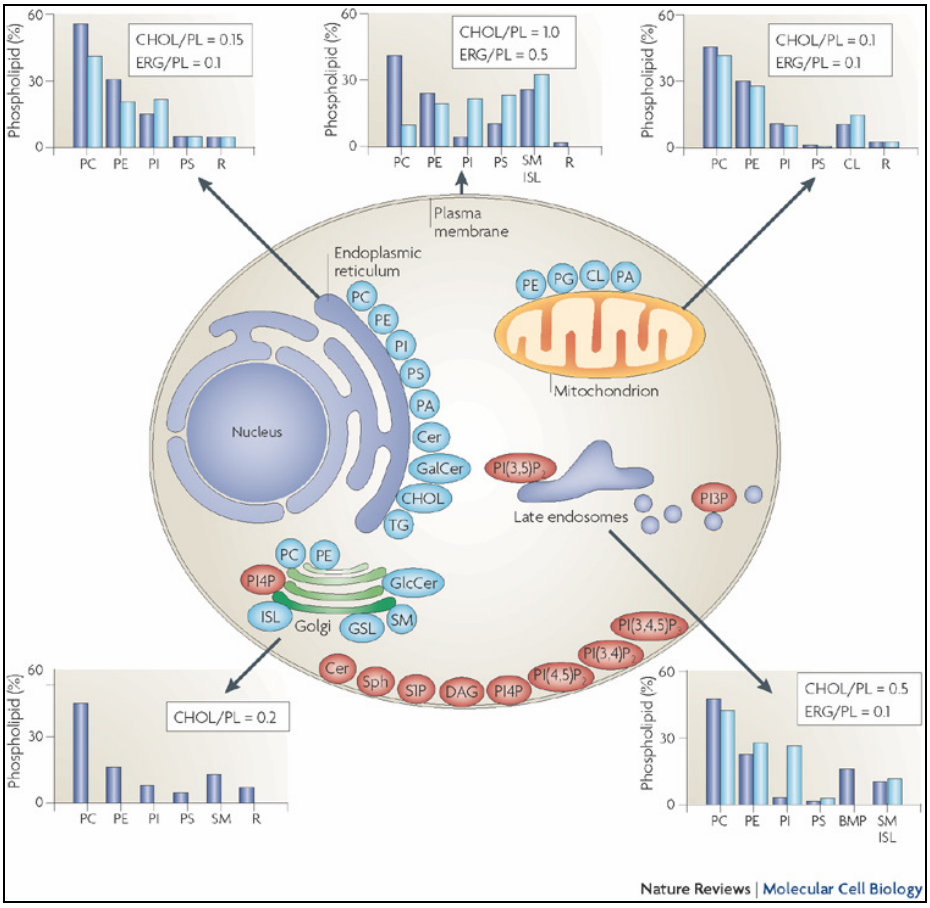


Figure 3. The lipid composition of different membranes varies throughout the cell. The lipid compositional data (shown in graphs) are expressed as a percentage of the total phospholipid (PL) in mammals (blue) and yeast (light blue). As a measure of sterol content, the molar ratio of cholesterol (CHOL; in mammals) and ergosterol (ERG; in yeast) to phospholipid is also included. The figure shows the site of synthesis of the major phospholipids (blue) and lipids that are involved in signalling and organelle recognition pathways (red). It should be appreciated that the levels of signalling and recognition lipids are significantly below 1% of the total phospholipid, except for ceramide (Cer). The major glycerophospholipids assembled in the endoplasmic reticulum (ER) are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). In addition, the ER synthesizes Cer, galactosylceramide (GalCer), cholesterol and ergosterol. Both the ER and lipid droplets participate in steryl ester and triacylglycerol (TG) synthesis. The Golgi lumen is the site of synthesis of sphingomyelin (SM), complex glycosphingolipids (GSLs) and yeast inositol sphingolipid (ISL) synthesis. PC is also synthesized in the Golgi, and may be coupled to protein secretion at the level of its diacylglycerol (DAG) precursor. Approximately 45% of the phospholipid in mitochondria (mostly PE, PA and cardiolipin (CL)) is autonomously synthesized by the organelle. BMP (bis(monoacylglycero)phosphate) is a major phospholipid in the inner membranes of late endosomes. PG, phosphatidylglycerol; $PI_{(3,5)}P_2$, phosphatidylinositol-(3,5)-bisphosphate; $PI_{(4,5)}P_2$, phosphatidylinositol-(4,5)-bisphosphate; $PI_{(3,4,5)}P_3$, phosphatidylinositol-(3,4,5)-trisphosphate; PI_4P, phosphatidylinositol-4-phosphate; R, remaining lipids; S1P, sphingosine-1-phosphate; Sph, sphingosine. (van Meer G *et al.*, 2008).

lysosomes. Electrostatic binding will occur in a manner dependent on both the charge of the membrane and that of the ligand, such that the most-negative membrane (i.e., the plasmalemma) will overwhelmingly accumulate the most cationic proteins, whereas less-positive proteins associate with the plasma membrane and, to a substantial degree, also with membranes of intermediate charge. Because the interaction is dynamic, changes in charge can redirect proteins from one target membrane to another. Thus, diminution of the plasmalemmal charge caused by phospholipid redistribution or metabolism, or phosphorylation of proteins like K-Ras can relocalize them to endocytic membranes, where they could catalyze a different set of reactions. Clearly, interaction with the surface charge of membranes is but one of the determinants of protein targeting, and other types of interactions must not be neglected.

To ensure the numerous functions we have told before can work properly, cells have to preserve their membranes fluidity, trying to respond to the variability of the external envinronment. The "homeoviscous adaptation" is the ability of cells and organisms to regulate the fluidity of their cell membranes by altering lipid composition. The maintenance of proper cell membrane fluidity is of critical importance for the function and integrity of the cell, essential for the mobility and function of embedded proteins and lipids, diffusion of proteins and other molecules laterally across the membrane for signaling reactions, and proper separation of membranes during cell division. A fundamental biophysical determinant of membrane fluidity is the balance between saturated and unsaturated fatty acids. Regulating membrane fluidity is especially important in poikilothermic organisms such as bacteria, fungi, protists, plants, fish and other 'cold-blooded' animals that cannot regulate their own body temperatures. The general trend is an increase in unsaturated fatty acids at lower growth temperatures and an increase in saturated fatty acids at higher temperatures. This compositional adaptation of membrane lipids serves to maintain the correct membrane fluidity at the new conditions. Although it is known and well defined its importance, few is already known about the mechanisms that cells exploit to follow membrane fluidity, and to respond in a fast way to changes that can happen in the outer envinronment. But only one seems to be the answer: to modify their fatty acid balance.

CHAPTER 2
MEMBRANE FATTY ACIDS

2.1 Physico-chemical characterization

Phospholipids, the main constituents of membranes, are composed by an hydrophilic polar head and two hydrophobic non-polar fatty acid tails (Figure 4), which properties will be discussed in this chapter. In chemistry, a fatty acid (FA) is a carboxylic acid often with a long unbranched aliphatic chain; it has a carboxylic acid at one end and a methyl group at the other end (Figure 5). Carbon atoms in a FA are identified by greek letters on the basis of their distance from the carboxylic acid. The carbon atom closest to the carboxylic acid is the alpha carbon, the next adjacent carbon is the beta carbon, etc. In a long-chain FA the carbon atom in the methyl group is called the omega carbon, because omega is the last letter of the greek alphabet. Carboxylic acids as short as butyric acid (4 carbon atoms) are considered to be FA, whereas FA derived from natural fats and oils may be assumed to have at least 8 carbon atoms. Most of the natural FA have an even number of carbon atoms, because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group.

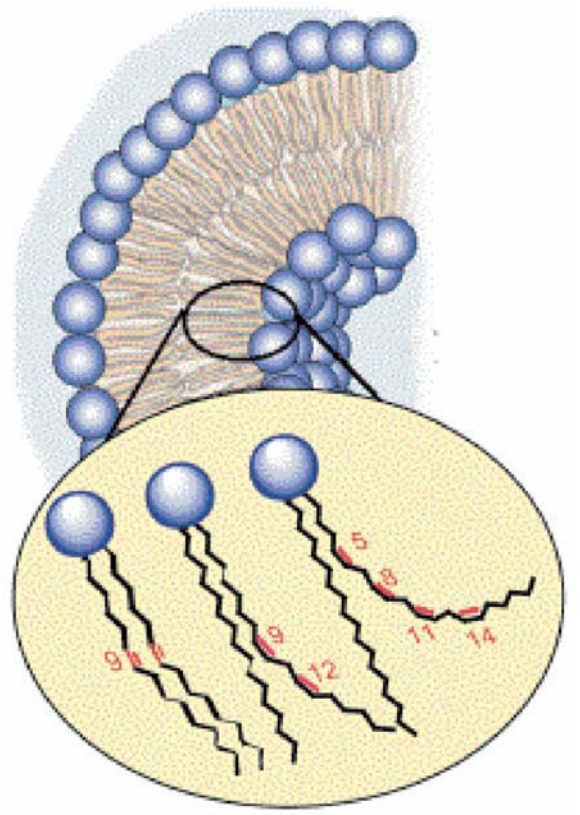


Figure 4. Membrane model with different FA residues. (Ferreri C *et al*., 2005).

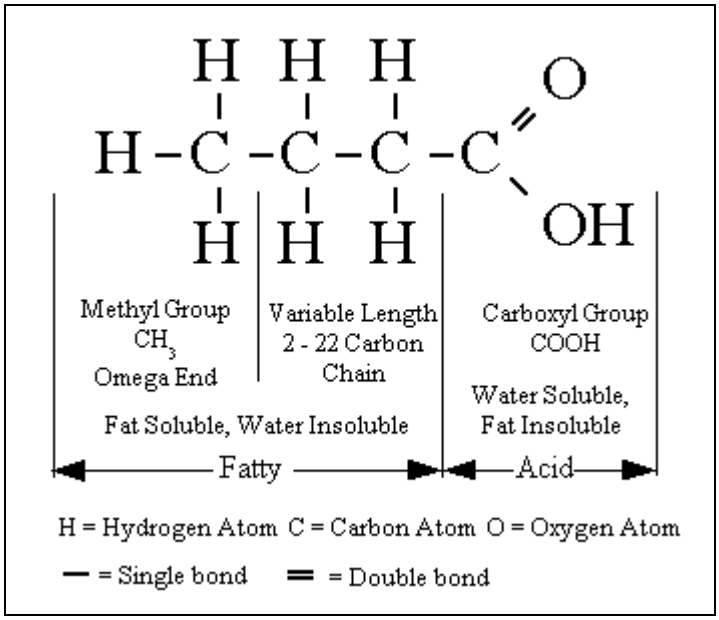


Figure 5. Chemical structure of a fatty acid.

2.1.1 Nomenclature

To describe precisely the structure of a FA molecule, it should be given the length of the carbon chain (number of carbons), the number of double bonds and also the exact position of these double bonds. This will define not only each FA physico-chemical properties, but also its biological reactivity and even of the lipid containing the FA studied. There are several different systems of nomenclature in use for FA; table 1 describes the most common systems. Hereinafter in this thesis, FA will be designated with their common names where possible, despite the accuracy of informations that a systematic nomenclature would give.

System	Example	Explanation
Trivial nomenclature	α-Linolenic acid	Common names are non-systematic historical names which are the most frequent naming system used in literature. Most common FA have trivial names in addition to their *systematic names*. These names do not follow any pattern, but are concise and generally unambiguous.
Systematic nomenclature	(9*E*,12*E*,15*E*)-Octadecatrienoic acid	Systematic names (or IUPAC names) derive from the standard *IUPAC Rules for the Nomenclature of Organic Chemistry*, published in 1979, along with a recommendation published specifically for lipids in 1977. Counting begins from the carboxylic acid end. Double bonds are labelled with *cis*-/*trans*-notation or *E*-/*Z*- notation, where appropriate. This notation is generally more verbose than common nomenclature, but has the advantage of being more technically clear and descriptive.
Δ^x nomenclature	*cis,cis,cis*-Δ^9,Δ^{12},Δ^{15}	In Δ^x nomenclature, each double bond is indicated by Δ^x, where the double bond is located on the *x*th carbon–carbon bond, counting from the carboxylic acid end. Each double bond is preceded by a *cis*- or *trans*- prefix, indicating the conformation of the molecule around the bond.
***n*−*x* nomenclature**	n−3 Ω-3	*n*−*x* (*n* minus *x*; also ω−*x* or omega-*x*) nomenclature does not provide names for individual compounds, but is a shorthand way to categorize FA by their physiological properties. Holman RT proposed in 1964 a new numbering system for the unsaturation of FA, the "omega nomenclature". A double bond is located on the *x*th carbon–carbon bond, counting from the terminal methyl carbon (designated as *n* or Ω) toward the carbonyl carbon. For example, α-linolenic acid is classified as a *n*−3 or omega-3 FA, and so it shares properties with other compounds of this type. The Ω −*x* or omega-*x* notation is common in popular literature, but IUPAC has deprecated it in favor of *n*−*x* notation in technical documents.
Lipid numbers	18:3	Lipid numbers take the form *C*:*D*, where *C* is the number of carbon atoms and *D* is the number of double bonds in the FA. This notation is ambiguous, as different FA can have the same numbers. Consequently, this notation is usually paired with either a Δ^x or *n*−*x* term.

Table 1. Different systems of nomenclature in use for FA. Examples of the different nomenclature of α-Linolenic acid are highlighted. (Fahy E *et al*., 2005).

2.1.2 Classification

Because FA are composed by a huge number of different families, there are many classifications available. The following FA classification is first based on the type of carbon chain: either straight (or normal), or branched, or containing a carbon ring. In each category, subdivisions are created according to the functional groups substituted on the carbon chain.

A - Normal fatty acids (straight chain)

Carbon chain without substituent

1 - Saturated fatty acids
2 - Monoenoic fatty acids
3 - Polyenoic fatty acids
    Methylene-interrupted
    Polymethylene-interrupted
    Conjugated
    Allenic acids
    Cumulenic acids
4 - Acetylenic fatty acids

Carbon chain with substituent

1 - Hydroxy fatty acids
2 - Dicarboxylic acids
3 - Fatty acid carbonates
4 - Divinyl ether fatty acids
5 - Sulfur containing fatty acids
6 - Fatty acid amides
7 - Methoxy and acetoxy fatty acids
8 - Keto fatty acids
9 - Aldehydic fatty acids
10 - Halogenated fatty acids
11 - Nitrated fatty acids
12 - Arsenic containing fatty acids

B - Branched-chain fatty acids

1 - Mono or multibranched chain fatty acids
2 - Branched methoxy fatty acids
3 - Branched hydroxy fatty acids (Mycolic acids)

C - Ring containing fatty acids

1 - Cyclopropane acids
2 - Cyclobutane acids
3 - Cyclopentenyl acids
4 - Furanoid acids
5 - Cyclohexyl acids
6 - Phenylalkanoic acids
7 - Epoxy acids
8 - Cyclic fatty peroxides
9 - Lipoic acid

Despite this huge variety, few of them are actually used by the human body and by vertebrates in general. Analysis of all these groups would require a long discussion, and is beyond the scope of this thesis. More informations are available online (http://www.cyberlipid.org/fa, http://www.lipidbank.jp). In the next paragraphs, properties of normal FA with straight chains, commonly used by human cells, will be anayzed. When different ones will come into discussion, they will be characterized at the moment.

Saturated Fatty Acids (SFA)

SFA do not contain any double bond or other functional groups along the chain. The term "saturated" refers to hydrogen, in that all carbons (apart from the carboxylic acid [-COOH] group) contain as many hydrogens as possible. In other words, the omega (ω) end contains 3 hydrogens (CH3-), and each carbon within the chain contains 2 hydrogen atoms. They have commonly straight chains and even carbon number (4-30) (Figure 6). They have the general formula: CH3(CH2)nCOOH. They are named from the saturated hydrocarbon with the same number of carbon atoms, with the final -e changed to -oic. Up to 6 (or 4) carbon atoms, organic acids are considered "short-chain organic acids", they have substantial solubility in water. Biochemically, they are more closely related to carbohydrates than to fats. From 8 (or 6) to 10 (or 12) carbon atoms, fatty acids are said to have a medium chain. FA which have 14 (or 12) and more carbon atoms are considered as long-chain FA.

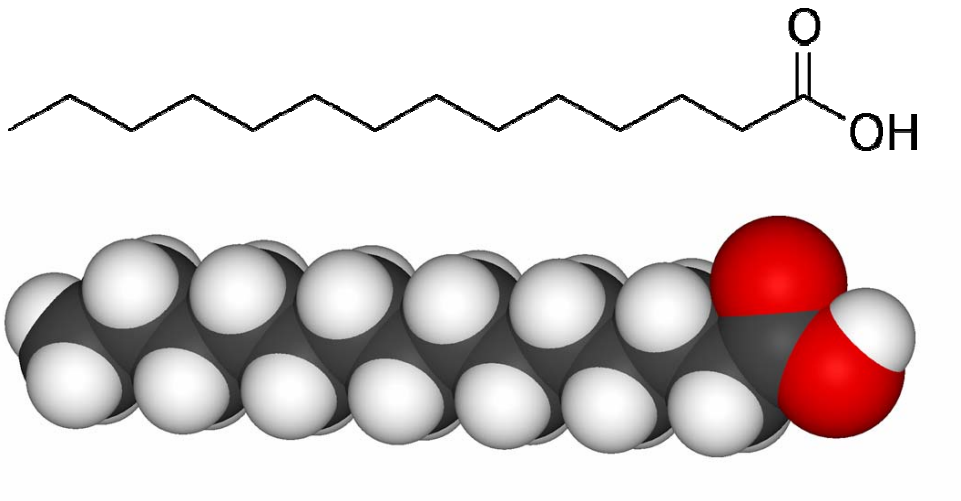


Figure 6. A two-dimensional representation and a space-filling model of the SFA myristic acid (14:0).

Most commonly-occurring SFA are found in table 2. With the exception of the short chain ones, all SFA have a high melting point, meaning that at normal temperature they are in solid state; this physico-chemical characteristic is due to their straight shape structure that cause the high packing that these FA are able to obtain. Normal FA exhibit appreciable solubility in water compared to the corresponding hydrocarbons due to the presence of the polar carboxyl group. The first members of the SFA series are miscible with water in all proportions at room temperature. The normal saturated FA are generally more soluble in chloroform and less soluble in acetonitrile than in any of the organic solvents investigated.

Common name	Systematic name	Shorthand designation	Melting point (°C)
Butyric acid	Butanoic acid	C4:0	-8
Caproic acid	Hexanoic acid	C6:0	-3
Caprylic acid	Octanoic acid	C8:0	16-17
Capric acid	Decanoic acid	C10:0	31
Lauric acid	Dodecanoic acid	C12:0	44-46
Myristic acid	Tetradecanoic acid	C14:0	58.8
Palmitic acid	Hexadecanoic acid	C16:0	63-64
Stearic acid	Octadecanoic acid	C18:0	69.9
Arachidic acid	Eicosanoic acid	C20:0	75.5
Behenic acid	Docosanoic acid	C22:0	74-78
Lignoceric acid	Tetracosanoic acid	C24:0	87.5-88.0

Table 2. The most commonly-occurring SFA.

Mono-Unsaturated Fatty Acids (MUFA)

Monounsaturated fats are FA that have a single double bond in the FA chain and all the other carbon atoms in the chain are single-bonded. They have the general structure: CH3(CH2)xCH=CH(CH2)yCOOH. The most frequently they have an even number of carbon atoms and the unique double bond may be in a number of different positions. FA fluidity increases with the increasing number of double bonds, because the double bond in the *cis* configuration insert a bend in the straight chain. This bend leads to a total spatial width of 0.72 nm for a *cis*-MUFA that narrows to 0.32 nm in saturated structures. The consequence in a three dimensional membrane is a higher molecular disorder due to the inhability of unsaturated FA to pack as SFA do. Therefore, MUFA have a higher melting temperature than PUFA but lower than SFA. MUFA are liquids at room temperature and semisolid or solid when refrigerated. Over a hundred naturally occurring monoene FA have been identified; common MUFA are very few though, being palmitoleic acid (16:1,*cis*-Δ^7), cis-vaccenic acid (18:1,*cis*-Δ^7) and oleic acid (18:1,*cis*-Δ^9). Palmitoleic acid has 16

carbon atoms with the first double bond occurring 7 carbon atoms away from the methyl group (and 9 carbons from the carboxyl end). It can be lengthened to the 18-carbon cis-vaccenic acid. Oleic acid has 18 carbon atoms with the first double bond occurring 9 carbon atoms away from the methyl group (Figure 7).

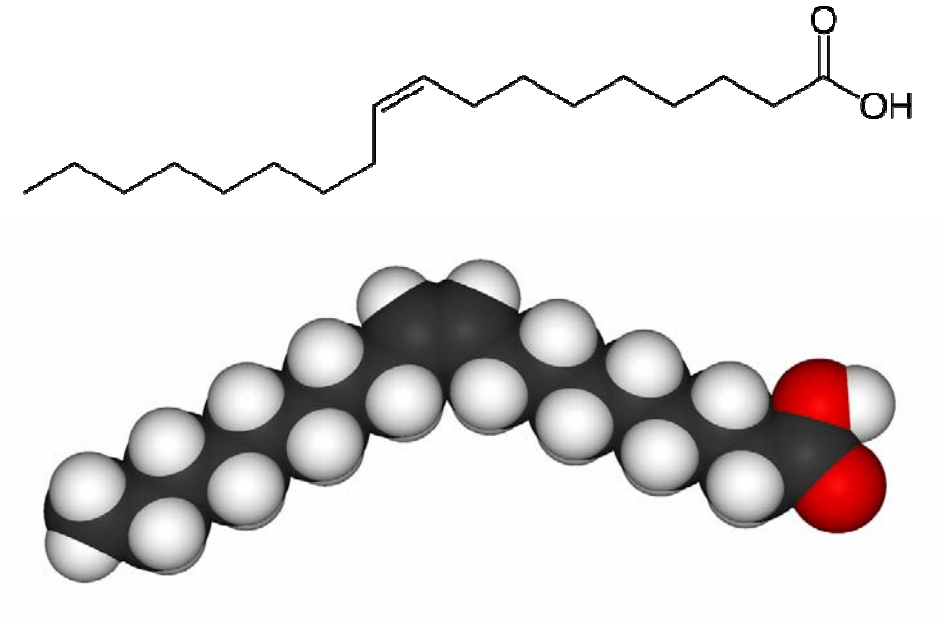


Figure 7. A two-dimensional representation and a space-filling model of the MUFA oleic acid (18:1).

Common name	Systematic name	Shorthand designation	Melting point (°C)
Palmitoleic acid	9-hexadecenoic	16:1	0.5
Vaccenic acid	11-octadecenoic	18:1	14.5-15.5
Oleic acid	9-octadecenoic	18:1	16.2

Table 3. The most commonly-occurring MUFA.

Poli-Unsaturated Fatty Acids (PUFA)

These FA have two or more *cis* double bonds which are the most frequently separated from each other by a single methylene group (methylene-interrupted polyenes), with a typical structure of the type -C-C=C-C-C=C-. Linoleic acid is a typical member of this group (Figure 8). Some other PUFA undergo a migration of one of their double bonds which are not again

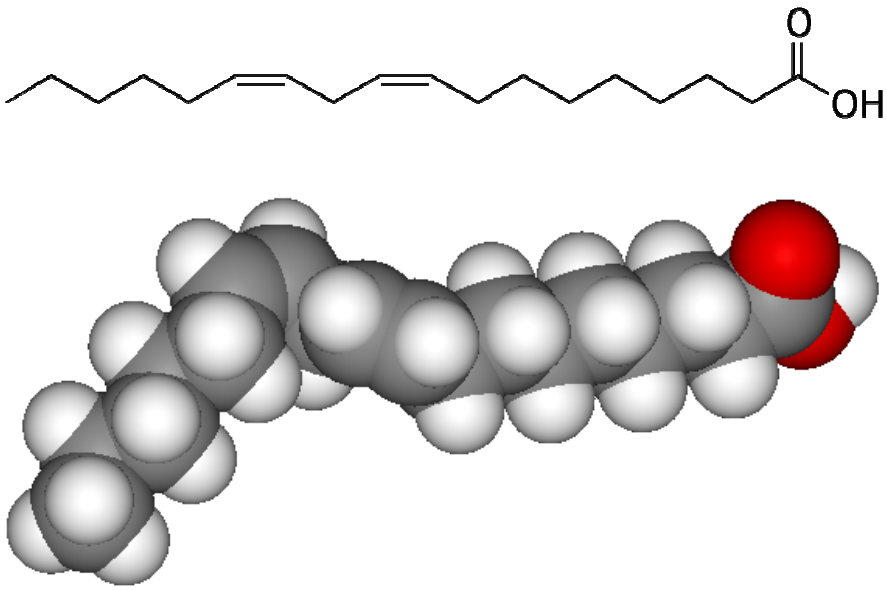


Figure 8. A two-dimensional representation and a space-filling model of the poliunsaturated fatty acid linoleic acid (18:2).

methylene-interrupted and are known as conjugated FA (-C-C=C-C=C-C-). Some unusual FA have not the regular structure with a methylene group between two double bonds but are polymethylene-interrupted polyenes (known also as non-methylene-interrupted FA, -C=C-C-C-C-C=C-). They are found in certain classes of bacteria, plants, marine invertebrates and insects. Rare FA have allenic (-C=C=C-) or cumulenic (-HC=C=C=CH-) double bonds; they are found in some higher plants. The greater the degree of unsaturation in a FA, the more vulnerable it is to lipid peroxidation. Antioxidants can protect unsaturated fat from lipid peroxidation. The differences in geometry between the various types of unsaturated FA, as well as between saturated and unsaturated fatty acids, play an important role in biological processes, and in the construction of biological structures such as cell membranes. Unsaturated fats have a more enlarged shape than saturated fats, because the double bond structures increase the bend of the acyl chain; moreover, the lack of the extra hydrogen atoms on the molecule's surface typically reduces the strength of the compound's

intermolecular forces. All this therefore causes the melting point of the compound to be significantly lower (Table 4). The most important FA can be grouped into three series with a common structural feature: $CH_3(CH_2)xCH=R$. x=4 for the n-6 series, x=1 for the n-3 series and x=7 for the n-9 series.

Common name	**Systematic name**	**Shorthand designation**	**Family**	**Melting point (°C)**
Linoleic acid	9,12-octadecadienoic	18:2	n-6	-5
γ-linolenic acid	6,9,12-octadecatrienoic	18:3	n -6	-11.3 to -11
Dihomo-γ-linolenic acid	8,11,14-eicosatrienoic	20:3	n -6	/
Arachidonic acid	5,8,11,14-eicosatetraenoic	20:4	n -6	-50
α-linolenic acid	9,12,15-octadecatrienoic	18:3	n -6	-11
Stearidonic acid	6,9,12,15-octadecatetraenoic	18:4	n -6	-57
EPA	5,8,11,14,17-eicosapentaenoic	20:5	n -6	-54
DPA	7,10,13,16,19-docosapentaenoic	22:5	n -6	/
DHA	4,7,10,13,16,19-docosahexaenoic	22:6	n -6	-44
Mead acid	5,8,11-eicosatrienoic	20:3	n -6	/

Table 4. The most commonly-occurring PUFA.

2.1.3 The geometry of the double bond: *Cis vs Trans*

Carbon atoms are tetravalent, forming four covalent bonds with other atoms, while hydrogen atoms bond with only one other atom. In SFA, each carbon atom is connected to its two neighbour carbon atoms as well as two hydrogen atoms. In unsaturated FA the carbon atoms that are missing a hydrogen atom are joined by double bonds rather than single bonds, so that each carbon atom participates in four bonds. The same molecule, containing the same number of atoms, with a double bond in the same location, can be either a *trans* or a *cis* FA, depending on the conformation of the double bond. For example, oleic acid and elaidic acid (Figure 9) are both unsaturated FA with the chemical formula $C_9H_{17}C_9H_{17}O_2$ (Taylor and Francis, 2007). They both have a double bond located

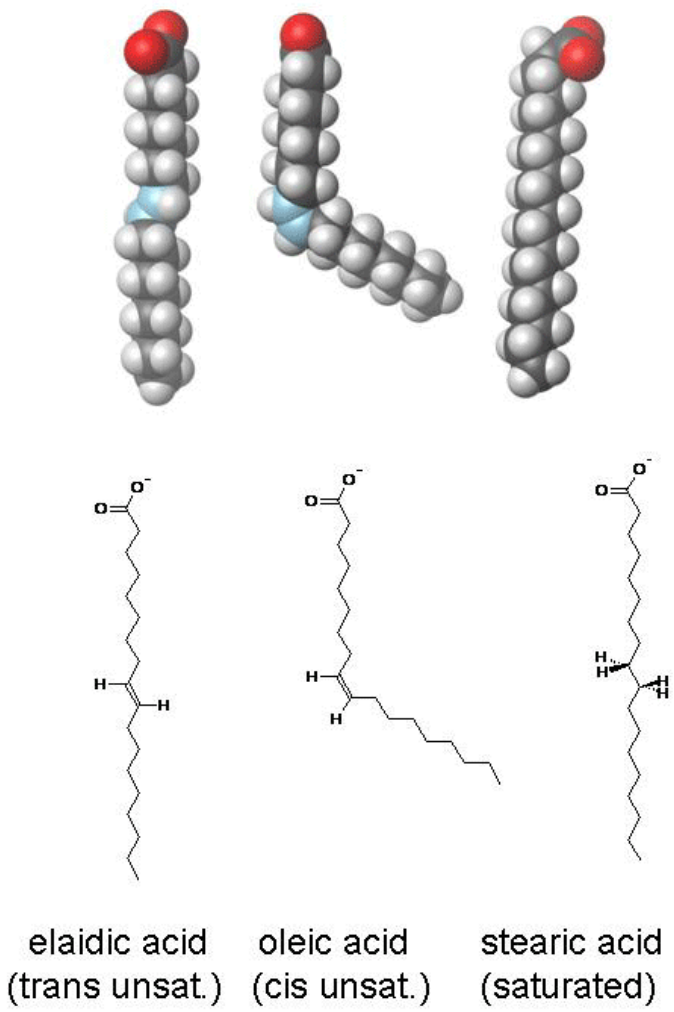


Figure 9. A space-filling model and a two-dimensional representation comparison between the *trans*-isomer and the *cis*-isomer of oleic acid and their respective 18:0 SFA stearic acid.

midway along the carbon chain. It is the conformation of this bond that sets them apart. The conformation has implications for the physical-chemical properties of the molecule. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond. The rigidity of the double bond freezes its conformation and, in the case of the *cis* isomer, causes the chain to bend and restricts the conformational freedom of the fatty acid. When a chain has many *cis* bonds, it becomes quite curved in its most accessible conformations. For example, OA, with one double bond, has a kink in it, whereas LA, with two double bonds, has a more pronounced bend. ALA, with three double bonds, favors a hooked shape. The effect of this is that, in restricted environments, such as when FA are part of a phospholipid in a lipid bilayer, *cis* bonds limit the ability of FA to be closely packed, and therefore could affect the melting temperature of the membrane. A *trans* configuration, by contrast, means that the next two hydrogen atoms are bound to opposite sides of the double bond. As a result, they do not cause the chain to bend much, and their shape is similar to straight SFA (Figure 8). The *trans* FA elaidic acid has different chemical and physical properties owing to the slightly different bond configuration. Notably, it has a much higher melting point, 45 °C rather than OA's 13.4 °C (Table 5), due to the ability of the *trans* molecules to pack more tightly, forming a solid that is more difficult to break apart (Taylor and Francis, 2007). This notably means that it is a solid at human body temperatures.

Common name	**Systematic name**	**Shorthand designation**	**Melting point (°C)**
Oleic acid	*cis*-9-octadecenoic	18:1	16.2
Elaidic acid	*trans*-9-octadecenoic	tr18:1	43.7
Vaccenic acid	*cis*-11-octadecenoic	18:1	14.5-15.5°C
Trans-vaccenic acid	*trans*-11-octadecenoic acid	tr18:1	44

Table 5. The comparison between the most commonly-occurring *trans*-fatty acids and their respective *cis*-configured ones.

A type of *trans* fat occurs naturally in the milk and body fat of ruminants (such as cattle and sheep) at a level of 2–5% of total fat. Natural *trans* fats, which include conjugated LA (CLA) and vaccenic acid, originate in the rumen of these animals. However, CLA is also a *cis* fat. Animal-based fats were once the only *trans* fats consumed, but by far the largest amount of *trans* fat consumed today

is created by the processed food industry as a side-effect of partially hydrogenating unsaturated plant fats (generally vegetable oils). These partially hydrogenated fats have displaced natural solid fats and liquid oils in many areas, notably in the fast food, snack food, fried food and baked good industries. In food production, the goal is not to simply change the configuration of double bonds while maintaining the same ratios of hydrogen to carbon. Instead, the goal is to decrease the number of double bonds and increase the amount of hydrogen in the FA. This changes the consistency of the FA and makes it less prone to rancidity, in which free radicals attack double bonds. Production of *trans* FA is therefore a side-effect of partial hydrogenation. Catalytic partial hydrogenation necessarily produces *trans*-fats, because of the reaction mechanism. In the first reaction step, one hydrogen is added, with the other coordinatively unsaturated carbon being attached to the catalyst. The second step is the addition of hydrogen to the remaining carbon, producing a saturated fatty acid. The first step is reversible, such that the hydrogen is readsorbed on the catalyst and the double bond is re-formed. Unfortunately, the intermediate with only one hydrogen added contains no double bond, and can freely rotate. Thus, the double bond can re-form as either *cis* and *trans*, of which *trans* is favored, regardless the starting material. Complete hydrogenation also hydrogenates any produced *trans* fats to give saturated fats. Most *trans* fats consumed today are created industrially in partial hydrogenation of plant oils — a process developed in the early 1900s and first commercialized as Crisco in 1911. Commercial hydrogenation is typically partial in order to obtain a malleable mixture of fats that is solid at room temperature, but melts upon baking or consumption. In most naturally occurring unsaturated fatty acids, the hydrogen atoms are in *cis* configuration. However, partial hydrogenation reconfigures most of the double bonds that do not become chemically saturated, twisting them to the *trans* configuration, that has the lower energy form, and is favored when catalytically equilibriated as a side reaction in hydrogenation.

An increasing number of studies have explored the presence of *trans* FA residues in living systems. This is a very lively field of interdisciplinary research spanning from chemistry to microbiology, pharmacology, biology, and, of course, medicine. The configuration of isolated double bonds in naturally occurring lipids of eukaryotes is *cis*. In modified fats, the structures of *trans* FA residues consist of geometrical and positional isomers having un-shifted and shifted double bonds, respectively, compared to natural *cis* compounds. Some *trans* geometrical isomers found in living organisms can only arise via an endogenous transformation of the naturally occurring *cis* structures and are correlated with radical stress produced during physiological and pathological processes. (Ferreri C *et al*., 2002; Zghibeh CM *et al*., 2004; Ferreri C *et al*., 2005; Kermorvant-Duchemin E *et al*., 2005; Zambonin L *et al*., 2006). Several free radicals, including the biologically relevant thiyl radicals (RS•) and nitrogen dioxide (NO_2•) are known to isomerize double bond (Chatgilialoglu C

and Ferreri C, 2005; Ferreri C and Chatgilialoglu C, 2005). Figure 10 shows the reaction mechanism, involving reversible addition of a thiyl radical to the double bond to form a radical-adduct. The reconstitution of the double bond is obtained by β-elimination of RS•, and the result favours *trans* geometry, the most thermodynamically stable configuration, by 0.6-1 Kcal/mol. It should be noted that (i) RS• acts as a catalyst for *cis–trans* isomerization and (ii) positional isomers cannot be formed because the mechanism does not allow a double-bond shift. The *cis–trans* isomerization by RS• is an efficient process and detailed kinetic data for the reactions are available in the case of methyl oleate with $HOCH_2CH_2S$• radical (Chatgilialoglu C *et al*., 2002, 2005).

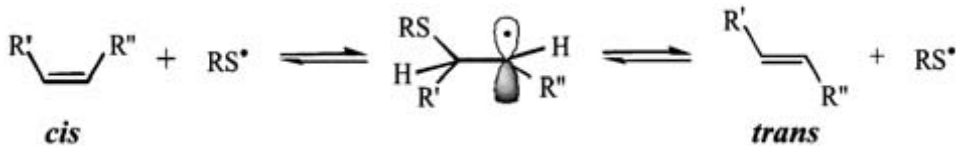


Figure 10. Reaction mechanism for the *cis–trans* isomerization catalysed by thyl radical. (Ferreri C *et al*., 2005).

Recently, using a biomimetic model of vesicle suspension which mimics the aqueous and membrane compartments of a cell, Lykakis IN *et al*. (2007) demonstrated the potential of sulfhydryl radicals ($HS•/S•^-$) derived from H_2S to access the hydrophobic fatty acid chains and attack the double bonds. The phospholipids produced in this way contained a high proportion of *trans* fatty acid residues. This model offers some insight into the chemical basis of the biological activity of H_2S, that will be discussed later on in this thesis. This chemistry is important in view of the intriguing role of the sulfhydryl radical induced *cis-trans* conversion of lipids, either as a damaging or a signaling pathway. On the other hand, the available kinetic data for the *cis–trans* isomerization by NO_2• suggest that this radical cannot be very efficient as an isomerizing species, and in a biological environment this reaction should not play a role (Chatgilialoglu C and Ferreri C, 2005). In the n-6 series of PUFA, *cis–trans* isomerization of methyl linoleate (Ferreri C *et al*., 2001), γ-linolenate (Ferreri C *et al*., 2001) and arachidonate (Ferreri C *et al*., 2002) catalysed by RS• has been studied in some detail. Each isolated double bond in PUFA behaves independently. Moreover, isomerization is a stepwise process with the formation of mono-*trans* isomers, followed by di-*trans* isomers and so on, the isomeric composition being regulated by the relative thermodynamic stability. It was also possible to demonstrate that the double bonds closest to the membrane polar region are the most reactive towards attack by diffusing thiyl radicals (Ferreri C *et al*., 2001). For

example, AA residues in vesicles were more reactive than OA and LA; two positions, the double bonds at positions 5 and 8 out of the four present in this FA, were transformed preferentially. From studies to date, AA residues in membrane phospholipids emerged as very important pointers to help distinguish endogenous *trans* isomers, formed by radical processes, from the exogenous *trans* isomers derived from dietary contributions (Ferreri C *et al*., 2002; 2004). The interplay between exogenous and endogenous pathways for isomerization of arachidonic double bonds is shown in Figure 11, where the biosynthesis from the precursor LA is detailed: the two double bonds of positions 11 and 14 are provided by dietary LA (which can be *cis* or *trans*, depending on the food), whereas the other two double bonds (positions 5 and 8) are formed by desaturase enzymes, which produce selectively *cis* unsaturation. *In vivo*, double bonds at positions 5 and 8 of AA, stored in membrane phospholipids, can only have a *cis* configuration, unless these positions are involved by an isomerization process occurring in membranes by diffusible thiyl radicals.

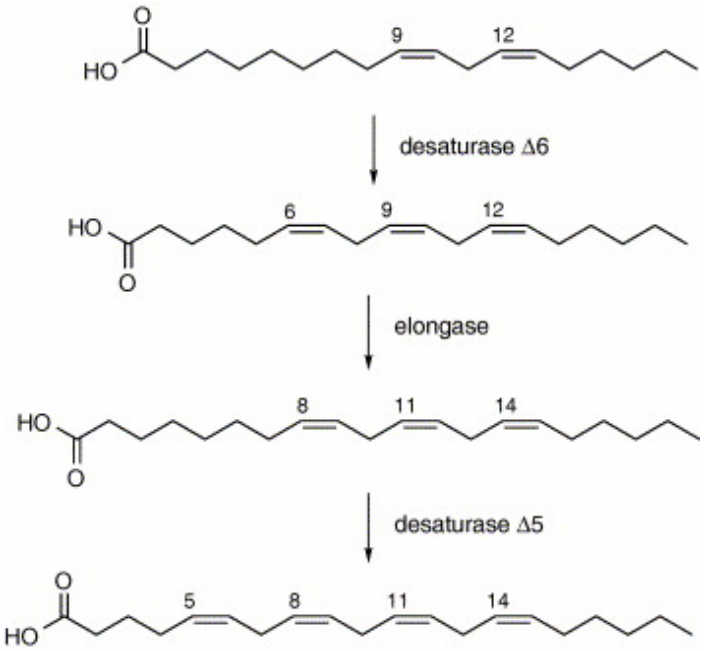


Figure 11. Enzymatic FA transformations from linoleic acid to arachidonic acid. (Ferreri C *et al*., 2002).

2.1.4 Membrane properties

PUFA accumulated into membrane phospholipids can potentially affect a variety of membrane physical properties including membrane thickness, lipid packing, fluidity, elasticity, permeability, flipflop, protein activity, fusion, blebbing and the structure and function of lateral and *trans*-membrane heterogeneities (domains) (Stillwell W and Wassall SR, 2003). The implication of many diverse observations, moreover, is that membrane perturbations are either the cause or the result of apoptosis. These are the main topics discussed below, with a major focus on DHA, the most unsaturated of human FA and therefore the most studied one.

Phase behaviour

Unlike saturated phospholipid membranes where the acyl chains pack near uniformly in all-*trans* configuration in the gel state, in membranes containing PUFA the packing is distorted by steric restrictions associated with the presence of multiple rigid double bonds (Figure 12). The consequent

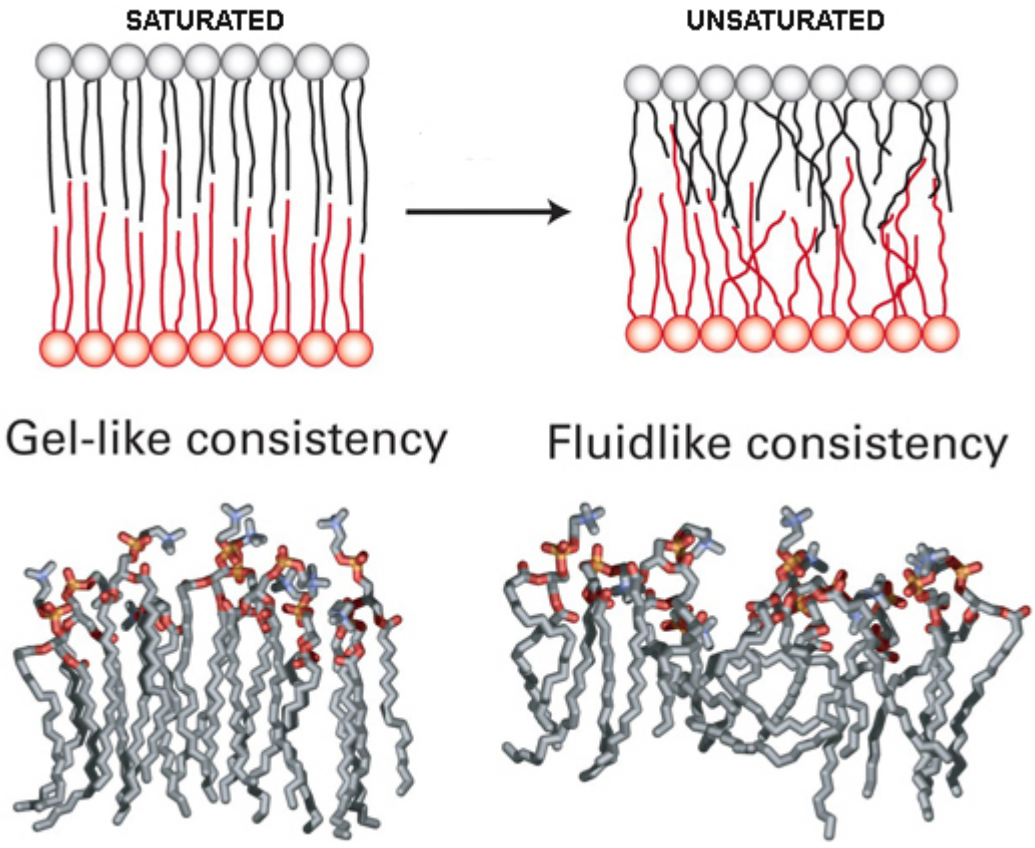


Figure 12. Differences between saturated and unsaturated phospholipid membranes.

reduction in intra- and inter-molecular van der Waals interactions produces a decrease in stability, manifest as a substantial depression in the melting temperature (Tm) of the gel to liquid crystalline phase transition. Differential scanning calorimetry (DSC) and 2H NMR have well documented this behaviour. In Figure 13, transition temperatures measured by Niebylski CD and Salem N (1994) for a series of PCs having SA in the sn-1 position and a FA with either 0, 1, 2, 3, 4, 5 or 6 double bonds in the sn-2 position are plotted against the number of double bonds.

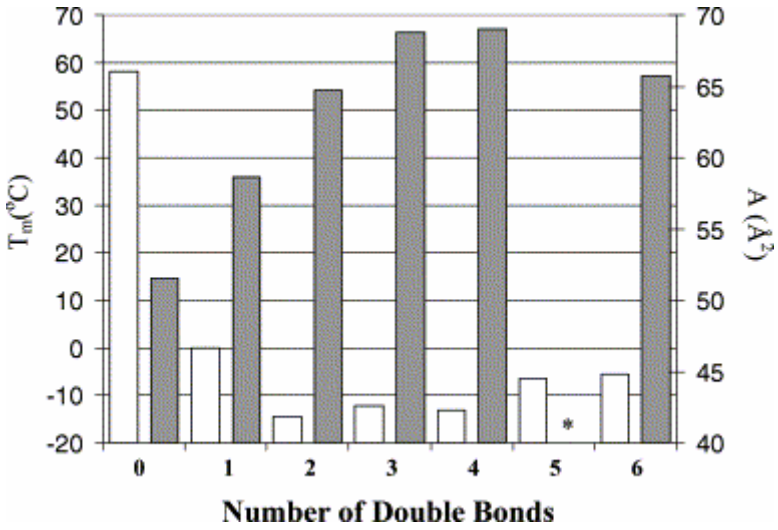


Figure 13. Plot of the chain melting transition temperature T_m (open bars) and of area/molecule A (closed bars) against the number of double bonds in the *sn*-2 chain, designated by X, for 18:0-XPC. The T_m and A values are taken from Niebylski CD and Salem N (1994) (* signifies data unavailable).

A common trend is seen in which the first double bond reduces Tm on the order of 60 °C and a further reduction of approximately 15 °C accompanies the second double bond. Beyond two double bonds, Tm is little affected and can even rise slightly. As a result, the DHA-containing PC melts at a higher temperature than does the ALA or AA containing species. This data emphasize that the relationship between phase behaviour and the number and position of acyl chain double bonds is not straightforward; preferential van der Waals interactions between saturated sn-1 chains on neighbouring molecules, perhaps indicating heterogenous lateral distribution, were proposed to be responsible.

Membrane thickness

As a first guess it can be probably assumed that membranes enriched in DHA with 22 carbons would be very thick. On the contrary, the rod outer segment membrane (where DHA levels approach 50% of the total acyl chains) is actually quite thin (Dratz EA *et al*., 1985). In agreement, on the basis of 2H NMR order parameters measured for the perdeuterated stearoyl *sn*-1 chain in PCs, it was inferred that the thickness of the bilayer is about the same with DHA as SA at the *sn*-2 position (Holte LL *et al*., 1995). A wedge shape becoming slightly fatter at the center of the heteroacid saturated-polyunsaturated bilayer is indicated for the saturated chain while, conversely, the DHA chain occupies an inverted wedge shape with higher volume density near the aqueous interface (Eldho NV *et al*., 2003). The origin is the tremendously flexible structure of DHA undergoing rapid inter-conversions between many torsional states such that the terminal methyl end often approaches the membrane surface (Saiz L and Klein ML, 2001; Feller SE *et al*., 2002; Huber T *et al*., 2002). As a result, the PUFA-containing phospholipid has an increased cross-sectional area and hence exhibits a decreased membrane thickness.

Fluidity

The addition of acyl chain double bonds is generally assumed to increase fluidity, on the basis of which it may be surmised that a membrane rich in DHA should be exceptionally fluid. The picture of DHA-related changes in membrane fluidity that arises from experimental studies, however, can appear quite contradictory as both increased fluidity and no change have been reported. Unfortunately this somewhat conceptual term means different things to different investigators and is often defined by the physical method employed. Also studies on relatively simple, protein-free lipid bilayer model systems are frequently compared to those from far more complex, heterogeneous biological membranes. As a result, contradictory reports of DHA's effect on membrane fluidity abound. High fluidity (disorder and rates of molecular reorientation) characterize DHA-containing membranes by fluorescence polarization of DPH (Straume M and Litman BJ, 1987; Stubbs CD, 1992; Salem N and Niebylski CD, 1995; Mitchell DC and Litman BJ, 1998). Salem and Niebylski (1995) made small unilamellar vesicles (SUV) from PCs containing SA in the sn-1 position and long chain FA with 0 to 6 double bonds in the sn-2 position. Steady state fluorescence polarization and time resolved correlation times of DPH were measured, showing a large increase in fluidity upon the addition of the first double bond, a smaller further increase with the second and a still smaller increase in fluidity with up to 4 double bonds. No further increase was measured for DHA with 6 double bonds. However, with time resolved anisotropy they reported a progressive increase in fluidity for each additional double bond through DHA. The interpretation offered was that there is an increase in acyl chain free volume with double bonds maximizing at DHA. While only minor

contradictions exist concerning the effect of double bonds on fluidity in model membranes, their effect on the fluidity of biological membranes is much less certain. Many dietary studies have reported increases in membrane fluidity from animals fed with DHA-rich fish oil diets (Kamada T, 1986; Ernst E, 1994), as well as for cells cultured in DHA-rich media (Brown ER and Subbaiah PV, 1994; Calder PC *et al*., 1994; Sobajima T *et al*., 1986; Yorek M *et al*., 1989). In contrast, some studies did not reveal any significant change in fluidity upon incorporation of PUFA, despite using the same techniques (Clamp AG *et al*., 1997; Wahnon R *et al*., 1992; Popp-Snijders C *et al*., 1986; Gibney MJ and Bolton-Smith C, 1988). For example, Treen *et al*. (1992) were unable to see a difference in fluidity (fluorescence polarization of DPH and TMA-DPH) with intact Y-79 retinoblastoma cells cultured in DHA-enriched media despite observing a 4 to 5 fold increase in cellular DHA levels. Employing a different fluorescence method, several reports have indicated DHA does increase fluidity as assayed by probes sensing lateral mobility (Clamp AG *et al*., 1997; Treen *et al*., 1992; Stillwell W *et al*., 1995). These observations are consistent with the hypothesis that PUFA, and in particular DHA, may play a major role in membrane lateral domain structure. The conclusion from these many experiments is that whatever PUFA's influence on membrane fluidity is, the changes in acyl chain mobility on biological membranes already rich in unsaturated FA are subtle and are less likely to be detectable. The increase in fluidity when DHA is introduced into model or biological membranes that already contain substantial unsaturation is consequently small or negligible.

Lipid packing and elasticity

Pressure-area curves for PC monolayer films at the air-water interface demonstrate that the cross-sectional area generally increases with increasing double bonds and is particularly large in the presence of DHA (Niebylski CD and Salem N, 1994; Dratz EA and Deese AJ, 1986). The area/molecule for a series of 18:0-XPCs, where X represents acyl chains with 0 to 6 double bonds, was shown to increase dramatically upon the addition of a first, second and third double bond, but does not change significantly with subsequent double bonds (Smaby JM *et al*., 1997) (Figure 13). Interestingly, this pattern of a large alteration caused by the addition of a first few double bonds but little further change with subsequent double bonds is qualitatively analogous to that reported for fluidity and for main melting transition temperature (Tm). Another property derived from pressure-area isotherms on lipid monolayers is the lateral compressibility modulus. The measurements indicate that monolayers become more compressible (lower modulus) with increasing number of double bonds (Smaby JM *et al*., 1997). The effect is maximal with DHA. A lower energy requirement to deform a DHA-containing membrane is the implication (Koenig *et al*., 1997). The values measured establish that the DHA chain is much more compressible than the saturated chain.

It was estimated that 75% of the compressibility of 18:0-22:6PC could be attributed to the DHA chain. On the other hand, control experiments on 18:0-18:1PC reveal similar compressibility for the saturated and monunsaturated chains. It is predicted that DHA's low compressibility modulus may facilitate structural transitions of certain membrane proteins. The details of the mechanism by which incorporation of DHA into the various phospholipid types affects membrane protein function is not yet clear. Some authors suggested modulation of three overlapping factors: curvature stress (Epand RM, 1998; Gruner SM, 1985), membrane thickness (Killian JA, 1998) and acyl chain packing free volume (Litman BJ and Mitchell DC, 1996). The packing free volume is of interest here since it is directly related to the tightness of lipid packing. The packing free volume increases with additional double bonds maximizing at DHA. Therefore DHA with its high disorder and associated large cross-sectional area would be expected to pack poorly with other membrane lipids. For more than a decade the effect of DHA on the visual process, particularly the rhodopsin MI to MII conformational transition, have been studied (Mitchell DC *et al*., 1992). The transition was shown to be dependent on lipid type, favoring lipids with small head groups and wide acyl tails (i.e. DHA-

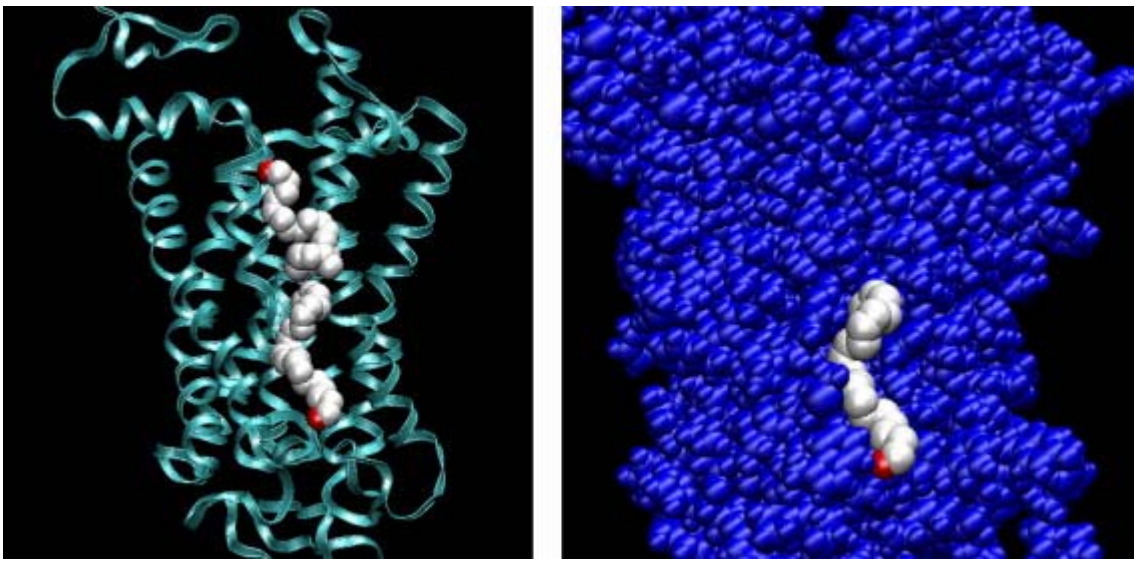


Figure 14. Molecular graphics of a snapshot from the simulation showing close contact between a DHA chain and rhodopsin. (Feller SE, 2008).

rich PCs) (Figure 14). In general, hexagonal phase-preferring lipids possessing high elastic curvature stress favor the MII conformation (Brown MF, 1994). In their MI to MII rhodopsin model, Mitchell and Litman (Mitchell DC *et al*., 1992; Mitchell DC and Litman BJ, 1997) found the packing free volume and MII formation increased in the order 16:0-18:1PC < 16:0-22:6PC <

22:6-22:6PC. It is not yet clear the relationship between elasticity, packing free volume and protein activity for the vast majority of membrane proteins.

Permeability

It stands to reason that membranes whose lipids are poorly packed should also be highly permeable and indeed there have been numerous reports linking PUFA to increases in membrane permeability. In an early report, Hendriks *et al*. (1976) showed that vesicles made from lipid etracts of rod outer segment membranes (DHA-rich) were 10 times more permeable to Na+ than were vesicles made from lipids extracted from other (DHA-poor) retinal membranes. Demel *et al*. (1972) demonstrated that DHA incorporated into the sn-2 position of PC enhanced permeability to glucose, erythritol and glycerol. Measuring bilayer permeability to erythritol and carboxyfluorescein, Stillwell *et al*. (1993) established that DHA enhanced bilayer permeability 2 to 3 fold more than OA in identical phospholipids. Huster *et al*. (1998) used 17O NMR to follow water permeability across lipid bilayers. They determined that 18:0-22:6PC membranes are about 4× more permeable than those made of 18:0-18:1PC, but are about 30% less permeable than those of 22:6-22:6PC. As a result of increased water penetration, DHA favors increased hydration of the head group and inter-chain region. In agreement, fluorescence measurements on lipid bilayers confirmed that water content in the bilayer hydrocarbon region increases with double bond content (Mitchell DC and Litman BJ, 1998). Increases in mitochondrial permeability have also been linked to DHA content (Stillwell W *et al*., 1997; Brand MD *et al*., 1994. The resultant proton leakage in turn decimates the essential trans-membrane H+ gradients, an event linked to mitochondrial-linked apoptosis (Newmeyer DD and Ferguson-Miller S, 2003).

Bilayer instability: vesicle fusion, exfoliation and flip-flop

The loose packing of PUFA-containing phospholipids implies that high levels of this FA may also lead to general membrane instability resulting in enhanced vesicle exfoliation ("blebs"), fusion and flip-flop. Perhaps not coincidentally, biological membranes that are naturally enriched in DHA (neuronal synaptosomes, rod outer segments and sperm) are partly characterized by their predisposition to undergo membrane vesicle formation and fusion. Ahkong *et al*. (1973) and later Meers *et al*. (1988) showed that PUFA promote the fusion of natural membranes. DHA as either the free FA or as part of a mixed chain PC (18:0-22:6PC) enhanced SUV fusion to a much larger extent than did PCs with other, less unsaturated FA (Ehringer W *et al*., 1990). When T27A tumor cells were fused with vesicles containing 18:0-22:6PC, the tumor plasma membrane became leakier (Stillwell W *et al*., 1993), had altered expression of surface proteins (Jenski LJ *et al*., 1995), altered domain structure (Stillwell W *et al*., 1993), decreased cell deformability (Zerouga M *et al*., 1997),

increased cytolysis by cytotoxic T-lymphocytes (Pascale AW *et al*., 1993) and decreased cell longevity (Jenski LJ *et al*., 1995). It is now well documented that membrane fusion is enhanced by the presence of PUFA, that promote inverted hexagonal phase and thus provide curvature stress to membranes (Ellens H, 1989). Since exfoliation is the reverse of fusion, Williams *et al*. (1998) isolated exfoliated vesicles (EV) from DHA-enriched T27A cells and measured their lipid content (phospholipid class, FA and cholesterol) and membrane molecular order. A comparison of these values between the DHA-induced exfoliated vesicles (EVs) and the parent plasma membrane led these authors to conclude that EV composition and structure was considerably different than that of the parent plasma membrane. After incubation in the presence of DHA, EVs exhibited higher levels of DHA and lower levels of cholesterol. These results imply that DHA-driven microdomains are either large enough or numerous enough to influence a sizable portion of T27A membrane surface and that EVs reflect plasma membrane lipid domains. Recently Armstrong *et al*. (2003) monitored the effect of PC acyl chain double bond content on trans-membrane flipflop. This study demonstrated that as the number of double bonds increases, so does the flip-flop rate. The increase was particularly marked in the presence of DHA. A half-life of 0.086 h was reported for bilayers composed of 22:6-22:6PC and 0.29 h for 18:0-22:6PC, whereas 18:0-18:1PC exhibited a much slower flip-flop of = 11.5 h. These authors concluded that PC membranes containing DHA support very fast flip-flop rates.

Membrane domains and lipid rafts

Lipid rafts are liquid ordered, sphingomyelin (SM)-rich/cholesterol-rich microdomains that are postulated to serve as platforms for protein activity by accumulating specific lipidated proteins such as the src family of kinases in the inner leaflet and GPI-anchored proteins in the outer leaflet of plasma membranes (Edidin M, 2003; Simons K and Ikonnen E, 1997; Brown DA and London E, 2000). The rafts are floating in a sea of liquid disordered, SM-poor/cholesterol-poor microdomains that must have its own array of proteins. In fact it is likely that numerous proteins may migrate between domains in response to alterations in lipid composition. Historically their resistance to dissolution in cold temperature detergents, particularly Triton X-100, has defined lipid rafts. While detergent resistant membranes mark the beginnings of raft studies, they are wrought with artifacts and are being replaced by gentler, detergent-free methods. DHA-phospholipids, being very fluid, accumulate in the detergent-soluble membrane fractions (Figure 15). Cholesterol seems to be at the heart of understanding the structure and function of rafts. For many years it has been known that the major lipid comprising the plasma membrane of most animal cells is cholesterol and the primary role of the sterol is to modulate the physical properties of membranes (Finegold L, 2003). The

situation is well-documented in membranes composed of saturated acyl chains (Finegold L, 2003), but is far less understood in unsaturated fatty acids containing membranes. Upon its incorporation into cells, PUFA are incorporated into membranes where they must be exposed to cholesterol; the interaction between these two species may profoundly affect membrane structure and function. Experiments showed that DHA-containing PCs were far less affected by cholesterol than were many other PCs (Finegold L, 2003; Shinitzky M, 1984). Cholesterol demonstrated little monolayer condensation and had little effect on bilayer permeability of DHA-containing PCs compared to less unsaturated PC (Demel RA *et al*., 1972; Evans RW and Tinoco J, 1978; Zerouga M *et al*., 1995). Using pressure-area techniques on monolayers, Smaby JM *et al*. (1997) measured elasticity compression moduli for various PCs. The in-plane elasticity moduli of the PC species with higher sn-2 double bond content were found to be less affected by increased levels of cholesterol. Their work predicts that cholesterol-unsaturated FA interaction results in localized regions in membranes that differ considerably in their in-plane elasticity. Partition coefficients determined for cholesterol in unilamellar vesicles follow the order 18:0-18:1PC > 18:1-18:1PC > 18:2-18:2PC > 16:0-22:6PC > 22:6-22:6PC (Niu SL and Litman BJ, 2002). Litman BJ and Mitchell DC (1996) have employed a model system monitoring the effect of acyl chain packing free volume (fv) on the M_I to M_{II} equilibrium (Keq) of rhodopsin to determine the effect of cholesterol on a protein's function in a DHA-rich membrane. Their results suggest that regulation of an integral membrane receptor (rhodopsin) function may be metabolically controlled by changes in phospholipid acyl chain composition and/or cholesterol content. They propose a primary role for phospholipid and a secondary role for cholesterol (Litman BJ and Mitchell DC, 1996). Many DSC experiments have demonstrated that cholesterol induces phase separations in PC bilayers (Stillwell W and Wassall SR, 2003; Shinitzky M, 1984; Ghosh D *et al*., 1973; Stillwell W *et al*., 1996). The addition of up to 50 mol% cholesterol to 22:6-22:6PC has little impact upon the temperature or enthalpy of the gel-to-liquid phase transition (Kariel N *et al*., 1991), whereas with 16:0-18:1PC or 16:0-22:6PC the transition is eliminated by $\geq$ 35 mol% cholesterol (Hernandez-Borrell J and Keough KMW, 1993). Regardless of unsaturation, the response to incorporation of cholesterol is a disruption of acyl chain packing in the organized gel state while in the fluid liquid crystalline state acyl chain motion is restricted (i.e. cholesterol increases the "fluidity" of solid membranes while decreasing the "fluidity" of fluid membranes) (Davis JH, 1993; Mitchell DC and Litman BJ, 1998; Jackman CS *et al*., 1999). The implication of the biophysical studies outlined above is that the solubility of cholesterol in phospholipid membranes may be dependent on double bond content. When forced to interact with PUFA chains in sn-1, sn-2 dipolyunsaturated PC membranes (Brzustowicz MR *et al*., 1999; 2000; 2002; Shaikh SR *et al*., 2003), cholesterol precipitates out of the membrane at

concentrations of a factor of 3–5 less than in the corresponding sn-1, sn-2 saturated-polyunsaturated membrane where close proximity to the PUFA chain may be avoided. Profoundly reduced affinity of the sterol for PUFA was thus quantified. It is the extraordinarily high disorder of PUFA chains that on steric grounds is incompatible with near approach from the rigid steroid moiety. Cholesterol will laterally organize within membranes to minimize contact with a DHA sn-2 chain and preferentially associate with a saturated sn-1 chain (Mitchell DC and Litman BJ, 1998; Litman BJ and Mitchell DC, 1996), and both experimental (Huster D *et al*., 1998) and computer simulated (Pitman MC *et al*., 2004) data lend further support. The interaction of cholesterol with DHA-containing PEs has received much less attention than with the equivalent PCs. Early studies found that cholesterol-lipid interaction follows the following sequence: SM >> PS, PG > PC >> PE (Van Dijck PW, 1979). In agreement, Niu and Litman (2002) determined partition coefficients in the order SM > PS > PC > PE for cholesterol in unilamellar vesicles. Although most PE localizes to the inner leaflet, nearly 17% is outer leaflet and so should directly act on rafts (Simons K and Vaz WL, 2004). Also PE is the major receptacle of DHA in most membranes. Comparing cholesterol solubility in sn-1 saturated, sn-2 unsaturated PE and PC bilayers with 18:1 acid or DHA at the sn-2 position, it was demonstrated that there is a greater DHA-associated reduction in solubility for PE than for PC bilayers, which we attribute to its smaller headgroup (Shaikh SR *et al*., 2003). A reduced affinity between cholesterol and PUFA may drive lateral phase separation into cholesterol-rich/PUFA-poor and cholesterol-poor/PUFA-rich membrane microdomains (Figure 15). There have been a few examples of DHA-induced lipid phase separations described for model bilayer membranes. Bilayers composed of 16:0-16:0PC and 18:0-22:6PC can phase separate (Stillwell W *et al*., 1993; Niebylski CD and Litman BJ, 1997). Surface elasticity measurements on monolayers, and DSC, detergent extraction and solid state 2H NMR measurements on bilayers support the idea that DHA-containing phospholipids enhance the lateral segregation of cholesterol into sterol-rich/SM-rich liquid ordered regions away from sterol-poor/DHA-PE- or DHA-PC-rich liquid disordered domains (Shaikh SR *et al*., 2002; 2001; 2004). Estimates of the size of PUFA-induced domains are small. From analyses of 2H NMR spectra, an upper limit of ~ 160 Å was placed on microdomain size in 18:0-20:4PC/20:4-20:4PC/cholesterol (1/1/2 mol) (Brzustowicz MR *et al*., 2002; Huster D *et al*., 1998). These values are much less than that typically quoted for lipid rafts ($\gtrsim$ 50 nm). As raft studies continue to evolve, however, the estimated raft size in biological membranes continues to decrease, making their direct observation less likely (Edidin M, 2001). Indirect observations supporting an effect of PUFA on lateral organization within membranes are abundant; there are examples that indicate DHA may affect membrane structure and hence function. The best studied DHA-membrane system is the rhodopsin model of Litman and Mitchell (Litman BJ and Mitchell

DC, 1996; Mitchell DC *et al*., 1992; Mitchell DC and Litman BJ, 1997). Their model is based on strong interactions between the saturated sn-1 chain of PCs containing DHA in the sn-2 position. The sn-1 chains are oriented towards the tightly packed domain interior with the sn-2 chains oriented toward the domain boundary (Litman BJ and Mitchell DC, 1996). The DHA-rich sn-2 chain is proposed to primarily determine the lateral packing of the system that is responsible for the MI to MII equilibrium. Cholesterol partitions into the saturated chain-rich domain interior where it reduces the acyl chain packing free volume.

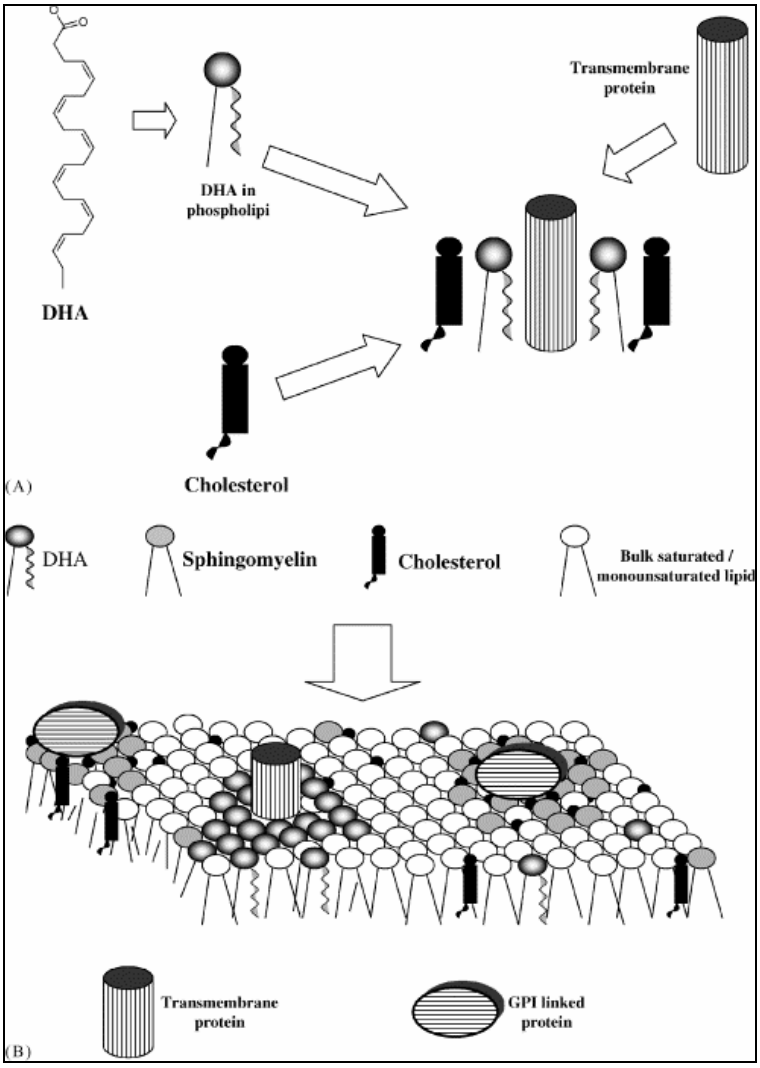


Fig. 15. (A) Cartoon demonstrating the incorporation of DHA into the *sn*-2 chain of a phospholipid and the association of this phospholipid with cholesterol. Also included is a transmembrane protein (e.g. rhodopsin) that has a high affinity for DHA. (B) Cartoon demonstrating membrane phase separation into DHA-rich/cholesterol-poor (liquid disordered) and DHA-poor/cholesterol-rich (liquid ordered) domains. Different proteins partition into each domain. The liquid ordered domains are often referred to as "lipid rafts". (Stillwell W and Wassall SR, 2003).

Asymmetry between leaflets

Biological membranes also exhibit substantial trans-bilayer heterogeneity. It is well documented that highly unsaturated species of PE and PS are found primarily on the inner leaflet of many membranes (Salem NJ *et al*., 1986; Salem N Jr *et al*., 2001). However DHA distribution is not absolute as rats fed a fish oil diet exhibited an increase in DHA-containing species of PC in the outer leaflet of erythrocyte membranes (Kuypers FA *et al*., 1988). Addition of PUFA to membranes has also been shown to alter trans-bilayer sterol localization (Sweet WD and Schroeder F, 1988; Schroeder F *et al*., 1991). Although the transmembrane distribution of cholesterol is not known with absolute certainty (Lange Y *et al*., 1981; Schroeder F *et al*., 1996), the outer leaflet of plasma membranes may contain more cholesterol and therefore be more rigid than the inner leaflet. This is consistent with the current idea of cholesterol and sphingolipid-rich lipid rafts residing in membrane outer leaflets (Brown DA and London E, 2000). Also, it is known that cholesterol has a higher affinity for sphingolipids than for other common membrane lipids (Simons K and Vaz WL, 2004; Van Dijck PMW *et al*., 1976; Leventis R and Silvius JR, 2001) and sphingolipids clearly are primarily located in the outer leaflet (Brown DA and London E, 2000). However, very rapid cholesterol flip-flop (Steck TL *et al*., 2002; Muller P and Herrmann A, 2002) makes precise cholesterol asymmetry measurements difficult. Upon addition of PUFA in culture, a decrease in molecular order and a redistribution of cholesterol with more than 70% going to the outer leaflet was reported by Schroeder and co-workers (Sweet WD and Schroeder F, 1988; Schroeder F *et al*., 1991). Dusserre E *et al*. (1995) reported cholesterol efflux from plasma membranes remained the same after incorporation of oleate, linoleate or arachidonate but increased with EPA and DHA. They suggested that incorporation of DHA into the membrane inner leaflet forces cholesterol into outer leaflet where it is more readily lost from the membrane.

Membane proteins, cell signaling and apoptosis

At present there is a large and ever expanding list of proteins whose activities have been shown to be affected by PUFA. Apart from the most studied protein rhodopsin, the important signaling protein, protein kinase C has also been shown to be activated by PUFA that exhibit large negative curvature stress (Slater SJ *et al*., 1994; Epand RM, 1992; Mosior M *et al*., 1996). The formation of membrane domains can be important for the activation of PKC and, furthermore, the activation can be inhibited by disrupting the domains (Giorgione J *et al*., 1995). Since nervous tissue is known to be highly enriched in DHA (Salem NJ *et al*., 1986), it was logical to investigate the effect of this FA on channel activity. Using patch clamp techniques, Poling JS *et al*. (1995; 1996) reported that non-esterified DHA interacts with an external channel domain and regulates the activity of certain voltage-gated K^+ channels in a Zn^{2+}-dependent fashion. PUFA have additionally been shown to

modulate L-type $Ca2^{+}$ channels (Pepe S *et al*., 1994; Xiao YF *et al*., 1997), suggesting a role in cell signaling. Hasler CM *et al*. (1991) reported that DHA inhibited gap junction intercellular communication by 18% while several other fatty acids had no measurable effect. Finally, Jenski LJ *et al*. have studied the expression of MHC I molecules as modified by DHA, demonstrating altered expression of MHC I, CD8, and CD90 (Thy-1) on murine lymphocytes and leukemia cells enriched in DHA through diet or cell culture (1995; 2000). These are but a few of a wide variety of reports linking PUFA to cell signaling events through membrane alteration.

The effect of FA on apoptosis has received considerable scrutiny. It is well established that many events associated with apoptosis are linked to membrane structure and function (Cohen GM *et al*., 1987). For example, externalization of PS to the outer leaflet of the plasma membrane (Van den Eijnde SM *et al*., 1997; Williamson P and Schlegel RA, 2002) and formation of blebs (Diep QN *et al*., 2000) are considered hallmarks of apoptosis. However, it remains a distinct dichotomy of opinions concerning whether PUFA enhances or inhibits apoptosis. The preponderance of reports coming from a wide variety of primarily cancer cell types (Shiina T *et al*., 1993; Calviello G *et al*., 1998; Minami M and Noguchi M, 1996; Tsai WS *et al*., 1998; Hatala MA *et al*., 1994) indicate that DHA induces apoptosis and it has been suggested that DHA's anticancer properties are not due directly to cytotoxicity, but rather to the FA's ability to induce apoptosis (Siddiqui RA *et al*., 2001; 2001; Connolly JM *et al*., 1999). Siddiqui RA *et al*. (2004) have recently reviewed the role of DHA in enhancing both the cytosol-linked and mitochondrial-linked apoptotic pathways. DHA caused a profound inhibition of cancer cell growth and increased apoptosis. Associated with this was an increase in N-SMYase activity (by ~ 40%) in the breast cancer tissues from mice raised on the n-3 containing diets and by 70–75% ($P < 0.05$) in the MDA cells that had been treated with DHA. The DHA-induced increase in N-SMYase activity was further analyzed by formation of ceramide, the product of sphingomyelin hydrolysis. Ceramide is a lipid that is regarded as a universal component of apoptosis (Jayadev S *et al*., 1995; Rose DP and Connolly JM, 1999). Extensive DHA-induced blebbing of the tumor cell membranes and appearance of PS on the outer membrane surface, both strong indicators of apoptosis , have also reported. The conclusion of these experiments was that inhibition of breast cancer growth in nude mice by fish oil or in culture by treatment with DHA appear to be mediated by generation of ceramide through enhanced N-SMYase activity. In sharp contrast to the many reports demonstrating that DHA induces apoptosis are fewer reports, usually on neuronal cells, indicating that DHA actually inhibits apoptosis (Akbar M and Kim HY, 2002; Kim HY *et al*., 2000; Kishida E *et al*., 1998). Clearly there is a discrepancy between the affect of DHA on neuronal *versus* other types of cells and the relationship between PUFA and apoptosis will require far more study.

2.2 Biological characterization

2.2.1 Metabolism and human biosynthetic pathways

FA are released by adipose tissue after lipolysis or by lipoproteins arising either directly from the intestine after a lipid-rich meal or from the liver. FA circulate in the plasma loosely bound to albumin and cross plasma membrane with the help of a Fatty Acid Transferase. In lipogenic cells, like hepatocytes and adipocytes, they can be synthetized from glucose (lipogenesis). Inside the cell they bind to a cell-specific cytosolic Fatty Acid Binding Protein and can be exchanged with FA of membrane phospholipids. Mainly in liver and adipose tissue they can be activated into fatty acyl-CoA and esterified to glycerol-3-phosphate to synthesize triacylglycerol. In many cell types, FA can be elongated and desaturated by specific enzymes, oxidized in mitochondria or peroxisomes, oxidized in microsomes, peroxidized, or participate in eicosanoid (prostaglandins, leukotrienes, thromboxanes) synthesis. All this FA methabolism (Figure 16) is discussed in the following part, with a major focus on PUFA human biosynthetic pathways.

FA are usually ingested as triglycerides, which cannot be absorbed by the intestine. They are broken down into free FA and monoglycerides by pancreatic lipase, which forms a 1:1 complex with a protein called colipase which is necessary for its activity. The activated complex can only work at a water-fat interface: it is therefore essential that FA are emulsified by bile salts for optimal activity of these enzymes. Most are absorbed as free FA and 2-monoglycerides, but a small fraction is absorbed as free glycerol and as diglycerides. Once across the intestinal barrier, they are reformed into triglycerides and packaged into chylomicrons or liposomes, which are released into the lymph system and then into the blood. Eventually, they bind to the membranes of hepatocytes, adipocytes or muscle fibers, where they are either stored or oxidized for energy. The liver acts as a major organ for FA treatment, processing chylomicron remnants and liposomes into the various lipoprotein forms, namely Very Low Density Lipoproteins (VLDL) and Low Density Lipoproteins (LDL). FA synthesized by the liver are converted to triglyceride and transported to the blood as VLDL. In peripheral tissues, lipoprotein lipase digests part of the VLDL into LDL and free FA, which are taken up for metabolism. This is done by the removal of the triglycerides contained in the VLDL. What is left of the VLDL absorbs cholesterol from other circulating lipoproteins, becoming LDL. LDL is absorbed via LDL receptors. This provides a mechanism for absorption of LDL into the cell, and for its conversion into free FA, cholesterol, and other components of LDL. Another type of lipoprotein known as High Density Lipoprotein (HDL), collects cholesterol, glycerol and FA from the blood and transport them to the liver. In summary, Chylomicrons carry diet-derived lipids to

body cells, VLDL carry lipids synthesized by the liver to body cells, LDL carry cholesterol round the body and HDL carry cholesterol from the body back to the liver for breakdown and excretion. When blood sugar is low, glucagon signals the adipocytes to activate hormone sensitive lipase, and to convert triglycerides into free FA. These have very low solubility in the blood, typically about 1 μM. However, the most abundant protein in blood, serum albumin, binds free FA, increasing their effective solubility to ~ 1 mM. Thus, serum albumin transports FA to organs such as muscle and liver for oxidation when blood sugar is low.

FA degradation is the process in which FA are broken down, resulting in release of energy. It includes three major steps: activation and transport into mitochondria, β-oxidation and electron transport chain. FA are transported across the outer mitochondrial membrane by carnitine-palmitoyl transferase I (CPT-I), and then couriered across the inner mitochondrial membrane by carnitine. Once inside the mitochondrial matrix, fatty acyl-carnitine reacts with coenzyme A to release the FA and produce acetyl-CoA. CPT-I is believed to be the rate limiting step in FA oxidation. Once inside the mitochondrial matrix, FA undergo β-oxidation. During this process, two-carbon molecules acetyl-CoA are repeatedly cleaved from the fatty acid. Acetyl-CoA can then enter the tricarboxylic acid cycle, which produces NADH and FADH. NADH and FADH are subsequently used in the electron transport chain to produce ATP, the energy currency of the cell.

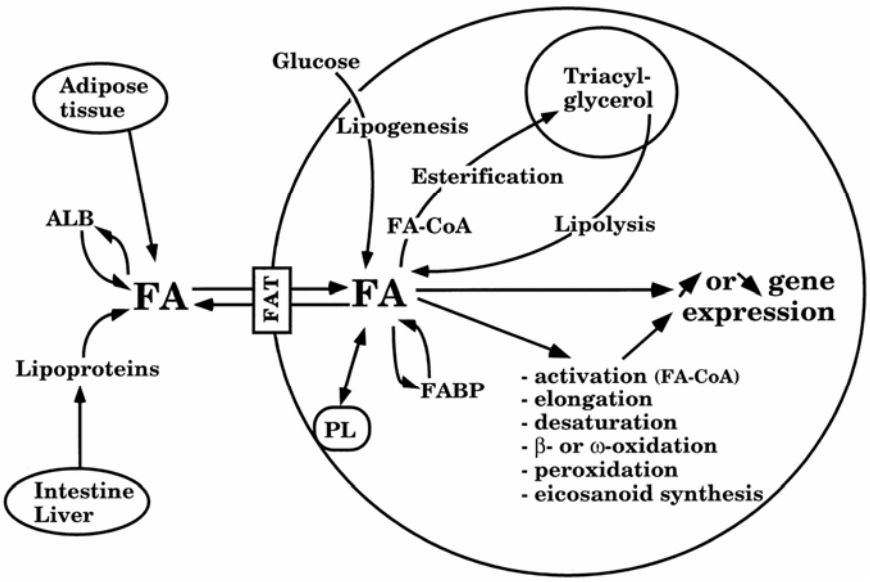


Figure 16. Major pathways of FA production, transport, and metabolism. (Duplus E *et al*., 2000).

SFA can be synthesized endogenously by the action of fatty acid synthases from acetyl CoA units by carbohydrate feeding, a process highly conserved through the evolution. In humans FA are predominantly formed in the liver, in the adipose tissue and in mammary glands during lactation. Acetyl-CoA is used as the primer and its carbon chain is elongated by the repeated condensation PA synthetases from various sources all require an acyl carrier protein (ACP) if they are the soluble system or include an ACP domain in their molecule if they are the multifunctional system. Although the molecular structures of the enzymes involved in FA synthesis and elongation are quite diverse between different pro- and eukaryotic species, the reaction mechanisms are essentially the same in all types of cells. Figure 17 illustrates the reactions involved in fatty acid synthesis and elongation. In an initial step, acetyl-CoA is carboxylated by the addition of CO_2 to malonyl-CoA, by the enzyme acetyl-CoA carboxylase (ACC). Biotin is an essential cofactor in this reaction, and is covalently attached to the ACC apoprotein, by the enzyme biotin:apoprotein ligase. ACC (EC 6.4.1.2) is a trifunctional enzyme, harboring a biotin carboxyl carrier protein (BCCP) domain, a biotin-carboxylase (BC) domain, and a carboxyl-transferase (CT) domain. In most bacteria, these domains are expressed as individual polypeptides and assembled into a heteromeric complex. In contrast, eukaroytic ACC harbor these functions on a single polypeptide. Malonyl-CoA produced by ACC serves as a two-carbon donor in a cyclic series of reactions catalyzed by FA synthase (FAS) and elongases. In most bacteria but also in mitochondria or in chloroplasts of eukaryotic cells, the reactions associated with SFA synthesis are catalyzed by dissociated, individual gene products (type II FAS systems), similarly to the initial ACC reaction. In contrast, in mammals or in yeast, the individual functions involved in cytosolic FA synthesis are represented as discrete domains on a single or on two different polypeptide chains, respectively.

Synthesis of fat however is reported to be relatively rare in people who consume more than 25% of their energy as fat (Hellerstein MK, 1999). Thus their adipose tissue stores tend to reflect dietary fat consumption except in the case of fasting or dietary cycling during which the long-term stores may be depleted. Endogenous alteration of dietary SFA can also occur. Lengthening the existing SFA, two carbons at a time, creates new FA via a common desaturase/elongase system (Figure 18) (Bazan HE et al., 1982). This generally occurs in the endoplasmic reticulum (Cinti DL *et al*., 1992). The third major alteration of FA within the body is through desaturation, resulting in the formulation of a double bond. Mammals convert SA to OA using a membrane-bound 18:0-CoA desaturase. However, their lack of a Δ^{12} desaturase prevents conversion of OA into LA [(n-9) to (n-6) conversion]. Lack of the Δ^{15} desaturase prevents the conversion of LA into ALA or the interconversion of n-6 and n-3 FA in man. Plants can perform both of the conversions. The diversity of PUFA synthesis relies on variations on desaturase and elongase biochemistry. Starting from

acetyl-CoA, the synthesis of DHA requires ~30 distinct enzyme activities and nearly 70 reactions, including four repetitive steps of the FA synthesis cycle and the energetically demanding desaturase reactions. However, nature has also solved the problem of PUFA synthesis using a fundamentally different pathway catalyzed by a specialized polyketide synthase (PKS) found in both prokaryotic and eukaryotic marine microbes (Metz JG *et al*., 2001). Polyketide synthases use the same basic four reactions of FAS, but the cycle is often abbreviated so that a highly derivatized carbon chain is produced, typically containing many keto and hydroxy groups as well as carbon-carbon double bonds in the *trans* configuration (Bentley R and Bennett JW, 1999).

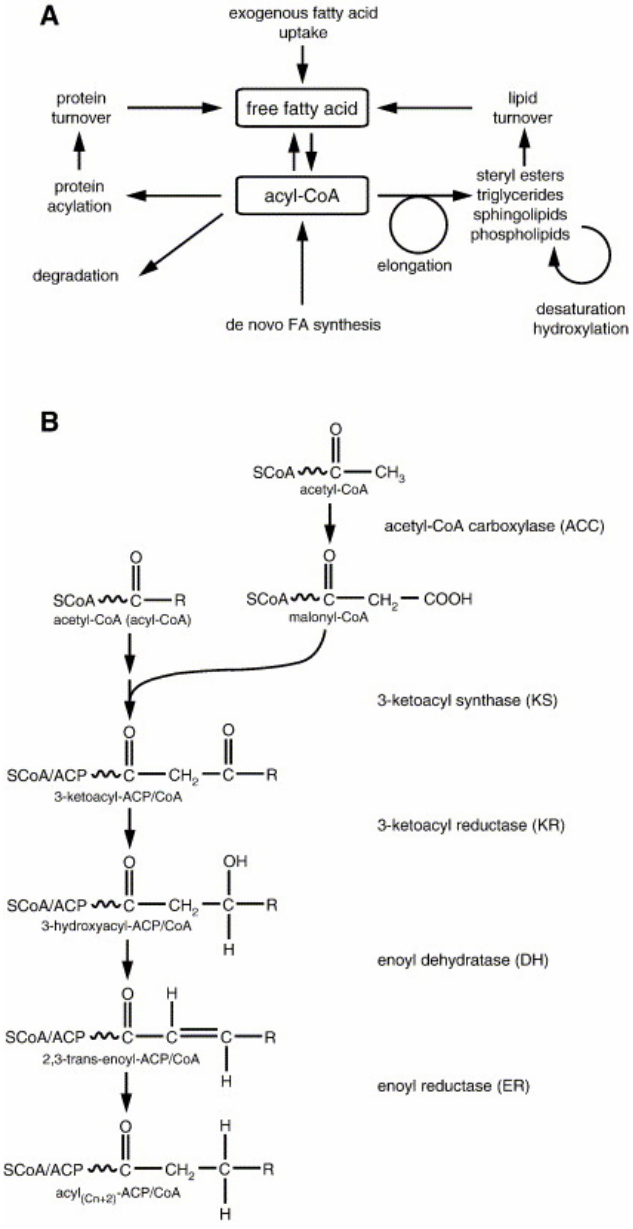


Figure 17. (A) Schematic representation of fatty acid metabolism. (B) Reaction schemes of fatty acid synthesis and elongation. (Tehlivets O *et al*., 2006).

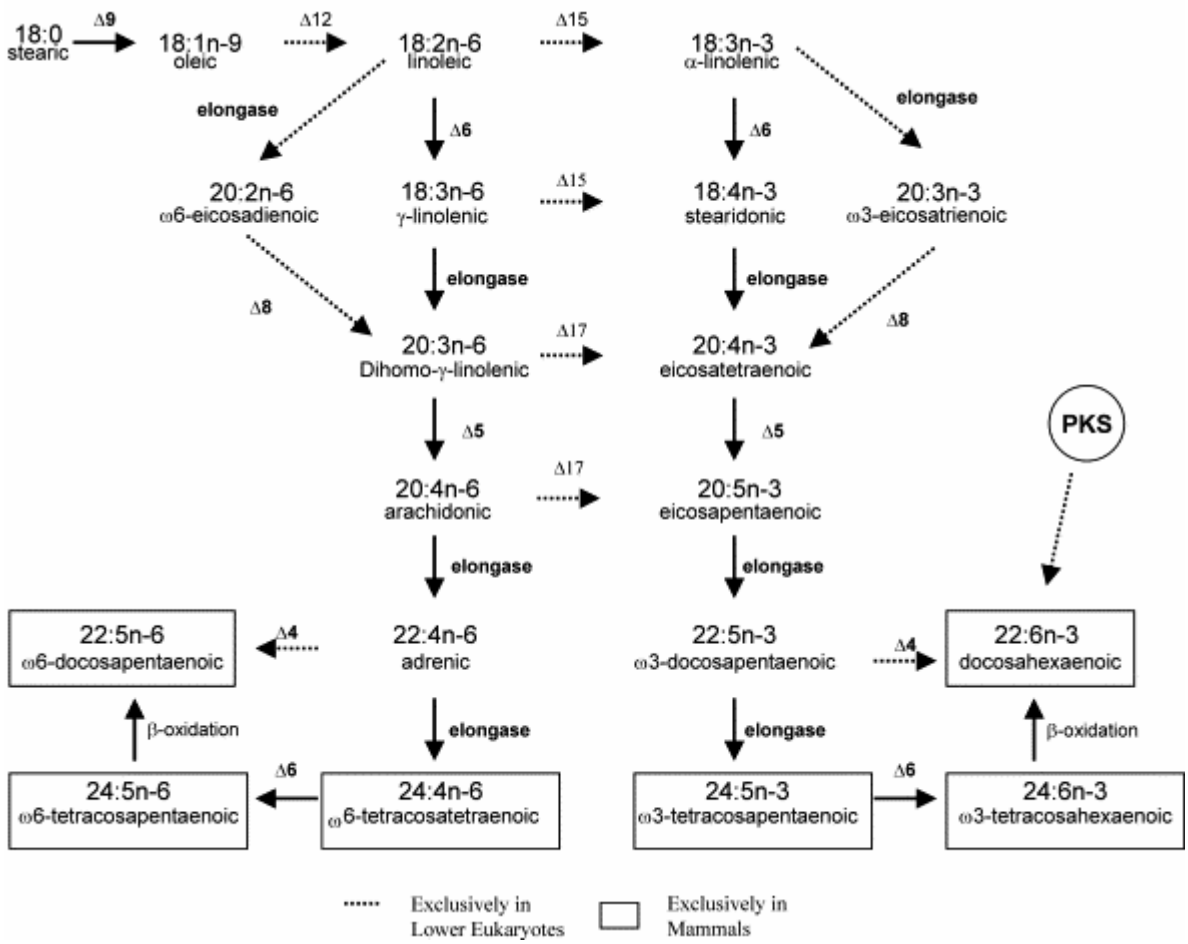


Figure 18. Biosynthesis of long-chain PUFA. Arrows with solid line (→) are found both in mammals are lower eukaryotes, while arrows with dotted line (•••>) are exclusively for lower eukaryotes. FA in □ indicate the pathway is exclusively in mammals. (Leonard AE *et al*., 2004).

For example, DHA is synthesized from ALA by the addition of a double bond by a Δ^6-desaturase to form stearidonic acid (SDA, C18:4, n-3); the elongation of SDA to form eicosatetraenoic acid (ETA, C20:4, n-3); the addition of another double bond by a Δ^5-desaturase to form eicosapentaenoic acid (EPA, C20:5, n-3); the elongation of EPA to form n-3-docosapentaenoic acid (DPA, C22:5, n-3); and the final addition of a double bond to form DHA (Sprecher H, 1982). The formation of DHA from EPA occurs via two different mechanisms in eukaryotes. In higher eukaryotes like mammals, EPA is elongated to n-3-DPA which is further elongated to n-3-tetracosapentaenoic acid (TPA, C24:5, n-3); a double bond is then added to n-3-TPA by a Δ^6-desaturase to form n-3-tetracosahexaenoic acid (THA, C24:6, n-3), and the THA is oxidized to DHA in peroxisomes (Ferdinandusse S *et al*., 2001**;** Sprecher H et al., 1995). However in lower eukaryotes EPA is elongated to n-3-DPA; a double bond is then added directly to n-3-DPA by Δ^4-desaturase to generate DHA (Qiu X *et al*., 2001). To synthesize long chain n-6 PUFA, n-6-docosapentaenoic acid

(DPA, C22:5, n-6) from LA, the same alternating desaturation and elongation steps (via either the Δ^4-desaturase route, or the β-oxidation route) are utilized.

Essential Fatty Acids (EFA) are PUFA, since they contain two or more double bonds and they are essential for survival of humans and other mammals, and they cannot be synthesized in the body; hence, they have to be obtained in our diet and, thus, are essential (Das UN *et al*., 1988; Das UN, 1991). The human body can synthesize the main SFA (PA and SA) and MUFA (POA and OA) that are found in cell membranes, but it becomes diet-dependent towards PUFA. The two main EFA for our body are the n-6 series precursor *cis*-linoleic acid (LA, 18:2) and the n-3 series precursor α-linolenic acid (ALA, 18:3). The main dietary sources of EFA are, for LA, cereals, eggs, poultry, most vegetable oils, whole-grain breads, baked goods, and margarine; sunflower, saffola, and corn oils are also rich in LA (Das UN, 2002; Ollis TE *et al*., 1999); and for ALA, canola oil, flaxseed oil, linseed and rapeseed oils, walnuts, and leafy green vegetables such as purslane. The average daily intake of EFA varies from country to country and again from region to region. However, in general, the intake is around 7–15 g/day in Europe and USA (Das UN, 2002; Ollis TE *et al*., 1999).

There are at least four independent families of PUFA, depending on the parent FA from which they are synthesized, but only two of them derive from EFA. They include: the n-3 series derived from ALA; the n-6 series derived from cis-LA; the n-9 series derived from oleic acid (OA, 18:1, n-9); and the n-7 series derived from palmitoleic acid (PA, 16:1, n-7). In the n-6 series, LA is converted to γ-linolenic acid (GLA, 18:3, n-6) by the action of the enzyme Δ^6 desaturase, and GLA is elongated to form dihomo-GLA (DGLA, 20:3, n-6), the precursor of the 1 series of prostaglandins (PGs). DGLA can also be converted to arachidonic acid (AA, 20:4, n-6) by the action of the enzyme Δ^5 desaturase. AA forms the precursor of 2 series of prostaglandins, thromboxanes and the 4 series of leukotrienes. In the n-3 series, ALA is converted to eicosapentaenoic acid (EPA, 20:5, n-3) by Δ^6 and Δ^5 desaturases. EPA forms the precursor of the 3 series of prostaglandins and the 5 series of leukotrienes. There is another sequence of FA derived from oleic acid (OA, 18:1 n-9). OA is not an EFA since it can be derived from simple precursors in mammals. All the three n-9, n-6, and n -3 series of unsaturated FA are metabolized by the same set of enzymes to their respective long-chain metabolites.

LA, GLA, DGLA, AA, ALA, EPA and DHA are all PUFA, but only LA and ALA are EFA (see Figure 19 for metabolism of EFA). AA and EPA also give rise to their respective hydroxy acids, which in turn are converted to their respective leukotrienes (LTs). Both PGs and LTs are highly biologically active, have pro-inflammatory action, and are known to be involved in various pathological processes, such as atherosclerosis, bronchial asthma, inflammatory bowel disease, and several other inflammatory conditions. In the present discussion, the term "PUFA" is used to refer

to all unsaturated fatty acids: LA, GLA, DGLA, AA, ALA, EPA, and DHA, while the term EFA refers to LA and ALA. Although the terms EFA and PUFA are used interchangeably for the sake of

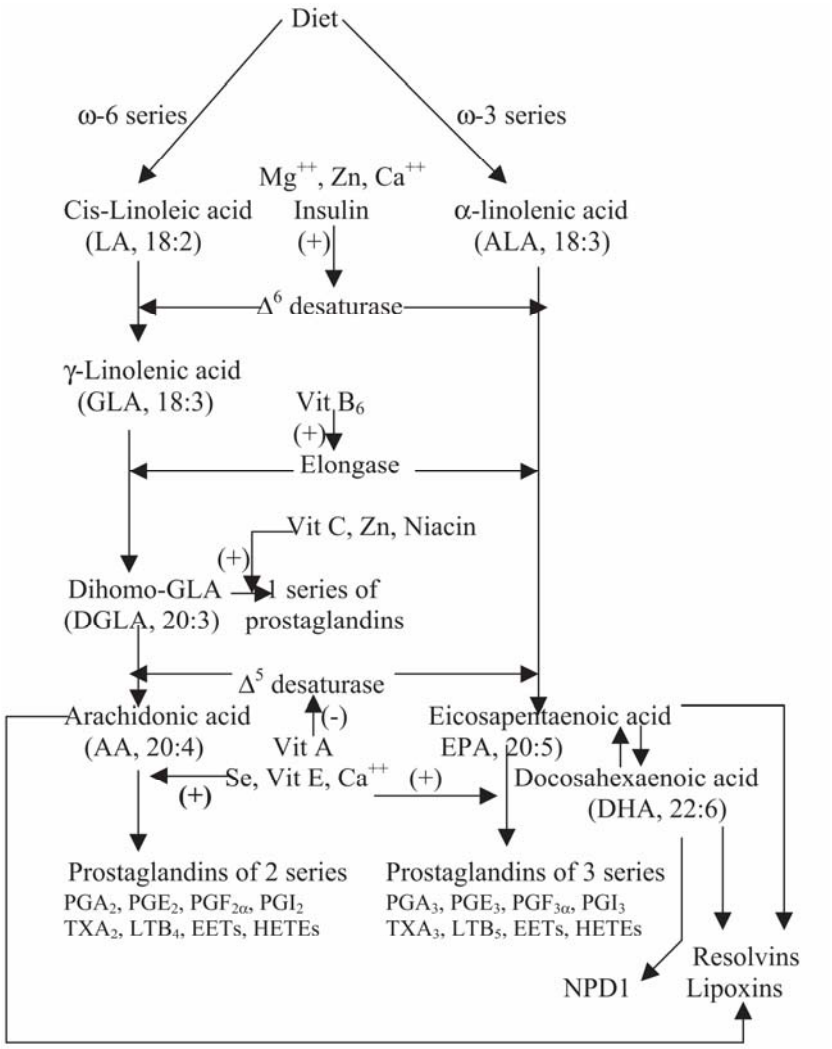


Figure 19. The metabolism of EFA and co-factors that enhance the activity of Δ^6 and Δ^5 desaturases and elongases and formation of PGs. (+) Enhancement of the activity of the enzyme or increase in the formation of the product. (–) Either inhibition of the activity of the enzyme or decrease in the formation of the product. (Das UN, 2006).

convenience, it should be understood that all EFA are PUFA but all PUFA are not EFA. It is known that many of the functions of EFA are also brought about by PUFA, and EFA-deficiency states can be corrected to a large extent by PUFA. This led to the suggestion that PUFA are "functional EFA". Since n-6 and n-3 derive from EFA and they appear to be the most important PUFA series in humans, further discussion here is centered on these two series of FA and their metabolites.

In the majority of the instances FA themselves appear to be active in influencing membrane properties and thus many biologically relevant cell responses. Studies revealed that EFA/PUFA themselves play a significant role in the pathobiology many clinical conditions, as described later in this chapter. This is in addition to the role of PGs and LTs in these conditions. The beneficial action of EPA/DHA when supplemented from external sources has been attributed to the displacement of AA from the cell membrane phospholipid pool and to the formation of less pro-inflammatory PGs (such as PGE_3, $PGF_{3\alpha}$, TXA_3), and LTs (such as LTB_5, LTC_5, and LTD_5) from them, and hence the favorable response. If the molecular mechanism(s) by which various stimuli are able to preferentially induce the release of AA, EPA and/or DHA and convert them to their respective products were known, then it may be possible to develop methods or strategies to treat various inflammatory conditions based on this knowledge. Armed with such knowledge, one may be able to preferentially divert the formation of anti- or less pro-inflammatory molecules from EPA/DHA, so that the disease process would be much less severe. In this context, it is interesting to note that AA, EPA and DHA could give rise to anti-inflammatory molecules such as lipoxins (LXs), resolvins and nitrolipids, discussed in the next paragraph. This suggests that PUFA form precursors to both pro- and anti-inflammatory molecules, and the balance between these mutually antagonistic compounds could determine the final outcome of the disease process.

Dietary LA and ALA are metabolized by the enzymes Δ^6 and Δ^5 desaturases to their respective metabolites as depicted in figure 16. OA, belonging to the n-9 series, also forms precursor to its own series of FA. However, OA is not an EFA since the body can synthesize it from simple precursors. LA, ALA, and OA are metabolized by the same set of Δ^6 and Δ^5 desaturases and elongases. The purpose of desaturases is to remove 2 hydrogens, whereas elongases is to add 2 carbons. As a result, these 3 series compete with one another for the same set of enzymes. It appears, however, that the enzymes prefer n-3 to n-6, and n-6 over n-9. Thus, in a given situation, the enzymes metabolize the FA with the following sequence of preference: n-3 > n-6 > n-9. Hence, under normal physiological conditions the metabolites of n-9 are formed only in trivial amounts in the cells. This has an important biological significance since presence of significant amounts of 20:3 n-9 suggests that there is deficiency of n-3 and n-6, and is used as an indicator of EFA deficiency. There are reports suggesting that this occurs in many, if not all, tumor cells. It should also be noted here that the activities of Δ^6 and Δ^5 desaturases are slow in humans ($\Delta^5 > \Delta^6$). Thus, the conversion of LA and ALA to their respective metabolites such as GLA and EPA may be inadequate under certain conditions. To bypass the block in the activities of Δ^6 and Δ^5 desaturases in some diseases and in ageing subjects, it may be necessary to directly supplement the metabolites such as GLA and DGLA (to bypass Δ^6 desaturase) and AA, EPA and DHA (to bypass Δ^6 and Δ^5

desaturases). A number of factors are known to influence the activities of desaturases and elongases involved in the metabolism of EFA (Das UN *et al*., 1988; Das UN, 1991; Horrobin DF, 1983; Mozaffarian D *et al*., 2004; Brenner RR, 1982). Saturated fats, cholesterol, *trans*-FA formed by vegetable oil processing, alcohol, adrenaline, and glucocorticoids inhibit Δ^6 and Δ^5 desaturases. Pyridoxine, zinc, and magnesium are necessary co-factors for normal Δ^6 desaturase activity. Insulin activates Δ^6 desaturase, whereas diabetics have reduced Δ^6 desaturase activity. The activity of Δ^6 desaturase falls with age. Oncogenic viruses and radiation inhibit Δ^6 desaturase activity. Total fasting and protein deficiency reduce the activity of Δ^6 desaturase. A fat-free diet and partial caloric restriction enhances Δ^6 desaturase activity. A glucose-rich diet inhibits Δ^6 desaturase activity. Peroxisome proliferator-activated receptor-α (PPAR-α) activator WY 14,643 significantly enhanced the transcription of hepatic Δ^6 desaturase by more than 500% (Takahashi Y *et al*., 2003). Dietary LA concomitantly increases the activity and mRNA levels of enzymes involved in FA synthesis and oxidation, and desaturation of PUFA in the mouse liver, which appears to be mediated by both the activation of PPAR-α and up-regulation of SREBP-1 (Tang C *et al*., 2003). Both Δ^6 and Δ^5 desaturases are regulated by SREBP-1c and PPAR-α, two reciprocal transcription factors for fatty acid metabolism, and that some of their lipogenic actions are brought about by their ability to regulate the producing PUFA (Matsuzaka T *et al*., 2002). Activities of Δ^6 and Δ^5 desaturases are decreased in diabetes mellitus, hypertension, hyperlipidemia, and metabolic syndrome X. It is known that *trans* fats interfere with the metabolism of EFA and promote inflammation, atherosclerosis and CHD (Das UN, 2002; Mozaffarian D *et al*., 2004; Cook HW, 1981). The pro-inflammatory action of *trans* fats can be attributed to their ability to interfere with the metabolism of EFA. Several PUFA, especially EPA and DHA, are known to inhibit the production of pro-inflammatory cytokines: interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-1, and IL-2 (La Guardia M *et al*., 2005; Das UN *et al*., 2001; Das UN, 2004). SFA and cholesterol also interfere with the metabolism of EFA and, thus, promote the production of pro-inflammatory cytokines, which explains their ability to cause atherosclerosis and CHD. This suggests that *trans* fats, saturated fats, and cholesterol have pro-inflammatory actions, whereas PUFA, such as GLA, DGLA, EPA and DHA, possess anti-inflammatory properties. By interfering with the metabolism of EFA, saturated fats, cholesterol and *trans* fats could reduce the formation of their long-chain metabolites GLA, DGLA, AA, EPA, and DHA that are essential for the formation of biologically active and beneficial prostacyclin PGI_2, PGI_3, LXs, and resolvins. Deficiency and/or absence of PGI_2, PGI_3, LXs and resolvins will lead to the initiation and progression of many pathologies.

2.2.2 Second-messenger like actions of PUFA

Some of PUFA functions require their conversion to eicosanoids and other products, that have second messenger like actions. Several hormones and growth factors activate phospholipase A_2 (PLA_2), which, in turn, induces the release of DGLA, AA, EPA, and DHA from the cell membrane lipid pool. These FA are utilized for the formation of various biologically active molecules and bring about their actions. In this paragraph a brief overview of second-messenger like actions of PUFA will be discussed.

As already mentioned above, PUFA not only form precursors for eicosanoids such as TXs, LTs, PGs, and PGI_2 but also gives rise to LXs and resolvins (Figure 20). LXs have potent anti-inflammatory properties (Claria J and Serhan CN, 1995; Chiang N *et al*., 1999). The interaction between endothelial cells and PMNs, leading to the formation of 15R-HETE and its subsequent conversion to 15-epimeric LXs by aspirin-acetylated COX-2, is a protective mechanism to prevent local inflammation on the vessel wall by regulating the motility of PMNs, eosinophils, and monocytes (Chiang N *et al*., 1999 ; Xiao G *et al*., 1997; Levy BD *et al*., 2001 ; Bandeira-Melo C *et al*., 2000; Serhan CN *et al*., 1995). Endothelial cells oxidize AA (and possibly EPA and DHA) via the P_{450} enzyme system to form 11,12-epoxy-eicosatetraenoic acid(s), which blocks endothelial cell activation (Claria J and Serhan CN, 1995; Serhan CN *et al*., 1995). These studies imply that COX-2 enzyme is essential for the formation of the beneficial LXs. In situation in which there is a deficiency or absence of LXs, interaction between PMN-endothelial cells occurs, leading to endothelial damage that may result in the development and progression of atherosclerosis, thrombus formation and coronary artery disease, and persistence of inflammation. Compounds similar to 15R-HETE and 15-epimeric LXs are also formed from EPA and DHA. Human endothelial cells, in the presence of IL-1β (which induces COX-2) and aspirin, converted EPA to 18R-HEPE, 18-HEPE, and 15RHEPE. Activated human PMNs, in turn, converted 18RHEPE to 5,12,18R-triHEPE and 15R-HEPE to 15-epi-LXA_5 by 5-lipoxygenase. Both 18R-HEPE and 5,12,18R-triHEPE inhibited LTB_4-stimulated PMN transendothelial migration, similar to 15-epiLXA_4. 5,12,18R-triHEPE competed with LTB_4 for its receptors and inhibited PMN infiltration. These results suggest that 5,12,18R-triHEPE suppress LT-mediated responses if present in adequate amounts at the sites of inflammation (Serhan CN *et al*., 2000). Murine brain cells expressing COX-2, when treated with aspirin, enzymatically transformed DHA to a 17R series of hydroxy DHA (HDHA) that, in turn, is enzymatically converted by PMNs to di- and tri-hydroxy containing docosanoids (Serhan CN *et al*., 2002). Small molecular weight compounds similar to HDHA are generated from AA and EPA. Thus, 15R-hydroxy containing compounds are formed from AA, the 18R series from EPA, and the

17R-hydroxy series from DHA. All these compounds have potent anti-inflammatory actions and are involved in resolution of the inflammatory process, and hence have been termed as "resolvins". Resolvins inhibit cytokine generation, leukocyte recruitment, leukocyte diapedesis, and exudate formation. Thus, LXs and resolvins formed from AA, EPA, and DHA have cardioprotective, neuroprotective, and other cytoprotective actions. Of the several 17-hydroxy-containing bioactive mediators derived from DHA, which were termed docosatrienes and 17S series resolvins, 10,17S-dihydroxydocosatriene, also called neuroprotectin D1 (NPD1), was found to reduce the infiltration of PMNs and possess potent anti-inflammatory and neuroprotective properties (Marcheselli VL *et al*., 2003; Hong S *et al*., 2003). NPD1 inhibited oxidative stress-induced apoptosis of human retinal pigment epithelial cells (Mukherjee PK *et al*., 2004). Both LXs and NPD1 enhanced wound healing (Gronert K *et al*., 2005), and promoted brain cell survival via the induction of anti-apoptotic and neuroprotective gene-expression programs that suppress Aβ42-induced neurotoxicity (Calon F *et al*., 2004; Lukiw WJ *et al*., 2005). It is likely that under physiological conditions, both COX-1 and COX-2 enzymes are utilized for the formation of beneficial eicosanoids such as PGE_1, PGI_2, and LXs, resolvins, and NPD1 in various tissues such that inflammation is prevented. Failure to produce adequate amounts of LXs, resolvins, and NPD1 or interference with their action, and a simultaneous increase in the production of pro-inflammatory PGs, TXs, and LTs, and cytokines, could lead to initiation and persistence of inflammation and tissue damage.

PUFA regulate the secretion of cytokines. PUFA (especially ALA, DGLA, EPA, and DHA), LXs and resolvins suppress IL-1, IL-2, IL-6, and TNF-α production by T cells (Endres S *et al*., 1989; Kumar GS and Das UN, 1994; Arita M *et al*., 2005; Dooper MM *et al*., 2003), and thus function as endogenous antiinflammatory molecules. Although, no studies have reported direct effect(s) of AA on the production of various cytokines, it is generally believed that PGE_2, PGF_{2a}, TXA_2 and LTs derived from AA have modulatory role on IL-6 and TNF-α production. In view of the different effects of PGE_2, $PGF_{2\alpha}$ and PGE_1 on the synthesis of IL-6 and TNF-α, the local levels of IL-6 and TNF-α at the sites o inflammation and injury may depend on the balance between DGLA and AA and the respective PG products formed from them. PGE_1 and $PGF_{1\alpha}$ (derived from DGLA) and TXB_2 (derived from AA) inhibit, whereas DGLA and AA *per se* do not have much influence on the growth of human lymphocytes *in vitro* at the doses tested. In contrast, PGE_1, PGE_2, $PGF_{2\alpha}$, and TXB_2 suppressed IL-2 production, and PGE_2, $PGF_{2\alpha}$, and TXB_2 enhanced IL-4 synthesis, whereas PGE_2, $PGF_{2\alpha}$, TXB_2, PGI_2, and $PGF_{1\alpha}$ increased TNF-α synthesis with no action on IL-6 synthesis (Kumar GS and Das UN, 1994). On the other hand, DGLA and AA enhanced, whereas EPA decreased, the synthesis of IL-4 in human lymphocytes *in vitro* with no action on IL-6 production, and were modulated by the doses of FA used (Kumar GS and Das UN, 1994; Trebble T *et al*.,

2003). DHA has been shown to suppress IL-1β and TNF-α production by stimulated human retinal vascular endothelial cells (Chen W *et al*., 2005). There is evidence to suggest that some of the suppressive actions of EPA and DHA on the production of pro-inflammatory cytokines and their anti-inflammatory action seems to be mediated by their ability to increase both PPAR-γ mRNA and protein activity (Li H *et al*., 2005). These results suggest that various PUFA and their products have different, and at times diametrically opposite, actions on the synthesis of various cytokines. Hence, the local concentrations of different PUFA and eicosanoids formed and the balance between these various modulators will ultimately determine the types and concentrations of cytokines formed and the degree of inflammation.

Previously, it was shown that PUFA inhibited leukocyte angiotensin-converting enzyme (ACE) activity (Sun CQ *et al*., 2003). This suggests that PUFA could function as endogenous regulators of ACE activity, and thus regulate the formation of Ang-II. PUFA enhance NO generation (Kumar KV and Das UN, 1997; Das UN, 2002; Okuda Y *et al*., 1997). Hence, when cell/tissue concentrations of PUFA are low, the activity of ACE will be high, leading to the formation of increased amounts of Ang-II, and the formation of endothelial NO (eNO) will be low. Plasma concentrations of PUFA and eNO are low in hypertension, diabetes mellitus, renal diseases, rheumatoid arthritis (RA), lupus, psoriasis, eczema, atopic and non-atopic dermatitis, atherosclerosis, insulin resistance, obesity, dementia, schizophrenia, bipolar disorders, Huntington's disease, Alzheimer's disease, peptic ulcer disease, and cancer (Das UN *et al*., 2001; Das UN, 1995; Das UN and Vaddadi KS, 2004).

Similar to statins, EFA and their metabolites (especially AA, EPA, and DHA) are useful in the treatment of hyperlipidemias, have anti-proliferative action on tumor cells both *in vitro* and *in vivo*, bind to DNA and regulate the expression of genes and oncogenes. More importantly, PUFA are also potent inhibitors of the HMG-CoA reductase enzyme (El-Sohemy A and Archer MC, 1999; Das UN, 2000). Statins have the ability to enhance plasma AA concentrations and decrease the ratio of EPA to AA significantly (Nakamura N *et al*., 1998).

PUFA have the ability to modulate brain growth and development, and neuronal differentiation. This action is in addition to the ability of PUFA to form an important constituent of neuronal cell membranes and involvement in memory formation and consolidation (Das UN, 2003a; 2003b). This explains why PUFA are useful in the prevention and treatment of dementia and Alzheimer's disease (Calon F *et al*., 2004; 2005; Lukiw WJ *et al*., 2005; Akbar M *et al*., 2005; Aravindakshan M *et al*., 2003; Das UN, 2004). The beneficial actions of PUFA in Alzheimer's disease, schizophrenia and dementia have been attributed to the formation of anti-inflammatory compounds such as LXs and resolvins.

PUFA show anti-biotic-like actions (Sun CQ *et al*., 2003; Das UN, 2006; Giamarellos-Bourboulis EJ *et al*., 2004) . For instance, LA rapidly killed cultures of *Staphylococcus aureus*, and hydrolyzed linseed oil (which contains both LA and ALA) can inactivate methicillin-resistant ones. ALA promotes adhesion of *Lactobacillus casei* to mucosal surfaces and, thus, augments their growth. *Lactobacilli*, in turn, suppress the growth of pathogenic bacteria like *Helicobacter pylori*, *Shigella flexneri*, *Salmonella typhimurium*, *Pseudomonas aeroginosa, Clostridium difficile*, and *Escherichia coli*. PUFA inactivate enveloped viruses and show anti-fungal properties. The anti-inflammatory and anti-bacterial, anti-viral, and anti-fungal actions of PUFA may explain some of their beneficial actions. In this context, it will be interesting to study whether local application or intravenous infusions of PUFA would help patients with various bacterial, viral and fungal infections to recover faster. Since neutrophils, T cells and macrophages release PUFA on stimulation, it is possible that this could be one of the defense mechanisms of the body to fight infections.

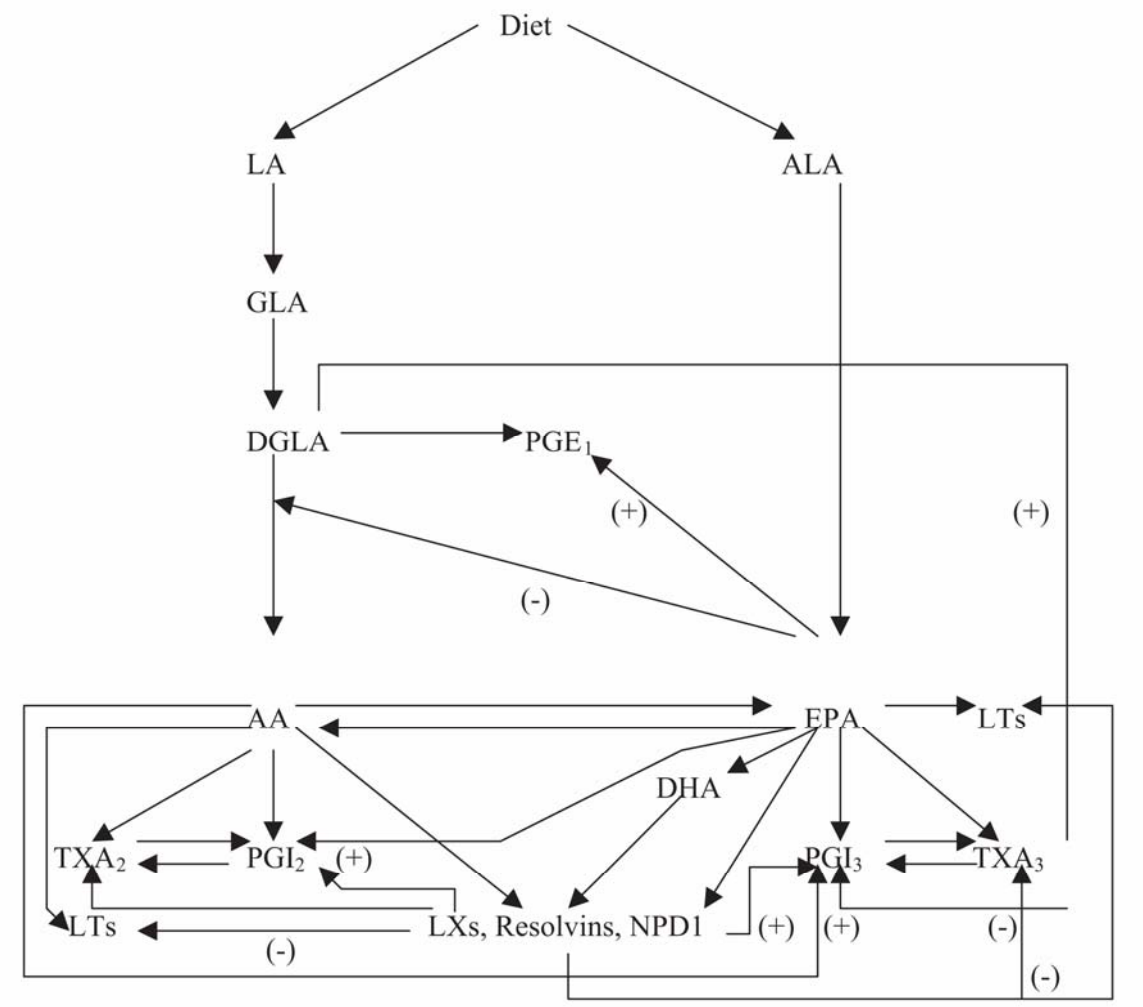


Figure 20. Interaction(s) between n-3 and n-6 FA and their effect on the formation of PGI_2, PGI_3, PGE_1, and LXs, resolvins and NPD1. (–) Inhibition or block in the synthesis, formation or release. (+) Enhancement in the formation or release. LXs, resolvins, and NPD1 enhance the formation and/or action of PGI_2 and PGI_3, suppress that of TXA_2 and TXA_3 and suppress the formation of LTs. (Das UN, 2006).

It is evident from the preceding discussion that PUFA and their products participate in the pathobiology of inflammation. The amount and type of PUFA released in response to inflammatory stimuli depends on the cell membrane phospholipid FA content. Since the EFA LA and ALA have to be obtained direct from diet, this suggests that dietary content EFA could be one factor that determines the degree of inflammation. Furthermore, direct intake of various PUFA alters the cell membrane FA composition, which, in turn, modulates cell/tissue response to infection, injury and inflammatory events. Increased dietary intake of GLA, DGLA, and EPA/DHA substantially decreases inflammatory response (Dooper MM *et al*., 2003; Trebble T *et al*., 2003; Chen W *et al*., 2005; Li H *et al*., 2005). For instance, GLA is rapidly elongated to form DGLA, which, in turn, is converted to PGE_1 and its (DGLA) 15-lipoxygenase product 15-hydroxy-8, 11, 13-eicosatrienoic acid (15-OH-20:3, n-6), which have anti-inflammatory actions (Miller CC *et al*., 1988; Tate G *et al*., 1989; Heitmann J *et al*., 1995). In summary, GLA, DGLA, EPA and DHA possess anti-inflammatory actions that can be attributed to decreased formation of pro-inflammatory eicosanoids and cytokines, and an increase in the production of beneficial eicosanoids such as PGE_1, PGI_2, PGI_3, HPETEs, eNO, LXs, resolvins and NPD1 and early resolution of inflammation. It is likely that when the cell membrane lipid pool is rich in GLA/DGLA/EPA/DHA and contains appropriate amounts of AA, a specific activation of $sPLA_2$ and $cPLA_2$ could occur in response to an inflammatory stimuli that leads to the formation of increased amounts of LXs, PGD_2 and $15deoxy^{\Delta 12-14}PGJ_2$, eNO, GSNO, PGE_1, PGI_2, PGI_3, and HPETE that dampen inflammatory process and enhance resolution of inflammation. On the other hand, AA serves as the precursor of several pro-inflammatory compounds, and hence in the presence of excess AA, inflammation is likely to be significant. In addition to their action on pro-inflammatory cytokines and adhesion molecules, it has also been shown that DGLA, EPA, and DHA can suppress NF-κB signaling (Borghaei H *et al*., 1997; Zhao Y *et al*., 2004; Schley PD *et al*., 2005; Weber C *et al*., 1995). In contrast, AA activates NF-κB, explaining its pro-inflammatory actions. In this context, it is important to note that PUFA could serve as endogenous ligands for PPARs, which could be yet another mechanism of action by which they are able to suppress inflammatory events (Li H *et al*., 2005; Kliewer SA *et al*., 1997). It is evident from the preceding discussion that EFA and their long-chain metabolites, eicosanoids, LXs, resolvins, and NPD1 have many biological actions and participate in several diseases processes (Figure 21). In this context, it is interesting to note that NO can react with PUFA to yield their respective nitroalkene derivatives that can be detected in plasma, and have been shown to induce vascular relaxation, inhibit neutrophil degranulation and superoxide formation, and inhibit platelet activation (Baker PRS *et al*., 2005; Coles B *et al*., 2002; Lima ES *et al*., 2005). These nitroalkene derivatives of various PUFA, which can be detected in substantial

amounts both in the plasma and urine, have endogenous PPAR-γ ligand activity and decay in the blood to release NO. These reports suggest that PUFA not only form precursors to various eicosanoids, resolvins, LXs, and NPD1, but also may react with various other molecules and form novel compounds that have biological activity. Since several biologically active molecules that have both pro- and anti-inflammatory actions are formed from PUFA, it is important to know the molecular triggers that facilitate their formation. Once the mechanisms of their formation is clear, it may be possible to device methods of selectively enhancing the synthesis of LXs, resolvins, NPD1, and nitroalkenes to suppress inappropriate inflammatory events. Synthesis of stable and more potent LXs and resolvins could be attempted, so that their usefulness in the management of several inflammatory conditions (Das UN, 2002; 2004; Emsley R *et al*., 2002). In view of their varied actions PUFA and their products may also form the basis for the development of many nutraceuticals and drugs.

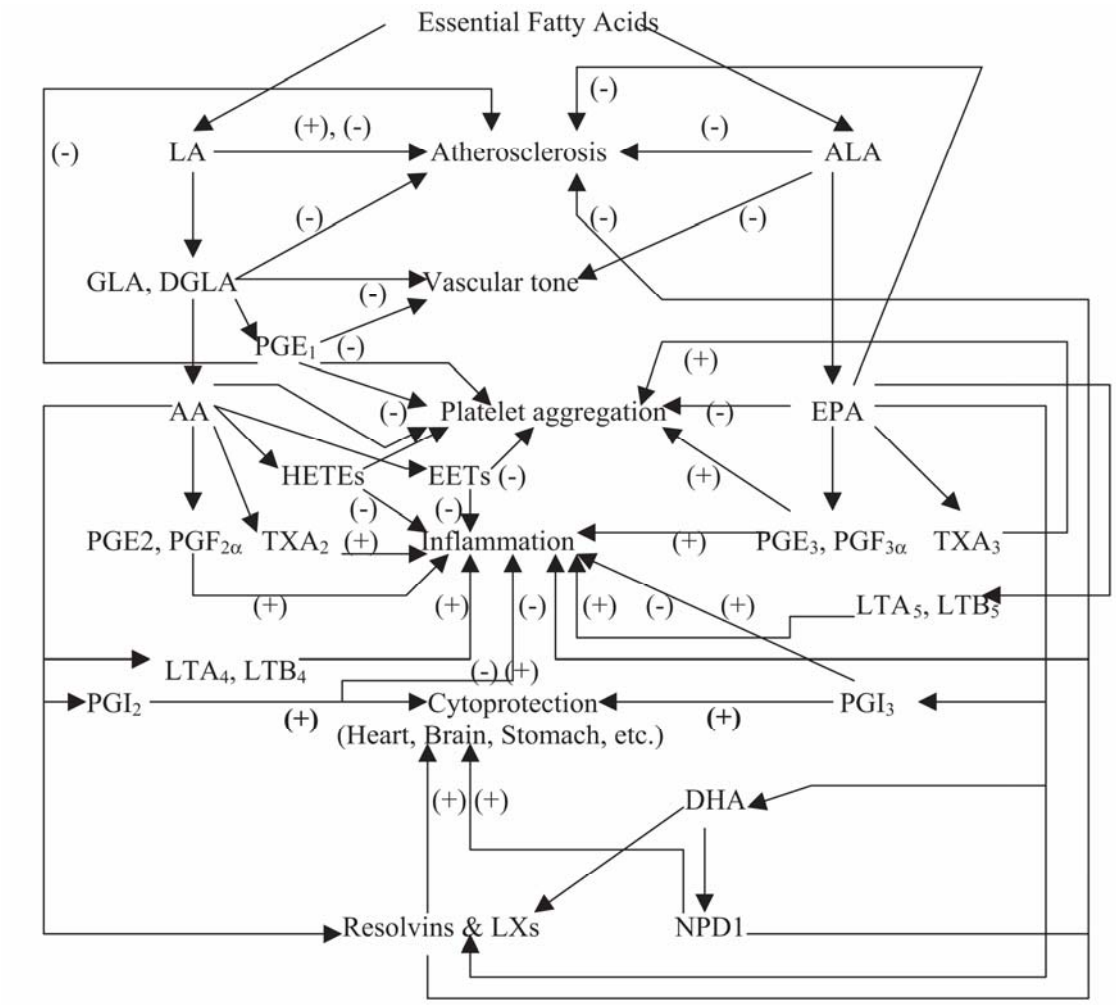


Figure 21. Actions of EFA, their long-chain metabolites, and various eicosanoids formed from them. Note that the scheme does not depict an exhaustive list of the actions of the various molecules mentioned, with only the representative major actions depicted. (+) Positive control or enhancement of the process. (–) Negative control or inhibition or decrease of the process. Some eicosanoids may have both positive and negative actions on some processes. (Das UN, 2006).

2.2.3 Composition of human cell membranes

On the basis of estimates from studies of paleolithic nutrition and modern-day hunter-gatherer populations, it appears that human beings evolved consuming a diet that was much lower in SFA than is today's diet (Eaton SB and Konner M, 1985). Furthermore, the diet contained small and roughly equal amounts of n-6 and n-3 PUFA (ratio of 1-2:1) and much lower amounts of *trans* fatty acids than does today's diet (Figure 22) (Simopoulos AP, 1995; 1999). Wild plants contributed with higher amounts of vitamin E, vitamin C and other antioxidants than cultivated plants, providing additional protection against cancer and atherosclerosis. The current western diet is very high in n-6 FA (the ratio of n-6 to n-3 FA is now 10 - 20:1). Comparing the n-6:n-3 intake of various populations (Simopoulos AP, 2001; 1998; Eaton SB *et al*., 1998; Sanders TAB, 2000; Sugano M and Hirahara F, 2000), it can be noted that the population of Crete obtained a higher intake of ALA from purslane and other wild plants, walnuts and figs, whereas the japanese obtained it from canola oil and soybean oil (Simopoulos AP, 2001).

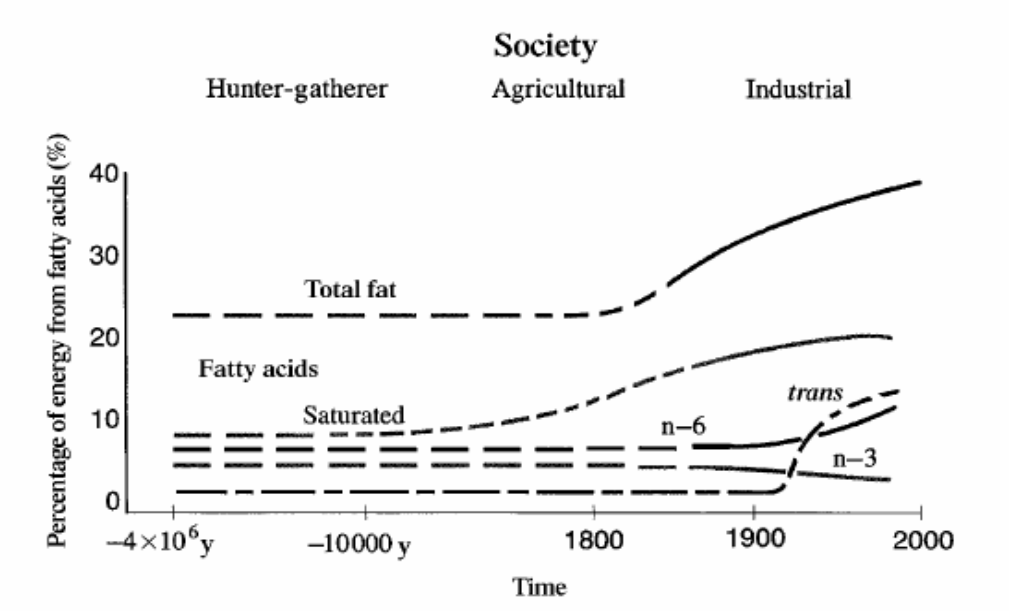


Figure 22. Percentage of energy from FA used by humans during history. (Simopoulos AP, 2000).

Intake of n-3 FA is much lower today because of the decrease in fish consumption and the industrial production of animal feeds rich in grains containing n-6 FA, that leads to the production of meat rich in n-6 and poor in n-3 FA (Crawford MA, 1968). The same is true for cultured fish (Van Vliet

T and Katan MB, 1990) and eggs (Simopoulos AP and Salem N JR, 1989). Even cultivated vegetables contain fewer n-3 FA than do plants in the wild (Simopoulos AP and Salem N JR, 1986; Simopoulos AP *et al*., 1995). In view of the fact that ALA is converted to EPA and DHA in human beings, it is important to consider terrestrial sources of n-3 FA in the food supply. In plants, leaf lipids usually contain large proportions of ALA, which is an important component of chloroplast membrane polar lipids. Mammals who feed on these plants convert ALA to EPA and DHA. Wild animals and birds who feed on wild plants are very lean with a carcass fat content of only 3.9 % and contain about five times more PUFA per gram than is found in domestic livestock. Most importantly, 4 % of the fat of wild animals contains EPA whereas domestic beef contains very small or undetectable amounts, since cattle are fed grains that are rich in n-6 FA and poor in n-3 FA, whereas a deer that forages on ferns and mosses contains n-3 FA in its meat. In summary, modern agriculture, with its emphasis on production, has decreased the n-3 FA content in many foods: green leafy vegetables, animal meats, eggs, and even fish.

EFA are principally present in dietary triacylglycerols, which should be hydrolyzed by lipases in gastric and intestinal lumen. DHA seems to be released more slowly than the others; its intestinal absorption is delayed but not decreased. Long-Chain PUFA (LCPUFA) are incorporated in noticeable amounts in chylomicron phospholipids. However, their uptake by tissues is no more rapid than uptake of shorter chain PUFA. In tissues, LA and ALA, which constitute the major part of dietary EFA, are converted into FA of longer and more unsaturated chain by alternate desaturation-elongation reactions. Liver is one of the most active organs and its role is critical in providing less active tissues, particularly the brain, with LCPUFA secreted in VLDL. In the liver, many nutritional, hormonal and physiological factors act on the PUFA biosynthesis. Dietary FA exert a great influence and are often inhibitory. Dietary ALA inhibits Δ^6 desaturation of LA. The desaturation products AA, EPA, and DHA inhibit Δ^6 desaturation of LA and Δ^5 desaturation of DGLA. With regard to hormones, insulin and thyroxin are necessary to Δ^6 and Δ^5 desaturation activities, whereas other hormones (glucagon, epinephrine, ACTH, glucocorticoids) inhibit desaturation. Concerning the physiological factors, the age of individuals is critical. In the fetus, the liver and the brain are capable of converting LA and LNA into longer-chain EFA, but these are also delivered by the mother, after synthesis in the maternal liver and placenta.

FA are the basic structural components of triglycerides and are also found in phospholipids and cholesterol esters. They are rarely available as free FA *in vivo*. Fats have important functions as storage units for energy, as structural units in membranes and as precursors to second-messengers. They can therefore be found in serum, membranes and adipocytes. The proportion to be found in each media appears to depend upon the type of FA. For example, LA concentrations appear to be

greatest in cholesterol esters, followed by phospholipids, and lowest in triglycerides. OA shows the inverse sequence. AA appears to be particularly controlled across tissues, and it can represent 27% of the platelet phospholipid content, as little as 2% of plasma and less than 1% of adipose tissue concentrations.

Fatty acid	Adipose tissue	RBC	Placenta	Liver	Testis	Brain cerebrum	Retina
Total SFA	27.2	43.1	39.4	42.0	39.6	45.9	48.2
Total MUFA	59.7	23.0	11.6	23.8	20.0	29.7	14.2
18:2*n*-6	10.5	9.3	9.5	17.5	5.6	0.6	1.4
18:3*n*-3	0.8	n.d.	–	0.3	0.7	tr.	n.d.
20:3*n*-6	0.2	1.5	4.3	1.6	6.7	1.2	2.1
20:4*n*-6	0.3	15.2	21.1	7.7	13.4	7.7	9.6
20:5*n*-3	tr.	0.7	0.1	0.4	–	tr.	0.1
22:4*n*-6	n.d.	1.6	1.6	0.3	2.1	5.4	1.8
22:5*n*-6	–	–	1.0	0.3	0.4	1.1	0.8
22:5*n*-3	0.2	1.8	1.3	0.5	–	0.2	1.3
22:6*n*-3	0.3	3.2	4.8	3.4	8.5	7.2	19.7
Total PUFA	13.1	33.3	44.4	32	30.7	23.4	37.2
Total LCPUFA	1.1	24.0	34.2	14.2	24.4	22.8	35.5
Total *n*-6	11.2	27.6	37.6	27.4	28.2	16.0	16.0
Total *n*-3	1.9	5.7	6.3	4.6	9.2	7.4	21.1
Total C22-FA	0.5	6.6	8.7	4.5	11.0	13.9	23.6
C20/C22	1.3	2.6	2.9	2.2	1.2	0.6	0.5
n-3/*n*-6	0.17	0.21	0.16	0.17	0.33	0.46	1.32

Table 6. The FA composition (wt.%) of various tissues from humans living in western societies. (Lauritzen L *et al*., 2001).

PUFA account for 21%–36% of the FA in the cell membrane, but the proportions of FA with 20 or 22 carbon atoms varies considerably between tissues (Table 6). The nervous system is the organ with the second largest concentration of lipids, only exceeded by adipose tissue. The adult brain contains approximately 50%–60% of its dry weight as lipid and approximately 35% of the lipids are PUFA, most of which are LCPUFA (EPA and DHA). The table gives only human values, but the general pattern is similar to other mammalian species, although there are also some species differences. AA is distributed in relatively large amounts in most tissues. DHA, however, is more specifc in its tissue distribution. Neuronal tissues, such as the brain and retina, and a few tissues outside the central nervous system (CNS), such as the testis, are especially high in DHA. Furthermore, within these tissues DHA is particularly abundant in the rod photoreceptors and in gray matter (Neuringer M *et al*., 1988; Tinoco J, 1982; Soderberg M *et al*., 1991), especially synaptic membranes (Kishimoto Y *et al*., 1979**)**. DHA can approach 50 mol% of the total phospholipid acyl chains in these membranes (Salem N *et al*., 1986). With very high levels of DHA,

it would be expected that di-DHA phospholipid species would be present and indeed they have been isolated from several tissues (Miljanich GP *et al*., 1979; Bell MV *et al*., 1997). The already high DHA levels in these membranes are not further augmented by diet and once incorporated, DHA is tenaciously retained at the expense of other FA (Salem N *et al*., 1986). Limited tissue distribution of high DHA levels implies a specialized but as yet undefined role for DHA in these cells. This relative specifc tissue distribution of DHA gives some indication of a possible important role in the membranes of these tissues. The DHA content of different phospholipid species varies considerably. DHA is especially abundant in PS and PE of membranes in general, as well as in synaptosomal membranes (Kishimoto Y *et al*., 1979; Breckenridge WC *et al*., 1972; Martinez M and Mougan I, 1998; Neuringer M *et al*., 1986; Salem N *et al*., 1986) and rod outer segment (ROS) membranes (Anderson RE, 1970). The highest DHA content is found in alkenyl,acyl-PtdEtn of synaptosomal membranes, where it attributes to almost 50% of the acyl group, and in PtdEtn and PtdSer in ROS, where DHA constitute 50% of the FA (Neuringer M *et al*., 1988; Aveldano MI, 1988). Upon defciency of n-3 FA, 22:5n-6 will accumulate to a certain degree instead of DHA (Neuringer M *et al*., 1986; Bourre JM *et al*., 1990; Guesnet P et al., 1997). Furthermore, membrane FA composition is not the only aspect of membranes that is affected by dietary n-3 FA deficency. In some cases a deficency-induced decrease in membrane DHA content was associated with a concomitant decrease in PS (Chalon S *et al*., 1998; Delion S *et al*.,1997; Garcia MC *et al*., 1998). Thus, there are several potential explanations for observed functional consequences of n-3 FA deficiency. A changed biological response can be attributed to a change in membrane phospholipid composition or FA composition. In sharp contrast to the few tissues containing high levels of DHA are the other tissues where DHA is often found below 5 mol% of the total phospholipid acyl chains. DHA in these tissues can be enriched 2–10-fold through dietary supplementation with foods rich in this n-3 FA (Salem N *et al*., 1986; Van Meter AR *et al*., 1994; Robinson DR *et al*., 1993). It is in these diet-affected cells that DHA probably supports many of its claimed health benefits. Here DHA is primarily found in the *sn*-2 chain with the *sn*-1 chain mainly composed of the saturated SFA PA and SA (Anderson RE and Sperling L, 1971).

2.2.4 Pathological conditions

PUFA appears to be the most active membrane FA, their actions ranging from the effect on membrane physico-chemical behaviour to the generation of the numerous biologically active compound previously described. Moreover, because of the current high ratio of n-6 to n-3 FA in western population, most of the studies regarding human pathological conditions that have been conducted in the last twenty years are based on n-3 supplementation. Mainly for these reasons, the scientific world have extensively studied and related all kind of diseases to PUFA, trying to fugure out how a PUFA or n-3 rich phenotype would behave or respond to a specific pathology; therefore it is easily explained why there is no literature concerning a SFA supplementation, because it is thought that a SFA-rich phenotype is the baseline of today experiments. In the following paragraphs, the FA studies concerning their role in main human diseases are grouped together and briefly discussed.

Cardiovascular Diseases

Epidemiological and interventional studies indicate that dietary n-3 PUFA reduce mortality due to coronary heart disease (CHD). They act at a low dose, since one or two meals with fatty fish per week is sufficient to provide protection when compared with no fish intake (Kris-Etherton P *et al*., 2001). Numerous experimental studies have indicated that low concentrations of exogenous n-3 PUFA reduce the severity of cardiac arrhythmia. This effect is probably responsible for the protective action of n-3 PUFA on CHD mortality. Inhibition of myocardial thromboxane synthesis may play a role in this effect (Charnock JS, 1991), as well as reduced cardiac responsiveness to α1-adrenergic stimulation (Reibel DK *et al*., 1988). The epidemiological data suggest that the benefit of dietary fish is centered on a reduction in sudden cardiac death. Fish and fish oil have been shown to reduce all-cause mortality and cardiovascular death in patients who had myocardial infarction (Burr ML *et al*., 1989; GISSI Investigators, 1999). n-3 PUFA supplementation significantly reduced death, particularly sudden death, but not reinfarction or stroke. An antiarrhythmic action of n-3 PUFA was supported by these findings (GISSI Investigators, 1999). At high doses, dietary n-3 PUFA have several beneficial properties in human (Kris-Etherton P *et al*., 2001): act favorably on blood characteristics by reducing platelet aggregation and blood viscosity, are hypotriglyceridemic, exhibit antithrombotic and fibrinolytic activities, exhibit antiinflammatory action, reduce ischemia/reperfusion-induced cellular damage. This effect is apparently due to the incorporation of EPA in membrane phospholipids.

The hypolipidemic effects of n-3 FA are similar to those of n-6 FA, provided that they replace saturated fats in the diet. An added benefit is shown by n-3 PUFA which in hypertriglyceridemic patients consistently lower serum triacylglycerol concentrations, whereas the n-6 FA do not and may even increase them (Phillipson BE *et al*., 1985). Studies in humans have shown that fish oils reduce the rate of hepatic secretion of very low-density lipoprotein and triacylglycerol and in normolipidemic subjects, n-3 FA prevent and rapidly reverse carbohydrate-induced hypertriglyceridemia (Nestel PJ *et al*., 1984).

The antithrombotic effects of fish oil are due to decreases in platelet aggregation, a decrease in TXA_2, increases in PGI_2 and PGI_3 production, decrease in whole blood viscosity and an increase in bleeding time (Simopoulos AP, 1994). Because of the increased amount of n-6 FA in the western diet, the eicosanoid metabolic products from AA, specifically PGE, TX, LT, are formed in larger quantity than those formed from n-3 FA, specifically EPA. The eicosanoids formed from AA are biologically active in small quantities and if they are formed in large amounts, they contribute to the formation of thrombi and atheromas, the development of allergic and inflammatory disorders, and cell proliferation. Thus a diet rich in n-6 FA shifts the physiologic state to one that is prothrombotic and proaggregatory, with increases in blood viscosity, vasospasm and vasoconstriction and decreases in bleeding time. n-3 PUFA helped to make scars harmless and stabilize the health in stroke patients who are at high risk of atherosclerotic plaques rupturing or forming clots. It was demonstrated that the proportions of EPA and DHA were higher in carotid plaque fractions in patients receiving fish oil compared with other groups. Fewer plaques from patients being treated with fish oil had thin fibrous caps and signs of inflammation and more plaques had thick fibrous caps and no signs of inflammation, compared with plaques in other groups. The number of macrophages in plaques from patients receiving fish oil was lower than in the other two groups. This finding suggests that within a short time, a modest level of dietary n-3 PUFA supplementation has a role in establishment of plaque stability, thus reducing the risk of neurological events in patients with advanced carotid atherosclerosis (Thies F *et al*., 2003).

Evidence from laboratory investigations, observational studies, and clinical trials indicates that supplementation of diet with high doses of n-3 PUFA can reduce blood pressure (Appel LJ *et al*., 1993). However, large quantities (3 g/day) are needed to see a minimal effect in non-hypertensive individuals and only very modest effects in hypertensive individuals. The most effective n-3 PUFA is DHA rather than EPA.

Diabetes

Cell membrane fluidity is determined by its lipid composition. Increased incorporation of SFA and cholesterol into the cell membrane phospholipids will render the membrane more rigid. In contrast, increased incorporation of unsaturated FA into membrane phospholipids will make it more fluid. Studies suggested that the number of receptors and their affinity to their respective hormones, growth factors or proteins depends on the fluidity of the cell membrane. For instance, increase in the rigidity of cell membrane reduces the number of insulin receptors and their affinity to insulin. This, in turn, causes insulin resistance. On the other hand, increase in cell membrane fluidity due to increase in the unsaturated FA content in membrane phospholipids increases the number of insulin receptors on the membrane and their affinity to insulin and thus, decrease in insulin resistance (Das UN, 1994; 2005; Coetzer H *et al*., 1994). This has important therapeutic implications in diabetes mellitus.

Type 2 diabetes is a multigenic, multifactorial disorder, characterized by hyperglycemia in the presence of insulin resistance, hypertriglyceridemia and the development of vascular complications. In 1993, Borkman *et al*. (1993) showed that hyperinsulinemia and insulin resistance are inversely associated with the amount of 20- and 22-carbon FA in muscle cell membrane phospholipids in patients with coronary heart disease and in normal volunteers. Such decreases in 20- and 22-carbon FA concentrations could occur from many reasons: low dietary intake of 20- and 22-carbon FA; high dietary intake of *trans* FA, which interfere with the desaturation and elongation of LA and ALA and thus lower AA, EPA, and DHA concentrations; genetic defects of Δ^5 and Δ^6 desaturase; genetic defects that interfere with the transport or binding of 20- and 22-carbon FA, such as intestinal FA binding protein; high dietary intake of LA, which leads to decreased production of AA and interferes with the desaturation and elongation of ALA to EPA and DHA; increased catabolism of AA, which reduces the number of available 20- and 22-carbon fatty acids; an increase in 20- and 22-carbon PUFA (ie, AA, EPA, and DHA), leads to increases in membrane fluidity; the number of insulin receptors and insulin action (Simopoulos AP, 1994; 1996; Yam D *et al*., 1996). The largest and longest reported placebo-controlled trial of the effect of n-3 FA on type 2 diabetes showed convincingly that n-3 FA intake, along with oral therapy for diabetes, can lower triacylglycerol concentrations, with no adverse effects on glycemic control (Connor WE *et al*., 1993). It is also known that the concentration of serum leptin (a hormone expressed and secreted in proportion to adipose mass) in patients with type 1 diabetes mellitus is influenced by the type of fat in the diet (Rojo-Martinez G *et al*., 2000). In particular it has been found that n-3 FA decreased leptin gene expression both *in vivo* and *in vitro*. The direct effects of PUFA on leptin promoter activity indicate a specific regulatory action of FA on leptin expression (Reseland JE *et al*., 2001).

Cancer

Although the role of individual FA in human cancer risk has hitherto been poorly investigated, some recent epidemiological and experimental data linked a high dietary intake of n-6 PUFA, especially in association with a low intake of n-3 PUFA, to increased risk for cancer of the breast, colon, and possibly prostate. n-6 PUFA enhance tumorigenesis and metastasis in experimental animals by several mechanisms, whereas n-3 PUFA can inhibit the growth of initiated cancer cells. Fat may regulate cellular functions by affecting the expression or activity of genes in the signal transduction pathway related to the control of cell growth and apoptosis. High intake of n-6 PUFA experimentally induces various physiological and metabolic effects (Rose DP, 1997; Hilakivi-Clarke L *et al*., 1998): increased ornithine decarboxylase activity in colon mucosa, resulting in enhanced epithelial polyamine levels and increased colon crypt cell proliferation; enhanced activities of protein kinases (i.e., protein kinase C) in rodent mammary gland and the increased number of estrogen receptor binding sites; increased prostaglandin concentrations; prostaglandins, thromboxanes, leukotrienes and hydroxy and hydroxyperoxy FA are involved in tumor initiation and promotion, cell proliferation, tissue invasion and metastatic spread. Tumor cells produce larger amounts of eicosanoids than their normal cell counterparts and eicosanoids ultimately derived from LA have been linked to increased growth and metastasis. The finding that OA and n-3 PUFA, specifically EPA, block the desaturase reaction, the first step from LA to eicosanoids, may partially explain their inhibitory effects on tumorigenesis. Dietary fats, specifically n-6 and n-3 PUFA, affect a variety of steps in the multistage carcinogenesis process, adding further weight to a causal effect. The effects may be direct or indirect and include (Jiang WG *et al*., 1998): peroxidation of conjugated double bonds in PUFA, leading to persistent oxidative stress and generation of reactive lipid peroxidation products (malondialdehyde, 4-hydroxyalkenals), which can induce DNA damage; conversion of essential FA to eicosanoids, short-lived hormone-like lipids derived primarily from dietary LA; interaction of FA with signal transduction pathways leading to altered gene expression; in the case of breast cancer, effects on unbound estrogenic hormone concentrations; effects on membrane (lipid)-bound enzymes such as cytochrome P450 that regulate xenobiotic and estrogen metabolism; structural and functional changes in cell membranes resulting in alterations in hormone and growth factor receptors. In initiated or preneoplastic cells, PLA_2, COX-2 and LOX are often constitutively overexpressed. This leads to increased release of AA and faster AA oxygenation, resulting in higher levels of n-6 eicosanoids, accompanied by generation of reactive oxygen species. These can cause DNA damage and trigger lipid peroxidation of PUFA in a self-perpetuating process, leading to various forms of exocyclic DNA base and protein modifications. In rapidly

dividing cells, the resulting genetic changes and disrupted signaling pathways may drive premalignant cells to genetic instability and malignancy.

Inflammation

It is evident from the preceding discussion that PUFA and their products participate in the pathobiology of inflammation. The amount and type of PUFA released in response to inflammatory stimuli depends on the cell membrane phospholipid FA content. Since the EFA LA and ALA have to be obtained direct from diet, this suggests that dietary content EFA could be one factor that determines the degree of inflammation. Furthermore, direct intake of various PUFA alters the cell membrane FA composition, which, in turn, modulates cell/tissue response to infection, injury and inflammatory events. Eicosanoids derived from AA and EPA have very similar molecular structures but markedly different biologic effects. As seen before, the EPA-derived eicosanoids are in general much less potent inducers of inflammation than the AA-derived eicosanoids (Shapiro AC *et al*., 1993). Consequently, a predominance of n-6 FA will result in a proinflammatory status with production of prostaglandins of the 2 series and leukotrienes of the 4 series. As the relative amount of n-3 FA increases, more prostaglandins of the 3 series and leukotrienes of the 5 series are produced. Increased dietary intake of GLA, DGLA, and EPA/DHA substantially decreases inflammatory response (Dooper MM *et al*., 2003; Trebble T *et al*., 2003; Chen W *et al*., 2005; Li H *et al*., 2005). For instance, GLA is rapidly elongated to form DGLA, which, in turn, is converted to PGE_1 and its 15-lipoxygenase product 15-hydroxy-8, 11, 13-eicosatrienoic acid (15-OH-20:3,n-6), which have anti-inflammatory actions (Miller CC *et al*., 1988; Tate G *et al*., 1989; Heitmann J *et al*., 1995) . GLA, DGLA, EPA and DHA possess anti-inflammatory actions that can be attributed to decreased formation of pro-inflammatory eicosanoids and cytokines (Lo CJ *et al*., 1999), and an increase in the production of beneficial eicosanoids such as PGE_1, PGI_2, PGI_3, HPETEs, eNO, LXs, resolvins and NPD1 and early resolution of inflammation. It is likely that when the cell membrane lipid pool is rich in GLA/DGLA/EPA/DHA and contains appropriate amounts of AA, a specific activation of soluble and cytosolic PLA_2 could occur in response to an inflammatory stimuli that leads to the formation of increased amounts of LXs, PGD_2 and 15deoxy$\Delta^{12–14}$ PGJ_2, eNO, GSNO, PGE_1, PGI_2, PGI_3, and HPETEs that dampen inflammatory process and enhance resolution of inflammation. They act both directly, by replacing AA as an eicosanoid substrate, by inhibiting AA metabolism, by giving rise to anti-inflammatory resolvins, and indirectly, by altering the expression of inflammatory genes through effects on transcription factor activation (Figure 23). Thus, long chain PUFA are potentially useful anti-inflammatory agents and may be of benefit in patients at risk of in a variety of acute and chronic inflammatory settings.

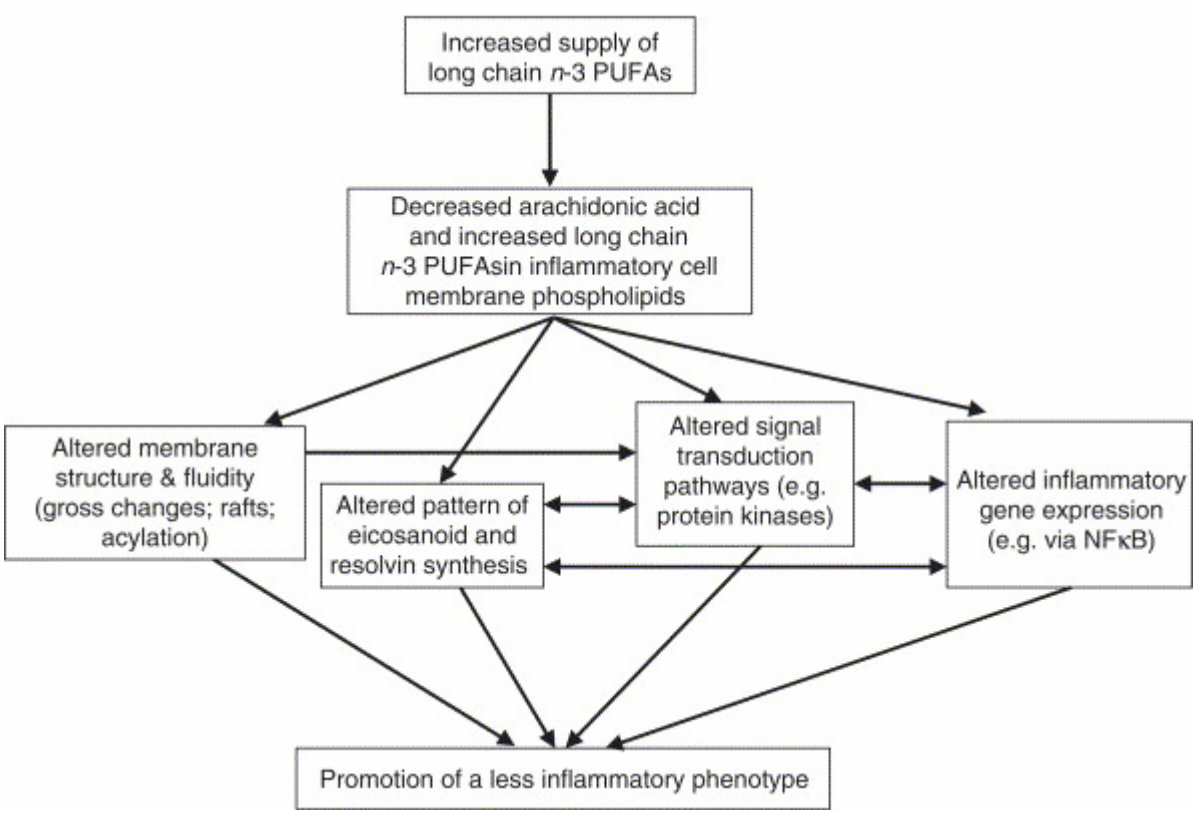


Figure 23. Representation of the cellular mechanisms by which long chain n-3 PUFA result in decreased inflammation. (Calder PC, 2006).

Autoimmune diseases

Among the FA, it is the n-3 PUFA which possess the most potent immunomodulatory activities, and among the omega-3 PUFA, EPA and DHA are more biologically potent than ALA. Some of the effects of n-3 PUFA are brought about by modulation of the amount and types of eicosanoids made, and other effects are elicited by eicosanoid-independent mechanisms, including actions upon intracellular signaling pathways, transcription factor activity and gene expression (Figure 24). Animal experiments and clinical intervention studies indicate that n-3 FA have anti-inflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases. Arthritis, Crohn's disease, ulcerative colitis and lupus erythematosis are autoimmune diseases characterized by a high level of IL-1 and the proinflammatory leukotriene LTB_4 produced by n-6 FA. There have been a number of clinical trials assessing the benefits of dietary supplementation with fish oils in several inflammatory and autoimmune diseases in humans, including rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus erythematosus, multiple sclerosis and migraine headaches. Many of the placebo controlled trials of fish oil in chronic inflammatory diseases reveal significant benefit, including decreased disease activity and a lowered use of anti-inflammatory drugs (Calder PC, 2007).

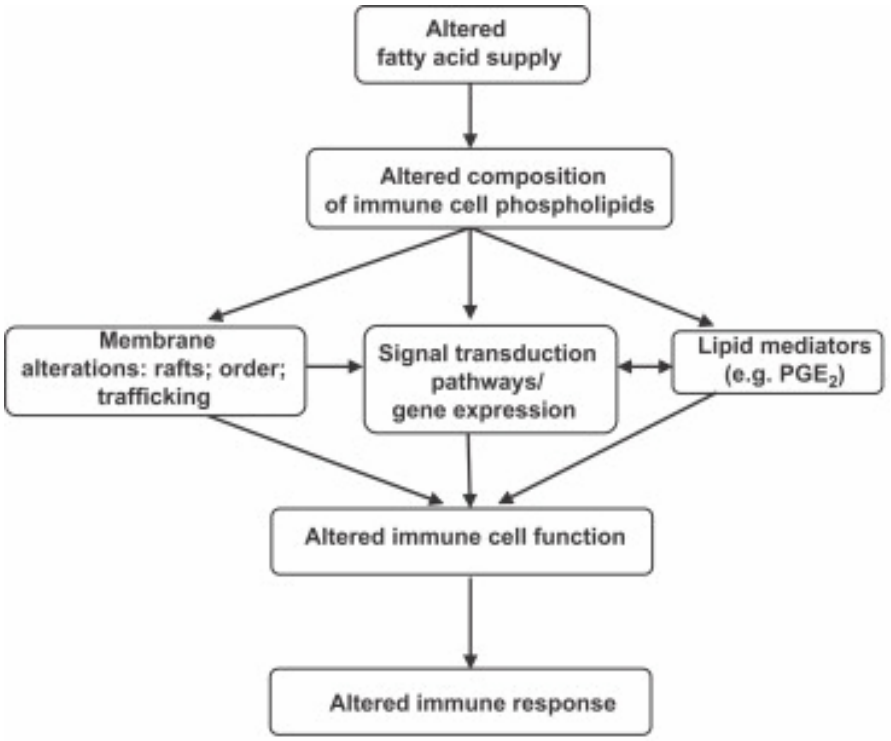


Figure 24. Representation of the mechanisms by which an altered supply of fatty acids could affect immune responses. (Calder PC, 2007).

Supplementation with n-3 FA can modulate the expression and activity of degradative and inflammatory factors that cause cartilage destruction during arthritis. *In vitro* study on bovine articular cartilage demonstrated that incorporation of n-3 FA into chondrocyte membranes results in a dose-dependent reduction in the expression and activity of proteoglycan degrading enzymes, and in the expression of inflammation-inducible cytokines (IL-1α and TNF- α) and COX-2, but not the constitutively expressed COX-1 (Curtis CL *et al*., 2000). These findings provide evidence that n-3 FA supplementation can specifically affect regulatory mechanisms involved in chondrocyte gene transcription and thus further advocate a beneficial role for dietary fish oil supplementation in alleviation of several of the physiological parameters that cause and propagate arthritic disease.

Altered AA metabolism plays a major role in the pathogenesis of cutaneous scaly disorders. Abnormally high levels of AA and its lipooxygenase products LTB_4 and 12-hydroxyeicosatetraenoic acid (12-HETE) are described in the lesions (plaques) of patients with psoriasis. Intravenous n-3 FA administration causes reduction of psoriasis, which may be related to changes in inflammatory eicosanoid generation (Mayser P *et al*., 2002).

LTB_4 and PGE_2, both products of AA metabolism, are increased in patients with ulcerative colitis. In ulcerative colitis LTB_4 is an important mediator of inflammation and has the ability to recruit additional neutrophils from the blood stream into the mucosa, exacerbating the disease process by

further increases of LTB_4. Four months of diet supplementation with fish oil in patients with ulcerative colitis resulted in reductions in rectal dialysate LTB_4 levels, improvements in histologic findings, weight gain and a reduction in the dose of prednisone administered (Stenson WF *et al*., 1992).

Neurodegenerative disorders

PUFA account for 21%–36% of the FA in the cell membrane, but the proportions of FA with 20 or 22 carbon atoms varies considerably between tissues. The nervous system is the organ with the second largest concentration of lipids, only exceeded by adipose tissue. The adult brain contains approximately 50%–60% of its dry weight as lipid and approximately 35% of the lipids are PUFA (Yehuda S *et al*., 1999), most of which are long-chain PUFA (EPA and DHA). Neuronal tissues, such as the brain, retina, and synaptic membranes are especially high in DHA (Sun GY and Sun AY, 1974). In animal models, this relative distribution gives some indication of a possible important role in the membranes of these tissues: the high concentration of DHA in synaptic membranes correlates with development of the synapses (Martin RE and Bazan NG, 1992); chronic AA deficient rats had altered dopaminergic transmission in the frontal cortex (Delion S *et al*., 1994); n-3 PUFA in neuronal membranes affect the activity of ion pumps and channels (Neuringer A *et al*., 1988); the precise FA composition of the membrane can affect the tertiary and quaternary structures of membrane-bound receptors such as cholinergic, adrenergic, dopaminergic and N-methyl-D-aspartate (NMDA) and associated neurotransmitter functioning (L'Hirondel M et al., 1995; Matsuo T *et al*., 1995; Methot N *et al*., 1995); deficiencies of EFA have been associated in animal models with disruption of neural integrity and function (Neuringer A *et al*., 1988; Yamamoto N *et al*., 1987), visual and cognitive deficit (Neuringer M *et al*., 1994). The presence of these FA as structural components of neuronal membranes influences cellular function both directly, through effects on membrane properties, and also by acting as a precursor pool for lipid-derived messengers. An adequate intake of n-3 PUFA is essential for optimal visual function and neural development. Furthermore, there is increasing evidence that increased intake of EPA and DHA may confer benefits in a variety of psychiatric and neurological disorders, and in particular neurodegenerative conditions. There is evidence to suggest that inflammation plays a significant role in the pathobiology of schizophrenia, Huntington's disease and Alzheimer's disease. In patients with schizophrenia, both circulating and cerebrospinal fluid (CSF) concentrations of pro-inflammatory cytokines are increased. The plasma phospholipid concentrations of EPA and DHA are decreased. Limited clinical trials showed that supplementation of EPA (especially ethyl EPA) are of significant benefit to these patients (Das UN and Vaddadi KS, 2004). Recent studies revealed

that a diet high in DHA slowed the progression of Alzheimer's disease in mice. Specifically, DHA reduced the harmful brain plaques that mark the disease. DHA protected against damage to the "synaptic" areas and enabled mice to perform better on memory tests (Calon F *et al*., 2004; Lukiw WJ *et al*., 2005; Calon F et al., 2005; Akbar M *et al*., 2005; Hashimoto M *et al*., 2005). These studies suggest that people who are genetically or otherwise predisposed to the disease may be able to delay it by increasing their DHA intake (Calon F et al., 2005; Mukherjee PK *et al*., 2004). Huntington's disease is an inherited neurodegenerative disorder due to a mutation in exon 1 of the *Huntingtin* gene that encodes a stretch of polyglutamine (polyQ) residues close to the N terminus of the Huntingtin protein. Aggregated polyQ residues are toxic to the neuronal cells. Transgenic R6/1 mice develop late-onset neurological deficits in a manner similar to the motor abnormalities of Huntington's disease seen in humans. These animals, when supplemented with PUFA, especially ethyl EPA, showed increased survival rates and decreased neurological deficits (Das UN and Vaddadi KS, 2004), suggesting that unsaturated FA may prevent or arrest polyQ aggregation. Based on these results, it is tempting to suggest that PUFA, in general, are useful in the treatment of various neurological diseases. It remains to be determined as to why and how a particular FA is useful only in a particular neurological condition but not in other conditions. For instance, DHA is useful in Alzheimer's disease, whereas ethyl EPA is of benefit in Huntington's disease and schizophrenia. Understanding the molecular mechanisms of action of EPA and DHA in these conditions may throw more light on the pathobiology of these diseases. Recent evidence also indicates that in addition to the positive effects seen in chronic neurodegenerative conditions, n-3 PUFA may also have significant neuroprotective potential in acute neurological injury. Thus, these compounds offer an intriguing prospect as potentially new therapeutic approaches in both chronic and acute conditions.

Bone growth, repair and osteoporosis

Bone is a multifunctional organ that consists of a structural framework of mineralized matrix and contains heterogeneous populations of chondrocytes, osteoblasts, osteocytes, osteoclasts, endothelial cells, monocytes, macrophages, lymphocytes and hematopoietic cells. Bone growth is regulated by complex interactions between an individual's genetic potential, environmental influences and nutrition. Evidences suggests that the high intake of n-6 and inadequate amount of n-3 FA in the diet contribute to the development of several pathologies, including those of the skeletal system (bone/joint diseases) (Watkins BA, 1998). Regarding the pathogenesis of osteoporosis and the interaction between various cells and mediators, many studies have been conducted to evaluate the benefit of different therapeutic options. Over the past several years evidence has been growing

on the effects of dietary FA on bone health. Bone mineral density and bone markers have been used in several animal studies to evaluate the beneficial effect of n-3 FA on bone health and the prevention of osteoporosis, with growing evidence on the benefits of dietary fats in bone health. A wide range of mechanisms may mediate the effects of dietary fats on bone, including alterations in calcium absorption and urinary calcium loss, prostaglandin synthesis, osteoblast formation, and lipid oxidation. There is increasing evidence of a lack of EFA in diet and its contribution to bone loss (Watkins BA *et al*., 2001). The modulatory effect of EFA on cytokines plays a pivotal role in the pathogenesis of osteoporosis (Das UN, 2000; Horowitz MC, 1993; Votta BJ and Bertolini DR,1994). Generally, animal studies support the beneficial effects of n-3 FA on bone health and osteoporosis; however, the dissimilar lipid metabolism in human and animals, the various study designs, and controversies over the human study outcomes make it difficult to draw a definite conclusion. Conclusive findings in humans are still lacking in this area and it needs to be further investigated.

Pregnancy and fetal development

PUFA are vitally important structural elements of cell membranes and, therefore, essential for the formation of new tissues, as occurs during pregnancy and fetal development. The central nervous system is particularly rich in AA and DHA and the cerebral accretion of these FA may have been decisive in the evolution of Homo sapiens (Broadhurst CL *et al*., 1998). The brain has its growth spurt in the third trimester of pregnancy and during early childhood. Therefore, an appropriate pre- and post-natal supply of PUFA is thought to be essential for normal fetal and neonatal growth (Innis SM, 1991), neurologic development and function, activity of retinal photoreceptors (Uauy R *et al*., 1996), and learning and behavior (Stevens LJ *et al*., 1995). Maternal concentrations of PUFA, especially DHA, are associated with sleep and wake states of newborns. Sleep and wake rhythm provides a tool for assessing the functional integrity of the central nervous system. It has been demonstrated that higher maternal plasma DHA during pregnancy is associated with more mature neonatal sleep-state patterning (Cheruku SR *et al*., 2002). In addition, intrauterine nutrition may influence the adult risk for chronic diseases (Barker DJP, 1994), suggesting that early nutrition has an imprinting effect on later life. This further emphasizes the importance of an adequate supply of essential PUFA during pregnancy, lactation, and infancy. However, prenatal and early postnatal DHA status is thought to have important consequences on the growth and function of the central nervous system and, consequently, on neurologic and cognitive development. Therefore, incomplete replenishment of maternal DHA stores after delivery may, at least in part, explain the observation that first-born children generally do better than their younger siblings on several developmental,

behavioral, and intelligence tests (Lucas A *et al*., 1992; Rodgers B, 1978; Gale CR and Martyn CN, 1996; Emken EA *et al*., 1994).

Viral, bacterial and fungal infections

PUFA show anti-biotic-like actions (Sun CQ *et al*., 2003; Das UN, 2006; Giamarellos-Bourboulis EJ *et al*., 2004). For instance, LA rapidly killed cultures of *Staphylococcus aureus*, and hydrolyzed linseed oil (which contains both LA and ALA) can inactivate methicillin-resistant *S. aureus*. ALA promotes adhesion of *Lactobacillus casei* to mucosal surfaces and, thus, augments their growth. *Lactobacilli*, in turn, suppress the growth of pathogenic bacteria like *Helicobacter pylori, Shigella flexneri, Salmonella typhimurium, Pseudomonas aeroginosa, Clostridium difficile,* and *Escherichia coli*. PUFA inactivate enveloped viruses and show anti-fungal properties. The anti-inflammatory and anti-bacterial, anti-viral, and anti-fungal actions of PUFA may explain some of their beneficial actions. In this context, it will be interesting to study whether local application or intravenous infusions of PUFA would help patients with various bacterial, viral and fungal infections to recover faster. Since neutrophils, T cells and macrophages release PUFA on stimulation, it is possible that this could be one of the defense mechanisms of the body to fight infections.

2.2.5 Evolution of FA biosynthetic machinery and of the pre- and post-biosynthetic homeostasis control

The appearance of FA was noted very early in our planetary history. It is hypothesized that life first emerged as a primitive, single cell organism termed the universal progenote from a series of prebiotic chemical reactions, postulated to have begun before 3.8 billion years ago (Figure 25) (Mojzsis *et al*., 1996). Chemical fossils, i.e. hydrocarbon condensates, FA, porphyrins, amino acids and perhaps trace amounts of carbohydrate assemblages, also attest to the timing of this ancient biogenesis. Han J and Calvin M (1969) found aliphatic hydrocarbons and long-chain FA, particularly palmitate, in sedimentary rocks dated at 3.4–3.7 billion years old. These were speculated to be the geochemical remains of early primitive microbes.

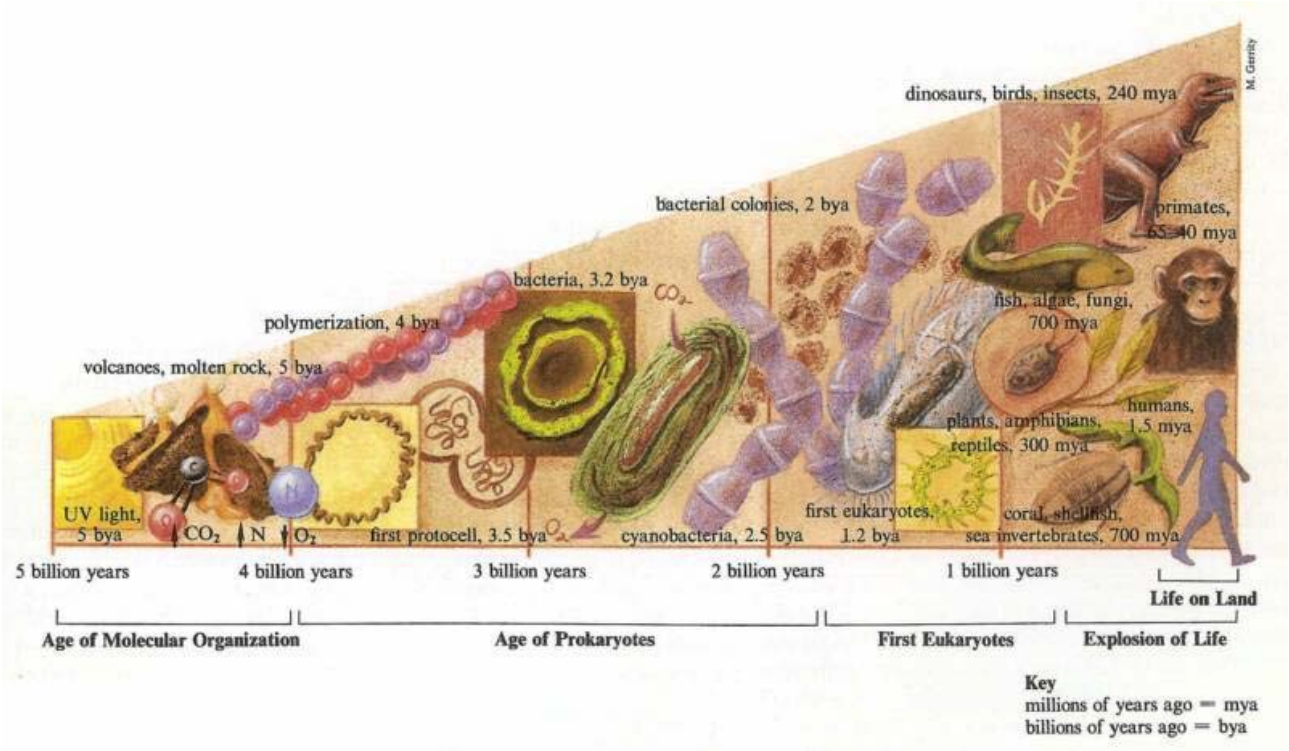


Figure 25. The evolution of life.

The role of FA in ancestral prokaryotes was presumably not initially directed toward energy metabolism, since the early planet was devoid of oxygen and, thus, the micro-organisms could not have participated in oxidative phosphorylation. Rather, they were anaerobic fermenters of short-chain carbon units, principally sugars. FA were used in the formation of critical membrane partitions to maintain cellular integrity against harsh environmental constraints. This was crucial to

the evolution of life on earth, as it resulted in the ability of cells and subcellular organelles to form and maintain their internal environment, which may differ to a large extent from the external media in which the cells and subcellular organelles are functioning (Deamer DW *et al*., 1996; Barenholz Y and Cevc G, 2000). Synthesis of long chain FA is a remarkable evolutionary achievement. On one hand, the formation of C–C-bonds is a highly energy-demanding process. Secondly, in the context of a rather hydrophilic cellular environment, the synthesis of hydrophobic hydrocarbon chains poses some critical biophysical constraints on the enzymology involved. FA are essential molecules in a cell, but may exert potentially harmful properties as membrane- and protein-perturbing detergents. Although different organisms use the same biosynthetic reaction sequence for the biosynthesis of FA, the structural organization of the enzyme activities is entirely different. The type I FA synthases (FAS I) in the cytoplasm of yeast, fungi, and animals, but also in certain bacteria like corynebacteria and mycobacteria, are megasynthases in which the enzymatic activities are confined to only one or two polypeptide chains. In prokaryotes, plants, but also in mitochondria of animal cells, the different reactions leading to FA formation are catalyzed by independent enzymes that belong to the so-called type II fatty acid synthase system (FAS II)(White SW *et al*., 2005). Branched-chain FA synthetase in bacteria also belongs to this group. Also the formation of very long chain FA by elongation of medium-chain-length precursors is catalyzed by individual enzymes that are localized in the endoplasmic reticulum, mitochondria, and peroxisomes. The overall reaction of FA synthesis is strikingly uniform throughout the systems of animals, plants, and microorganisms and was already discussed previosly. Instead, for the insertion of double bonds in the aliphatic chains there are two major mechanisms by which living organisms synthesize PUFA: mostly of them use an oxygen-dependent FA desaturation pathway, whereas many prokaryotes, including *E. coli*, synthesize PUFA anaerobically (Mansilla *et al*., 2004). The fatty acyl desaturases, which introduce double bonds into fatty acyl chains, encompass a family of enzymes, representatives of which are found in all eukaryotes (Tocher *et al*., 1998; Pereira et al., 2003; Sperling *et al*., 2003), as well as some prokaryotes such as cyanobacteria, bacilli, mycobacteria and pseudomonas (Mansilla and de Mendoza, 2005). The reaction catalysed by these enzymes is an oxygen-dependent desaturation of the full-length FA chain, either as an acyl-thioester or as a phospholipid FA moiety, and requires a specific electron transport chain.

Through 3–3.5 billion years of evolution, biological membranes maintained this feature of being a lipid bilayer, which proteins are embedded in, connected to, or associated with (Barenholz Y and Cevc G, 2000). However, lipid composition of the biological membranes changed dramatically from membranes of archaebacteria, in which the membrane is composed of one lamella of molecules, each having two headgroups and two very long hydrocarbon chains, through eubacteria,

in which the matrix of the membrane is already a lipid bilayer as we know it today. While in eubacteria the number of lipid species is small and versatility is poor, in membranes of mammalian cells, which developed much later in the evolution ladder, it includes more than 2000 species of lipid molecules, including sphingolipids and sterols (Barenholz Y and Cevc G, 2000). The appearance of sterols in biological membranes is, without doubt, one of the important steps in membrane evolution. From bacterial hopanoids to mammalia membrane-active sterols, evolution developed a wide range of these related-structure molecules. The effect of membrane-active sterols on controlling membrane permeability by reducing average fluidity and free volume is well established. Recently it became clear that cholesterol also has a key role in the lateral organization of membranes and free volume distribution. The latter two parameters seem to be involved in controlling membrane protein activity and raft formation. Such an effect allows for the fine tuning of membrane lipid composition, organization/dynamics, and function. It is clear today that large changes in environment (or medium) in which cells live, and the formation of multicellular organisms in which cells face more than a single environment at one time, as well as the changes in environment during the lifetime of cells, led to their ability to modify the lipid composition of membranes by one or more mechanisms in order to survive and/or optimize their interaction with the environment. This led to the development of such a huge repertoire of membrane lipids which differ in their headgroup and hydrocarbon chains (Barenholz Y and Cevc G, 2000). The briefly overview of next paragraphs mainly focus on how FA are exploited through the multiple living beings of the earth, bearing in mind that the animal reign, to which the human race belongs, has already been discussed in the previous part.

ARCHAEABACTERIA

The archaea, divided into five broad physiological (halophiles, thermophiles and acidophiles) or metabolic (nitrifiers and methanogens) groups, remain the most enigmatic of life's three domains. The representatives of this domain were first distinguished from bacteria based on differences in tRNA and rRNA, the cytoplasmic membrane and the cell-wall composition, together with their restriction to unusual habitats (Woese CR *et al*., 1978). Archaeal cells have an outer cell membrane that serves as a barrier between the cell and its environment. Within the membrane is the cytoplasm, where the living functions of the archeon take place and where the plasmid DNA is located. Around the outside of nearly all archaeal cells is the cell wall, a semi-rigid layer that helps the cell maintain its shape and chemical equilibrium. All three of these regions may be distinguished in the cells of eubacteria and eukaryotes, but similarities are merely structural, not chemical.

The general capacity of archaea to dominate or outcompete bacteria under conditions of chronic energy stress is the crucial factor that distinguishes them, and the primary biochemical basis for this adaptation is membrane composition, with numerous secondary adaptations among the metabolic pathways (Reviewed by Valentine DL, 2007). Archaeal membranes are less permeable to ions than bacterial membranes; their membranes reduce the amount of futile ion cycling *in vivo* and provide an energetic advantage to them, with less energy lost during the maintenance of a chemiosmotic potential. The advantages of the archaeal membrane have been clearly shown in hyperthermophiles, halophiles, acidophiles and liposomes (Van de Vossenberg JL et al., 1998; Van de Vossenberg JL et al., 1995; Macalady JL et al., 2004; Mattai JC et al., 2001; Van de Vossenberg JL et al., 1999). Archaeal membranes are made of molecules that differ strongly from those in other forms of life, from which is evidence that archaea are related only distantly to bacteria and eukaryotes (Hulbert AJ and Else PL, 2005). Membrane proteins are located within the lipid matrix to support its structural stability and enhance surface adhesion (Baumeister and Lembcke, 1992). There are many fundamental differences between the archaeal membrane and those of all other cells (Figure 26): (1) ether linkage, (2) chirality of glycerol and (3) isoprenoid chains and branching of side chains.

- The first important distinction between the Archaea and the Eubacteria is the chemical structure of lipids composing the cytoplasmic membrane. Bacterial lipids typically consist of FA esterified to a glycerol moiety, whereas archaeal lipids typically consist of isoprenoidal alcohols that are ether-linked to glycerol. Ether bonds are more chemically-resistant then ester bonds, which might contribute to the ability of some archaea to survive at extremes of temperature and in very acidic or alkaline environments (Mattai JC *et al*., 2001). eubacteria

and eukaryotes do contain some ether lipids, but in contrast to archaea these lipids are not a major part of their membranes.

- Archaeal lipids are unique because the stereochemistry of the glycerol group is the reverse of that found in other organisms. This suggests that archaea use entirely different enzymes for synthesizing their phospholipids than bacteria and eukaryotes do; since such enzymes developed very early in life's history, this suggests that the archaea split off very early from the other two domains (Hulbert AJ and Else PL, 2005).
- The lipid tails of archaeal phospholipids are chemically different from those in other organisms. Archaea do not use FA to build their membrane phospholipids. Instead, they have side chains of 20 carbon atoms built from isoprene. Isoprene is the simplest member of a class of chemicals called terpenes. By definition, a terpene is any molecule built by connecting isoprene molecules together. Archaeal lipids are based upon the isoprenoid sidechain and are long chains with multiple side-branches and sometimes even cyclopropane or cyclohexane rings (Van de Vossenberg JL *et al*., 1998). Although isoprenoids play an important role in the biochemistry of many organisms, only the archaea use them to make phospholipids. These branched chains may help prevent archaean membranes from becoming leaky at high temperatures (Van de Vossenberg JL *et al*., 1999). Not only are the side chains of achaeal membranes built from different components, but the chains themselves have a different physical structure. Because isoprene is used to build the side chains, there are side branches off the main chain. The major part of eubacteria species and all the eukaryote ones do not have these types of side branches, and this creates some interesting properties in archaeal membranes. For example, the isoprene side chains can be joined together. This can mean that the two side chains of a single phospholipid can join together, or they can be joined to side chains of another phospholipid on the other side of the membrane. This fusion may make their membranes more rigid and better able to resist harsh environments (Jarrell HC *et al*., 1998). No other group of organisms can form such transmembrane phospholipids. Another interesting property of the side branches is their ability to form carbon rings. This happens when one of the side branches curls around and bonds with another atom down the chain to make a ring of five carbon atoms. Such rings are thought to provide structural stability to the membrane, since they seem to be more common among species that live at high temperatures. They may work in the same way that cholesterol does in eukaryotic cells to stabilize membranes. It's interesting to note that cholesterol is another terpene.

- Small amounts of FA have been detected in some archea, and they are actively synthesized using the enzyme fatty acid synthase (Pugh EL and Kates M, 1994). However, their principal function seems to be the acylation of membrane proteins, with the principal FA produced being PA and SA with lesser amounts of myristic and OA (Pugh EL and Kates M, 1994).

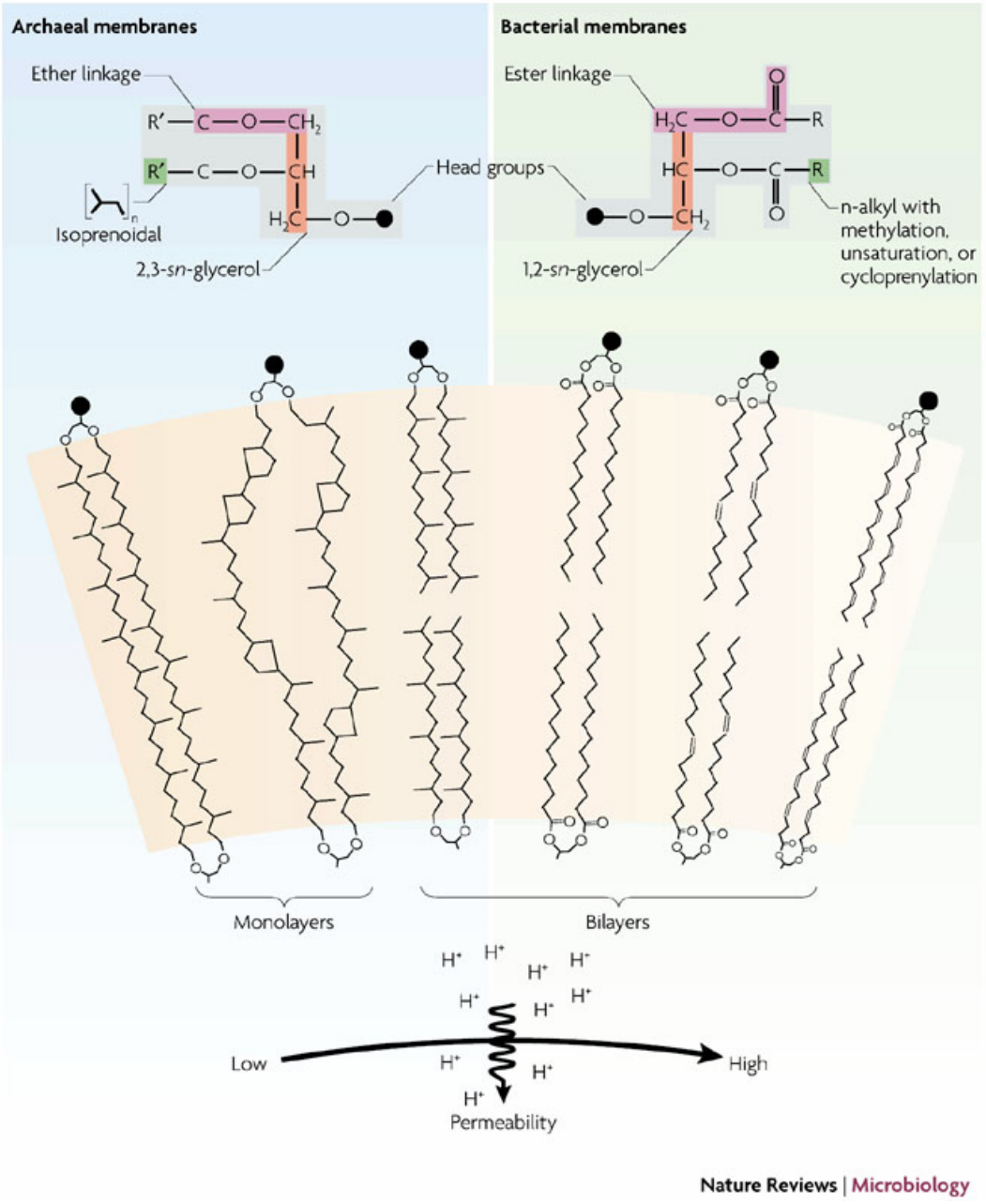


Figure 26. The basic chemical structures of archaeal (left) and bacterial (right) membrane lipids are shown to illustrate the typical chemical differences. Examples of intact membrane structures used by archaea (left) and bacteria (right) are shown below, including the monolayers that are produced by some archaea and the highly unsaturated membranes produced by some bacteria. The arrow (bottom) indicates a general trend of increasing permeability to ions such as protons and sodium. (Valentine DL, 2007).

If archaeal membranes provide a more effective barrier against ions, why do eukaryotes have membranes that are most similar to bacterial membranes? One explanation is that low-permeability membranes have lower rates of lateral diffusion (Jarrell HC *et al*., 1998; Baba T *et al*., 2001), a factor which is thought to be important for photosynthesis, respiration and signal transduction (Valentine RC and Valentine DL, 2004). The use of more laterally mobile membranes might be an adaptation to enhanced rates of energy production at the expense of energy conservation, with highly unsaturated membranes being an extreme case (Valentine RC and Valentine DL, 2004). Chemical modification of the membrane, such as the inclusion of sterols, might be a dynamic approach to control membrane permeability while maintaining advantages afforded by the bacterial membranes (Haines TH, 2001).

EUBACTERIA

Bacterial survival depends on membrane lipid homeostasis and on the ability to adjust lipid composition to acclimatize the bacterial cell to different environments. Bacterial membranes consist of proteins that are embedded in a lipid matrix that closely approximates a phospholipid bilayer. Although there is a considerable diversity of phospholipid structures in the bacterial world, most membrane phospholipids are glycerolipids that contain two FA chains. These phospholipid acyl chains determine the viscosity of the membrane, which in turn influences many crucial membrane associated functions, such as the passive permeability of hydrophobic molecules, the active solute transport and the protein–protein interactions. The adjustments in FA composition that maintain the biophysical properties of membranes are referred to as homoviscous adaptation, and are interpreted as a mechanism that modifies the permeability of the phospholipid bilayer to minimize energy expenditure and optimize growth. The ability of eubacteria to modify their membrane composition in response to environmental changes, such as in temperature, osmolarity, salinity and pH, is determined by the biophysical properties of the membrane, defined in large part by the FA structures that are incorporated into the component phospholipids. Phospholipid FA are present in various chain lengths of between 14 and 20 carbons, and many contain a single *cis* double bond, or *iso* or *anteiso* methyl branches (Figure 27).

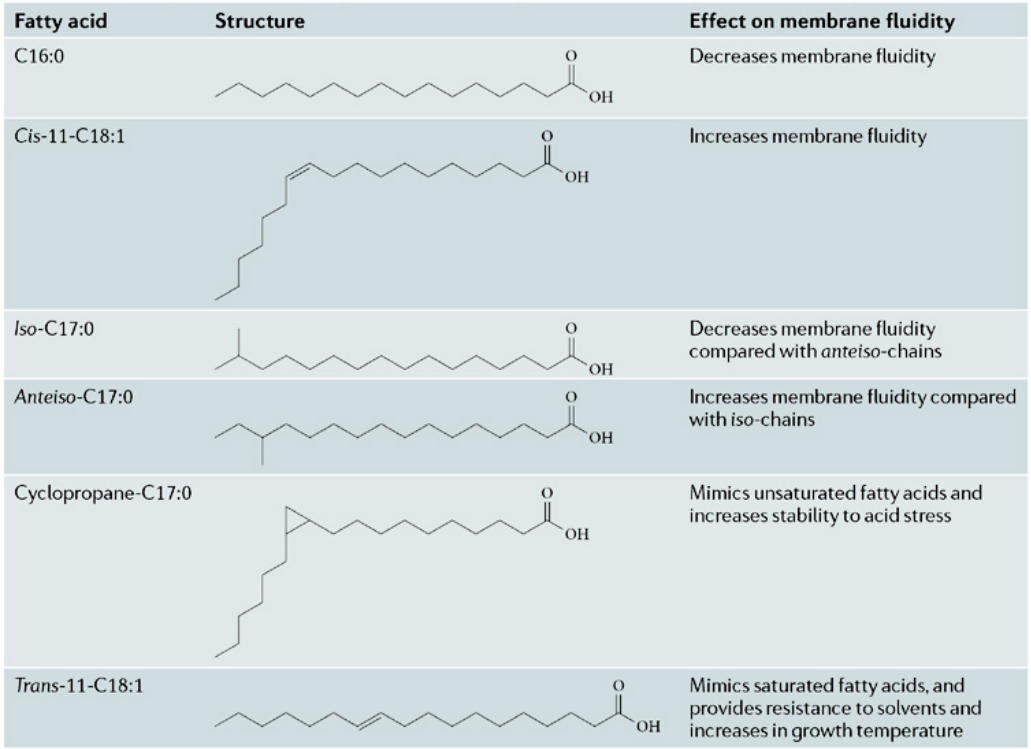


Figure 27. Chemical structures of eubacteria FA diversity. (Zhang YM and Rock CO, 2008).

Eubacteria have evolved mechanisms to control the formation of new FA and modify the structure of existing ones, and these allow them to adjust membrane viscosity to match environmental requirements. The *de-novo* FA biosynthetic pathway is a major focal point for the regulatory events that control membrane homeostasis. However, eubacteria are often subjected to abrupt changes in their extracellular environment that necessitate the modification of existing FA to rapidly adapt to the new conditions. These membrane alterations are important not only for free-living eubacteria, but also for pathogens that must also adapt to the host environment.

The FA biosynthetic machinery

FA synthesis in eubacteria is achieved by a highly conserved set of genes in which each gene encodes an individual step in the type II FA biosynthetic pathway. All of the proteins in this pathway are located in the cytosol, and each has been purified and biochemically characterized. Furthermore, the structure and the catalytic mechanism of each enzyme is known in considerable detail (White SW *et al*., 2005). Similar dissociated FA synthase II (FASII) pathways are found in chloroplasts, mitochondria and apicoplasts. The central player in the FASII pathway is the acyl carrier protein (ACP), a low-molecular-mass protein that carries all of the FASII pathway intermediates. The FA chains are attached as thioesters to the terminus of the prosthetic group of ACP, which is covalently attached to a serine. An acyl chain is accommodated by a hydrophobic binding cavity that is formed by the interior surfaces of the four helices of the ACP and varies in size depending on the nature of the acyl chain (Roujeinikova A *et al*., 2007). The protein–ACP interface is highly conserved in many eubacteria. Protein–protein interactions in the FASII system have considerable plasticity, because mutations that compromise protein–ACP interactions *in vitro* do not always lead to deficiencies in FA synthesis *in vivo* (De Lay NR and Cronan JE, 2007).

Bacterial membrane FA can be divided into two major families on the basis of their biosynthetic relationships. One is the straight-chain FA family, which includes PA, SA, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic, and 2- or 3-hydroxyl FA. These FA occur most commonly in eubacteria. They are synthesized from acetyl coenzyme A (acetyl-CoA) as the primer and malonyl-CoA as the chain extender, followed, in some cases, by a modification of the FA products. The other is the branched-chain FA family, which includes *iso*, *anteiso*, and ω-alicyclic FA with or without a substitution (unsaturation and hydroxylation). The occurrence of these FA in eubacteria is not nearly as common as that of the straight-chain FA family, but is still very significant (Brennan PJ, 1988; Kaneda T, 1977; Lechevalier MP; 1977; O'Leary WM and Wilkinson SG, 1988; Wilkinson SG, 1988). These FA are synthesized in certain eubacteria from *iso*, *anteiso*, or cyclic primer and malonyl-CoA with or without a subsequent modification. The

clear difference between these two families of cell membranes exists in the mechanism that controls their fluidity. The fluidity of membranes composed of straight-chain FA is adjusted to the proper level by the inclusion of MUFA, whereas that of membranes with branched-chain FA is controlled mainly by 12- and 13-methyltetradecanoic acids. Thus, eubacteria with the straight-chain membrane system usually require unsaturated FA for growth, but these FA are nonessential for eubacteria with the branched-chain membrane system. Banched-chain FA in bacteria are synthesized by a mechanism very similar to that of straight-chain FA synthesis in *E. coli*, including the involvement of ACP in the synthetic reactions. The only difference between the two systems appears to be the substrate specificity of acyl-CoA:ACP transacylase (Kaneda T, 1991).

Controlling membrane homeostasis

Control at the level of FA biosynthesis is crucial for membrane homeostasis, because the biophysical properties of membranes are determined in large part by the composition of the FA that are produced by *de-novo* biosynthesis. There is no noteworthy alternative fate for FA in most eubacteria, and all of the long-chain acyl-ACPs that are produced by the biosynthetic pathway are incorporated into the membrane. Almost all of the metabolic energy that is used to produce membrane lipids is expended in the formation of FA, and therefore their production must be precisely controlled to support membrane biogenesis and prevent the wasteful expenditure of ATP. Although the biophysical properties of membranes can be changed by altering the ratio of the polar head groups in membrane phospholipids, eubacteria seem to use biochemical and genetic mechanisms to modify the composition of the FA that are produced by FASII. A feedback control of the total membrane lipid content and of the membrane phospholipid head-groups composition is achieved by the biochemical regulation of enzymes, and its discussion is bejond the scope of this thesis. The most intensively investigated and unifying theme in membrane phospholipid homeostasis research is the adjustments in the structure of the fatty acyl chains that are attached to glycerol backbones. The structures of these FA determine the biophysical properties of the membrane bilayer: the straightchain SFA are linear and pack together efficiently to produce a bilayer that has a high phase transition and low permeability properties, while the *cis*-UFA introduces a pronounced kink in the chain, which disrupts the order of the bilayer and results in lower transition temperatures and higher permeability. The composition of the branched-chain FA affects membrane fluidity owing to the disruptive effect of the methyl group on acyl-chain packing. The *anteiso* FA promote a more fluid membrane structure than the *iso* FA, because the methyl branch is further from the end of the FA. Eubacteria that produce these FA modify their *iso:anteiso* ratio in response to temperature and pH stress. These membrane alterations have been well studied

in *Listeria monocytogenes*, a pathogen that can survive at low temperatures (Zhu K *et al*., 2005; Giotis ES *et al*., 2007). Bacteria that can introduce double bonds into growing acyl chains can adjust membrane fluidity by regulating the production of cis-UFA (Cronan JE and Gelmann EP, 1975; Cronan JE and Rock CO, 1994). The proportion of cis-UFA is determined by competition for intermediates at a specific branch point in the pathway. The introduction of the double bond at the 10-carbon intermediate and the subsequent elongation steps give rise to the two major UFA that are found in eubacteria: POA and VA. There is a biochemical mechanism that allows the elongation cycle to respond to the environmental temperature; *E. Coli*, for example, at low temperatures, promotes the production of VA, whereas at higher temperatures the enzyme is inactivated and the formation of VA is reduced (Garwin JL *et al*., 1980; Ulrich AK *et al*., 1983).

Modifying existing phospholipids components

De-novo biosynthesis is finely tuned by environmental signals to produce acyl chains with the properties that are required to optimize membrane function. However, eubacteria are often subjected to abrupt changes, for example in temperature and organic-solvent concentration, that necessitate the immediate modification of existing membrane phospholipid acyl chains to optimize growth under the new conditions. This section describes some biochemical processes that alter the structure of the FA and the biophysical properties of membranes (Figure 28).

- **Phospholipid acyl desaturases.** When eubacteria are exposed to suboptimal growth temperatures, their membrane lipids become more rigid, leading to subnormal functioning of cellular activities (Phadtare S, 2004; Mansilla MC and De Mendoza D, 2005). Adaptation to such new conditions involves an increase in the proportion of UFA in their membrane. The resulting increase in UFA content causes membrane lipid fluidity to return to its original state, or close to it, with concurrent restoration of normal cellular activity at the lower temperature (Mansilla MC *et al*., 2004; Phadtare S, 2004). Desaturases introduce a *cis* double bond into pre-existing FA (Figure 28). An interesting system that has been uncovered in *B. subtilis* is the regulation of membrane fluidity by a molecular thermosensor (Aquilar PS, 2001). Specifically, expression of a membrane-bound, oxygen-dependent phospholipid acyl-chain desaturase (DesA; also known as delta-5-desaturase) introduces a *cis* double bond at the 5-position of acyl chains that are attached to existing phospholipids. Transcription of the desA gene is controlled by a two-component regulator that senses changes in membrane properties which are due to abrupt temperature change. It will be interesting to determine exactly how the membrane component of this transcriptional

regulatory system senses membrane biophysical properties and transmits the signal to the transcriptional apparatus.

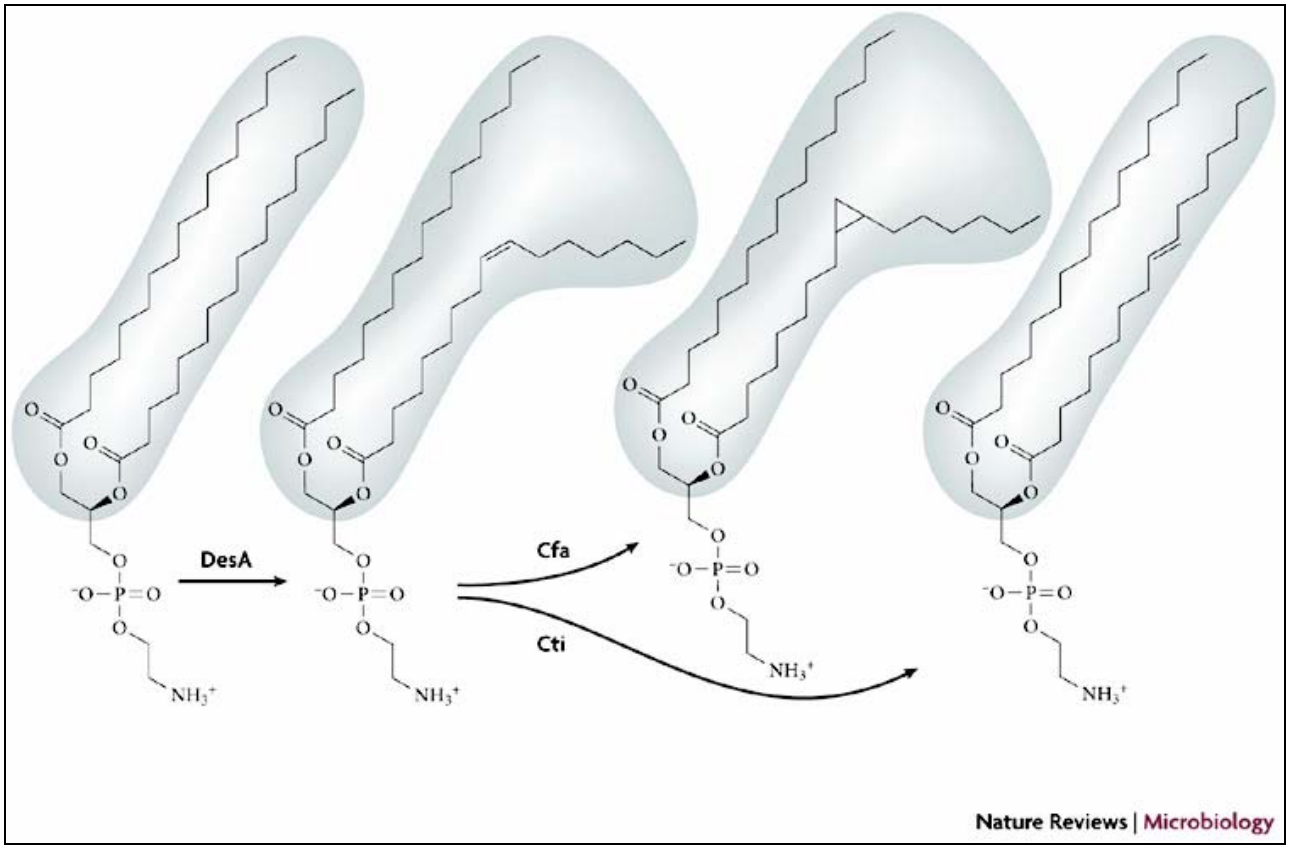


Figure 28. Eubacteria have several enzymes that respond rapidly to environmental changes by altering the structure of existing phospholipids. PE modifications are shown as an example. The phospholipid acyl-chain desaturase DesA introduces a double bond at the Δ^5 or Δ^9 position into a SFA, which increases membrane fluidity. The rigid *cis* double bond introduces a pronounced kink in the acyl chain. Phospholipids that contain *cis*-UFA occupy a greater molecular volume and do not pack as densely in the bilayer as phospholipids that contain SFA. Cyclopropane fatty acid synthase (Cfa) introduces a methyl group across the double bond to form a cyclopropane ring. The cyclopropane FA have similar biophysical properties to the UFA, but render the membrane more stable to environmental insults, such as acid stress. Cyclopropane fatty acid formation is universally activated in eubacteria that contain UFA as they enter stationary phase. In some eubacteria, phospholipid *cis–trans* isomerase (Cti) can replace the *cis* double bond with a *trans* double bond. *Trans* FA have properties that resemble SFA and give rise to membranes that have higher transition temperatures. Cti can be activated by an increase in growth, temperature or the addition of membrane-destabilizing organic solvents. (Zhang YM and Rock CO, 2008).

- **Formation of cyclopropane FA**. The conversion of pre-existing *cis*-UFA to cyclopropane FA is widespread in eubacteria (Figure 28). The required methylation reaction is carried out by cyclopropane FA synthase (Cfa), which uses *S*-denosylmethionine as the methyl donor to

create the cyclopropane group (Grogan DW and Cronan JE, 1997). The cyclopropane bond retains the *cis* double-bond configuration of the acyl chain (Figures 27 and 28), and therefore phospholipids that contain cyclopropane FA have similar biophysical properties to those that are composed of *cis*-UFA (Grogan DW and Cronan JE, 1997). The cyclopropanation of phospholipid *cis*-UFA begins as cells enter the stationary phase of growth and continues until virtually all of the *cis*-UFA have been converted into their cyclopropane derivatives. There is no mechanism to reverse the cyclopropanation of phospholipid FA, and their content is diluted by the synthesis of *cis*-UFA that occurs when cells re-enter logarithmic growth. The cyclopropane bond is more stable than a double bond and is physiologically useful; for example, cyclopropanation increases the resistance of *E. Coli* to acid stress (Chang YY and Cronan JE, 1999). In addition, eubacteria are more sensitive to freeze drying if they lack 17-carbon cyclopropane FA (Munoz-Rojas J *et al*., 2006). The inactivation of a Cfa homologue in *M. tuberculosis,* in which a cyclopropane ring is normally introduced into α-mycolates, results in *M. tuberculosis* being unable to establish a persistent infection (Glickman MS, 2000), which indicates that cyclopropane FA have an important role in the survival of bacterial pathogens. Although there are only a few examples, the common theme of cyclopropane FA function is to enhance the viability of slow-growing or quiescent cells in hostile environments.

- **Fatty acid *cis–trans* isomerization**. A few eubacteria have evolved a mechanism to convert existing *cis*-UFA in phospholipids to *trans*-UFA to adapt to environmental challenges (Figure 28). FA that have the *trans* configuration retain a slight kink in their conformation, and therefore do not pack as closely as SFA, but the bilayer packing of phospholipids that contain *trans*-UFA mimics the physical properties of phospholipids that contain SFA. The *cis–trans* isomerization reaction leads to membranes that have higher phase-transition temperatures, increased rigidity and decreased permeability to solutes. The isomerization reaction is carried out by a periplasmic, haem-containing *cis–trans* isomerase (Cti)(Holtwick R, 1987; Junker F and Ramos JL, 1989). Cti is constitutively expressed and increases *trans*-UFA formation in response to solvents or temperature change (Halverson LJ and Firestone MK, 1986; Kiran MD *et al*., 2005). It is, therefore, unclear how Cti activity is regulated by the properties of the membrane bilayer. As the *trans*-UFA cannot be returned to the *cis* conformation by Cti when the environmental stress is removed, *cis* FA must be replenished by *de-novo* biosynthesis or an as-yet-unidentified process.
- **Modification of surface charge**. Bacterial membranes have an overall high density of negative charge, a property that is exploited by the innate immune system to disrupt

microbial membranes by using various cationic peptides. The formation of lysine adducts of the acidic membrane phospholipids is an important modification that protects pathogens from attack by the innate immune system (Peschel A, 2002). These lipids are formed by the reaction of a charged lysyl-tRNA with PG to form lysyl-PG (Oku Y, 2004). This modification decreases the negative charge of the membrane bilayer, which prevents the intercalation of defensins into the bilayer and increases resistance to neutrophil killing and other cationic antimicrobial agents (such as vancomycin) (Peschel A *et al*., 2001; Staubitz P and Peschel A, 2002; Thedieck K *et al*., 2006; Nishi H *et al*., 2004).

EUKARYOTES

Protists

Currently, the term protists is used to refer to unicellular eukaryotes that either exist as independent cells, or, if they occur in colonies, do not show differentiation into tissues. This kingdom is nowadays still not well defined taxonomically, and is therefore composed by numerous species with nothing in common but being unicellular eukaryotes. Characterization of this group of living beings is hard to achive and literature is quite elusive on this subject, in particular when lipid biosynthesis, metabolism and homeostasis are discussed. The main feature that characterize protists is the high production large amounts of C20-22 PUFA (Uttaro AD, 2006). That's why scientific research on has mainly taken the pathway of biotechnological exploitation for them, in order to develop better industrial strains for PUFA production, and therefore few is known about the basic biology of protist lipidomics. In mammals, a number of polyunsaturated 20- and 22-carbon acids of both the n–3 and n-6 groups are synthesized from dietary ALA and LA, respectively, by repeated chain elongation and introduction of double bonds. Higher animals are dependent on the exogenous precursors because they are unable to introduce double bonds on the methyl terminal side of existing double bonds and, therefore, cannot synthesize LA from OA, or ALA from LA. These latter reactions are typical of higher plants which do not, however, convert the 18-carbon acids to longer-chain FA, or introduce more double bonds towards the carboxyl group. Protists are not so limited. Previous reports (Korn ED *et al*., 1965) have emphasized the potential usefulness of protists in delineating the mechanisms of biosynthesis of unsaturated FA and, conversely, the possibility of applying this information to the taxonomy and phylogeny of the protists. Fungi and marine microalgae are currently used to produce nutraceutic oils and other protists and algae are being studied because of the variability in their enzymes involved in PUFA biosynthesis. Such enzymes could be used as source for the production of transgenic organisms able to synthesize designed oils for human diet or, in the case of parasitic protozoa, they might be identified as putative chemotherapeutic targets. All but one or two of reactions of PUFA synthesis occur in some organisms while one entire sequence may be missing from others. All protists so far examined are able to synthesize LA. They differ, however, in many other respects and it is these differences which one must consider in grouping the protists according to their abilities to synthesize PUFA (Uttaro AD, 2006). *Acanthumoeba*, for example, synthesizes LA from OA, and also converts LA to AA by a pathway differing in one step from that previously described in the rat (Korn ED *et al*., 1965). *Euglena*, a chlorophyll-containing phytoflagellate thought to be similar to those organisms that were the common origin of higher plants and animals, synthesizes at least 59 FA, including those PUFA typical of higher plants, higher animals, and algae (Korn ED *et al*., 1965). *Mortierella*

alpina is a representative of oleaginous fungi, able to accumulate large amounts of C20 PUFA as triacylglycerols. It has been used for years in the industry to produce AA-rich oils (Ratledge C, 2004; Sayanova OV and Napier JA, 2004; Pereira SL *et al*., 2003). *Tetrahymena* and *Acanthamoeba* have been long utilized as poikilothermic model organisms for studying the regulation of membrane fluidity by FA desaturation. Another interesting observation is the high proportion of UFA present in parasitic protozoa, like *trypanosomatids*, where they can represent up to the 80% of the total FA of the cell (Mellors A and Samad A, 1989; Haughan PA and Goad LJ, 1991). This fact could indicate that the biosynthetic pathways for UFA are essential in these flagellates. This is not unexpected considering the complexity of their life cycles, during which cells have to adapt to dramatic changes of temperature and morphology and, therefore, highly fluid membranes may be advantageous. This makes it attractive to evaluate the possibility of using these pathways as chemotherapeutic targets. Many microalgae serve as primary food for fish and consequently as the major PUFA source for them and fish-eating sea animals. *Isochrysis* and *Nannochloropsis* are used to enrich rotifers in EPA and DHA, which are then used in aquaculture to enrich the diet of fishes (Ratledge C, 2004; Sayanova OV and Napier JA, 2004; Pereira SL *et al*., 2003). Moreover, there is only one article describing the capacity of *Giardia lamblia* to desaturate oleate to 18:2 and 18:3 derivatives (Ellis JE *et al*., 1996). The desaturation level is increased in encysting trophozoites presumably to enable future cysts to cope with unfavourable environmental conditions.

The main reactions present in different combinations in protists are (Korn ED *et al*., 1965):

- The mechanism of synthesis of OA. Many protists synthesize OA by direct desaturation of SA while others use the uncharacterized, in-direct pathway of higher plants. The third pathway to OA present in many bacteria has not been demonstrated in protozoa.
- The ability to convert LA to ALA. If this reaction is missing, the entire n-3 group of PUFA will not be synthesized.
- The ability to convert LA and ALA to longer-chain FA by chain elongation and further desaturations.
- The utilization of ALA or 20:2 as the intermediate between LA and 20:3.
- The ability to synthesize 16:3 and 16:4.

Future studies on the basic biology of these organisms, including biodiversity, environmental adaptations and genome research, are likely to point out new directions for better understanding the unique lipidomics of these lower eukaryotes, leading to a better exploitation in applied industrial biotechnology.

Fungi

Due to the wide variability present in this kingdom of living beings, fungi lipids appear to be hard to analyze in a sistematic manner, and this is behind the scope of this thesis; a main overview will only be discussed here. Many fungi possess the same FA but produce different relative concentrations of each, while some fungi differ in both the FA produced and in the relative concentrations of others. Each *phylum* has distinctive FA profile; for example, all *zygomycetes* produce a greater number of FA than the dikaryotic fungi (*ascomycetes* and *basidiomycetes*) do. Significant differences in FA composition are also found at the intraspecific level. Fungi produce fewer different FA than bacteria do (Lechevalier H and Lechevalier MP, 1988), and in the past FA have been considered to have little taxonomic value for this group of organisms. However, multiple work has shown that FA profiles can be used to differentiate and identify genera, species, and strains of yeasts and yeast-like organisms (Augustyn OPH *et al*., 1990; Brondz I *et al*., 1989; Westhuizen JPJ *et al*., 1987). Fungi contain FA of carbon chain lengths ranging from 12 to 24. The most common and abundant FA are PA, SA, OA and LA, which often made up greater than 95% of the total fatty acid content of the dikaryotic fungi. In zygomycetes, oomycetes, and sterile forms, 18:3-$\Delta^{6\text{-}9\text{-}12}$ and AA are also present in relatively high concentrations. Other FA (12:0, 14:0, 15:0, 16:1, 17:0, 17:1, 20:0, 20:2, 20:5, and 24:0) are commonly detected but usually represented less than 5% of the total FA content. The lipid composition of the membrane under anaerobic conditions is different from that of cells grown under aerobic conditions. Anaerobically, the plasma membrane contains more SFA, less total sterol, less ergosterol and less squalene (Nurminen T *et al*., 1975). These differences can be explained by the inability of the cell to synthesize these compounds without oxygen. Culture age is known to influence FA composition in fungi (Losel DM, 1988; Weete JD, 1980). Smit *et al*. (1987) reported that in the yeasts they studied, a high degree of variation in relative FA concentrations was observed during the exponential and early stationary growth phases and relatively stable FA concentrations were obtained from cultures harvested in the late stationary phase. Also the temperature at which a fungus is cultured affects its FA composition. The degree to which FA composition is altered by temperature, however, is dependent on the fungus. In general, the same FA are present in a fungus when grown at different temperatures, but their relative amounts are different. The relative amount of PA decreases with decreasing temperature, and the amount of LA increased with decreasing temperature. Changes in the other common and abundant FA, SA and OA, are very low. In general, as the temperature of culture conditions is decreased, the degree of unsaturation in FA is increased (Dexter Y and Cooke RC, 1984; Losel DM, 1988; Sumner JL *et al*., 1969). Unsaturated FA play an essential role in the biophysical characteristics of cell membranes and determine the proper function of membrane-

attached proteins. Increasing the unsaturation index of yeast lipids fluidizes their membrane and alters the stress response of yeast cells. Production of heterologous desaturases in *S. cerevisiae* (Rodriguez-Vargas *et al*., 2007) increases the content of dienoic FA, especially LA, the unsaturation index, and the fluidity of the yeast membrane. The total FA content remaines constant, and the level of MUFA decreases. Changes in the fluidity of the lipid bilayer affect tryptophan uptake and/or the correct targeting of tryptophan transporters (Rodriguez-Vargas *et al*., 2007). Production of dienoic FA increased the tolerance to freezing of wild-type cells preincubated at 30°C or 15°C. Thus, membrane fluidity is an essential determinant of stress resistance in *S. cerevisiae*, and engineering of membrane lipids has the potential to be a useful tool of increasing the tolerance to freezing in industrial strains (Rodriguez-Vargas *et al*., 2007). Increased unsaturation of yeast lipids may also affect the protein sorting mediated by lipid rafts and the final destination of key membrane proteins. Association with lipid rafts, a sphingolipid- and sterol-rich membrane domain (Simons K and Ikonen E, 1997), plays an essential role in the correct localization of proteins such as Tat2p (Umebayashi K and Nakano A, 2003), Ole1p (Tatzer V *et al*., 2002), or Pma1p (Bagnat M *et al*., 2000 and 2001), the major plasma membrane H^+-ATPase in *S. cerevisiae*. Mutations in Pma1 result in cold sensitivity and Na^+ resistance phenotypes (Kaminska J *et al*., 2000, Withee JL *et al*., 1998) shared by Trp^- FAD2-overexpressing cells. Thus, changes in the unsaturation index of lipids could affect the localization of key proteins and thereby cause pleiotropic phenotypes. Thus, the ability of cells to alter the degree of unsaturation in their membranes is an important factor in cellular acclimatization to environmental conditions. *S. cerevisiae* and *Schizosaccharomyces pombe* (Weete JD, 1974; McDonough VM and Roth TM, 2004) are unusual fungi, however, in that they form only MUFA. Approximately 70–80% of their total glycerolipid acyl chains consist of the $\Delta^{(cis)-9}$ species 14:1, POA and OA when cells are grown under a wide range of physiological conditions. MUFA in *S. cerevisiae* and other fungi are formed from saturated fatty acyl CoA precursors by the Ole1p Δ^9-FA desaturase; most other fungi also express membrane bound Δ^{12} and Δ^{15} desaturases. These enzymes introduce additional double bonds into the Δ^9 or $\Delta^{9,12}$ unsaturated acyl chains of phospholipids (Pugh EL and Kates M, 1975; 1979; 1973; Domergue F *et al*., 2003) to produce high levels of LA and ALA (Weete JD, 1974). In *Neurospora crassa* and *Candida albicans*, for example, LA and ALA are produced by sequential desaturation of OA and in cells grown at 30°C the dienoic LA is the most abundant FA (Martin CE *et al*., 1981; Oh CS and Martin CE, 2006); at low growth temperatures, however, ALA is the predominant species. Some fungi will also elongate LA and ALA and introduce additional double bonds through the activity of $\Delta^{4,5,6}$ and Δ^{17} desaturases to form very long chain (≥ C20) PUFA such as AA (Michaelson LV *et al*., 1998; Sakuradani E *et al*., 1999) and EPA (Domergue F *et al*., 2002; Hornung E *et al*., 2005) acids. Minor species include C14

and C26 FA, which play essential functions in protein modification or as components of sphingolipids and GPI anchors, respectively.

Fungi FA derive from three different sources: external supply, endogenous lipid (protein) turnover, and *de-novo* synthesis and elongation. Since yeast laboratory media typically do not contain FA, net gain of FA during cellular growth solely depends on *de-novo* synthesis. However, exogenous FA can be readily taken up by yeast, and incorporated into lipids, which might be relevant processes in the context of a yeast cell's life in their natural habitat. Phospho- and neutral lipids undergo rapid turnover, required for adjusting specific acyl-compositions of membrane phospholipids, or during lipolysis. Almost all subcellular organelles are involved in FA metabolism; thus, maintenance of fatty acid homeostasis requires regulation at multiple levels.

The most studied pathway of FA synthesis in fungi is the OLE1p of *S. cerevisiae* (Figure 29). OLE1p introduces a double bound in the Δ^9 position of FA esterified to CoA (Stukey *et al*., 1989; Martin *et al*., 2002) and it is localized into the endoplasmic reticulum (ER), where most of the lipid

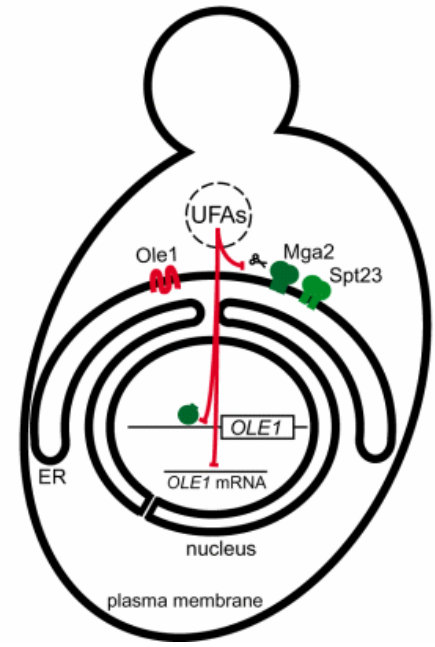


Figure 29. The OLE1p pathway of *S. cerevisiae*. (Aguilar PS and de Mendoza D, 2006).

biosynthetic machinery resides. In this organelle, saturated PA and SA acyl-CoA precursors are desaturated, yielding POA and OA respectively, which are then distributed throughout the membranes of the cell systems and comprise more than 70% of the total FA. OLE1p is a chimeric protein consisting of an amino terminal desaturase domain fused to cytochrome b5. It catalyzes the

formation of the double bond through an oxygen-dependent mechanism that requires reducing equivalents from NADH. These are transferred to the enzyme via NADH cytochrome b5 reductase to the Ole1p cytochrome b5 domain and then to the diiron-oxo catalytic center of the enzyme. The OLE1 gene is highly regulated in response to various environmental signals, and its transcription is transiently activated at low temperature (Nakagawa *et al*., 2002). It is also induced under hypoxic conditions (Kwast *et al*., 1998). As the desaturation reaction utilizes oxygen as an electron acceptor, OLE1 induction under hypoxia might be a response to UFA depletion under such limiting substrate conditions (Nakagawa *et al*., 2001; Vasconcelles *et al*., 2001). The control of OLE1 gene expression appears to be mediated through the ER membrane proteins Spt23p and Mga2p. N-terminal fragments of these proteins are released by an ubiquitin/proteasome mediated proteolysis system and translocated to the nucleus where they appear to act as transcription coactivators of OLE1. OLE1 is regulated through Spt23p and Mga2p by multiple systems that control its transcription and mRNA stability in response to diverse stimuli that include nutrient FA, carbon source, metal ions and the availability of oxygen. The differences between Mga2p and Spt23p suggest that both proteins have evolved complementary, overlapping roles in the regulation of OLE1 (Zhang *et al*., 1999). Nevertheless, OLE1 does not appear to be essential in acclimation to low temperature, since its overexpression does not confer growth advantages at 10°C. Overexpression of OLE1 is toxic and reduces growth at 30°C (Kajiwara S *et al*., 2000). Thus, the functional role of OLE1 is unclear, and the mechanism by which *S. cerevisiae* alters membrane fluidity in response to different types of stress remains unknown.

Plants

Structural membrane glycerolipids of all plant cells contain almost exclusively 16-carbon and 18-carbon FA, with up to three methylene-interrupted double bonds (PA, POA, SA, OA, LA, ALA, and in some species 16:3). In contrast with the conservative FA composition of plant membrane lipids, tremendous FA diversity exists in the seed storage lipids. To date, >300 naturally occurring FA have been described in seed oils (Badami RC and Patil KB, 1981; Van de Loo FJ *et al*., 1993), and it has been estimated that thousands more could be present throughout the plant kingdom. The structures of these FA can vary in chain length from 8 to 24 carbons, they can have double bonds in unusual positions, or novel functional groups, such as hydroxy, epoxy, cyclic, halogen or an acetylenic group on their acyl chain (Figure 30). In many cases, unusual FA are the predominant FA in the seed oil of a plant species. The reason for such a great diversity of seed oil constituents is unknown, but plants can tolerate high levels of unusual FA in storage lipids because they are sequestered into oil bodies and have no structural function. The special physical and chemical

properties of many unusual FA might explain why they are excluded from the membrane lipids of seeds, and are absent from other parts of the plant. It is believed that they would perturb the structural integrity of the membrane bilayer and have deleterious effects on the cell. Consequently, storage and membrane lipids have different FA compositions.

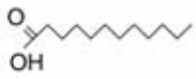
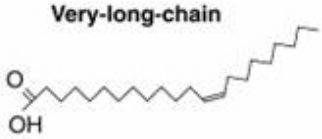
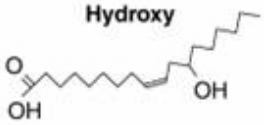
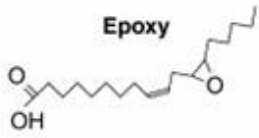
Fatty acid class and example	Name	Principal biosynthetic enzyme	Plant family	% in lipids	
				TAG	Membrane
Medium-chain 	Lauric (12:0)	Thioesterase	Lauraceae Lythraceae *Cuphea wrightii* Arecaceae Palm oil (*Elaeis guineensis*)* *Cocos nucifera**	54	2.9
Very-long-chain 	Erucic (22:1)	β-Keto-acyl-CoA synthase	Brassicaceae *Crambe abyssinica* Oilseed rape* *Arabidopsis thaliana* Limnanthaceae *Limnanthes douglasii*	60	3.0
Hydroxy 	Ricinoleic (18:1-OH)	Diiron hydroxylase	Euphorbiaceae *Ricinus communis**	85	5.0
			Brassicaceae *Lesquerella* spp.		
Epoxy 	Vernolic (18:1⊃O)	Epoxidase	Asteraceae *Crepis palaestina* *Vernonia* spp. Euphorbiaceae	57	2.0
Double bond position 	Petroselinic (18:1Δ6)	Souble desaturase	Apiaceae Carrot *Coriandrum sativum* Araliaceae	70–75	10.0–20.0

Figure 30. Examples of some of the unusual FA made by plants. Current commercial sources are indicated by an asterisk. (Millar AA *et al*., 2000)

In plants, *de-novo* FA biosynthesis occurs in the plastid where acetate (C2) is elongated by the sequential addition of further C2 units while attached to a soluble acyl-carrier-protein (ACP) (Ohlrogge J and Browse J, 1995). For common FA formation, the growing acyl chain is terminated when it is 16 or 18 carbons long, by the action of an acyl-ACP thioesterase, which cleaves the acyl

group from the ACP to produce a free FA. The synthesis of these unusual FA involves just one additional or alternative enzymatic step from primary lipid metabolism (Voelker TA *et al*., 1992; Van de Loo FJ *et al*., 1995; Lee M *et al*., 1998; Millar AA and Kunst L, 1997; Cahoon EB *et al*., 1992). However, although only one enzyme is needed for their synthesis, other specialized FA biosynthetic enzymes or components are required for their high accumulation (Suh MC *et al*., 1999). All the enzymes identified to date that are involved in unusual FA biosynthesis are structurally related to enzymes of primary lipid metabolism. Thus, it appears that housekeeping genes have been recruited and specialized for the development of enzymes, which either have altered substrate specificities, or can catalyse closely related but modified reactions (Shanklin J and Cahoon EB, 1998). Many of the unusual FA are found in taxonomically dispersed families (Figure 30), implying that the recruitment of enzymes for the synthesis of these unusual FA might have occurred a number of independent times during angiosperm evolution. What selective advantages are associated with high levels of accumulation of any of the given unusual FA is unknown. It is puzzling how these different FA compositions become established and are maintained. This question is particularly pertinent as the production of storage and membrane lipids occurs simultaneously and involves common precursors (Ohlrogge J and Browse J, 1995). Castor bean (*Ricinus communis*), for example, produces seed oil containing nearly 90% ricinoleic acid (18:1-OH), an unusual hydroxy FA (Figure 30). Analysis of the membrane lipids from developing castor bean seeds indicates that even during its most active period of biosynthesis this FA accounts for only a small proportion of the total FA in the membrane (Bafor M *et al*., 1991). This observation is striking because it is a membrane lipid, phosphatidylcholine, which serves as the substrate for 18:1-OH synthesis. Thus, the mechanisms that edit out unusual FA from membrane lipids and then channel them to storage lipids must be extremely selective and efficient. How strict this editing is, probably depends on the structure of the particular unusual FA. For example, the structure of petroselinic acid (18:1-Δ^6) deviates from common acyl groups only by the position of the double bond. Therefore, 18:1-Δ^6 can accumulate in membrane lipids to a much greater extent than FA such as 18:1-OH, which contains a polar oxygenated functional group that would be incompatible with the hydrophobic environment of the membrane. The membrane-editing ability of plants is also important for biotechnology, as many of the unusual FA represent valuable feedstocks for the chemical industry. However, plants that accumulate these FA are often not amenable to agriculture. Therefore, there is an enormous interest in generating transgenic crop plants engineered to accumulate high levels of specific unusual FA. Two hypotheses have been proposed to explain the targeting of unusual FA to storage lipids and their exclusion from membrane lipids: enzyme specificity and compartmentation. However, it is highly likely that there is no single sorting

mechanism in operation in any given plant species, and that multiple mechanisms are working in concert. Because plants accumulating unusual FA have evolved independently, it is likely that these mechanisms, or their relative importance, differ significantly in different species.

Since plants lack mobility they cannot avoid exposure to stresses in their environment, but must adapt to them in other ways. Plants have evolved both constitutive and inducible means to resist stresses. FA are crucial components of cellular membranes, suberin, and cutin waxes that provide structural barriers to the environment (Beisson *et al*., 2007). They contribute to inducible stress resistance in both biotic (pathogens) and abiotic (temperature, salt, drought, heavy metals) stress (reviewed by Upchurch RG, 2008). This is obtained through the remodeling of membrane fluidity (Iba K, 2002), the release, through lipase activity, of ALA (Grechkin A, 1998), and as modulators of plant defense gene expression (Kachroo *et al*., 2001) and seed colonization by fungi (Calvo *et al*., 1999). The ability to adjust membrane lipid fluidity by changing levels of UFA is a feature of stress acclimating plants provided mainly by the regulated activity of FA desaturases. Modification of membrane fluidity results in an environment suitable for the function of critical integral proteins, such as the photosynthetic machinery, during stresses. Free ALA is itself a stress signal and the precursor for phyto-oxylipin biosynthesis (Blèe E, 2002). Mounting evidence suggests that chloroplast OA levels are critical for normal pathogen defense responses in *Arabidopsis*, including programmed cell death and systemic acquired resistance (SAR; Kachroo *et al*., 2001). Stearoyl-ACP desaturases (SADs) are cytoplasmic enzymes that catalyze the conversion of SA to OA, a key step regulating the cellular PUFA content. OA and LA levels, in part, regulate fungal development, seed colonization, and mycotoxin production by *Aspergillus* spp. (Wilson *et al*., 2004; Xue *et al*., 2006). The release of ALA from plant membrane lipids by stress activated lipases is thought to provide the substrate for lipoxygenase and subsequent octadecanoid (oxylipin) pathway synthesis of jasmonic acid and methyl jasmonate (Padham *et al*., 2007; Wasternack C, 2007). Jasmonic acid and methyl jasmonate participate in the signal regulation of a number of plant processes including wound and pathogen defense responses. Efforts have been successful to identify and characterize FA-deesterifying lipases that are activated by pathogen attack and/or environmental stress. Results suggest that both A_1 and A_2 phospholipases (Grechkin A, 1998; Padham *et al*., 2007) are involved in ALA mobilization form membrane lipids. Plants often encounter the abiotic stresses of low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. More complete knowledge of FA unsaturation, mobilization, and regulation processes may significantly aid the development of effective strategies for managing abiotic and biotic stresses in plant exploitation.

CHAPTER 3
RIBOSOME-INACTIVATING PROTEINS

3.1 General characteristics

Ribosome-inactivating proteins (RIPs) are a group of proteins that share the property of damaging ribosomes in an irreversible manner, acting catalytically, i.e. enzymatically. Two potent toxins, ricin and abrin, from the seeds of *Ricinus communis* and *Abrus precatorius*, respectively, were known since more than a century, but the denomination of RIPs was introduced much later (Stirpe F, 1982), after it had been discovered that they damaged ribosomes. Subsequently, it was found that other similar toxins and other much less toxic plant proteins inactivated ribosomes, all with an apparently similar mechanism. Currently, RIPs are divided into two broad groups, type 1 RIPs, consisting of a single peptidic chain of 30 kDa, approximately, and type 2 RIPs which, as found by Olsnes S and Pihl A (1973), consist of an enzymatically activeA chain similar to type 1 RIPs, linked to a slightly larger (35 kDa, approx.) B chain, which has the properties of a lectin with specificity for sugars with the galactose structure (Lord JM *et al*., 1994).

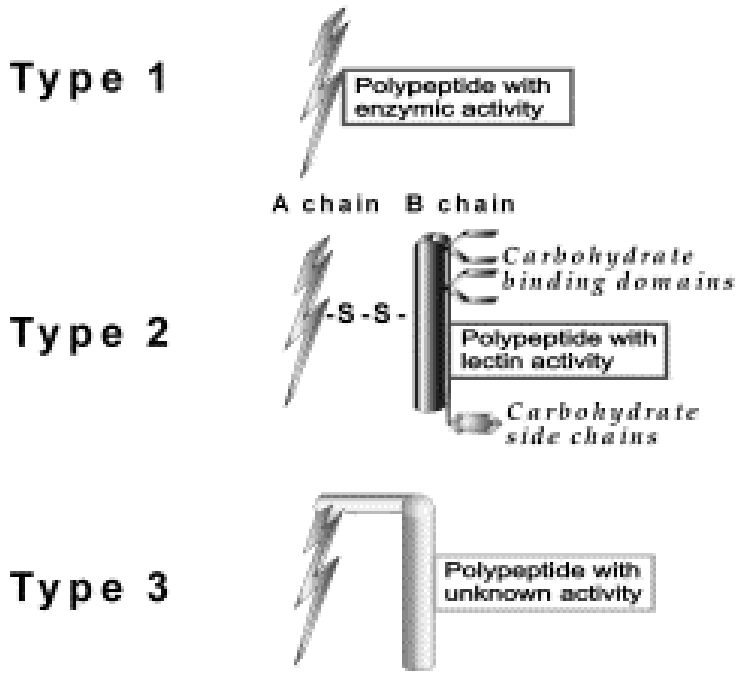


Figure 31. Schematic rapresentation of the structure of ribosome-inactivating proteins. (Stirpe F, 2004).

It was proposed to include in a type 3 group two other proteins. One is a maize b-32 RIP, which is synthesized as a proenzyme, activated after the removal of a short internal peptide segment leaving two segments of 16.5 and 8.5 kDa (Walsh TA *et al*., 1991). The other one is JIP60, a RIP from barley in which a segment similar to type 1 RIPs continues with another segment of similar size without a known function (Reinbothe S *et al*., 1994). More recently, it was suggested to include only the latter into this group (Peumans WJ *et al*., 2001). It seems unjustified to introduce a new denomination for presumably few and different cases, and it is preferable to consider these two proteins as peculiar type 1 RIPs. RIPs could be more appropriately differentiated into type 1 and type 2, respectively, on the basis of the absence or presence of a lectin-like chain, which makes a significant functional difference. A schematic representation of RIPs structure is in figure 31.

RIPs were initially detected in plants, mostly in *Angiopermae*, both mono- and dicotyledons, and also in mushrooms (Yao QZ *et al*., 1998; Lam SK and Ng TB, 2001) and in an alga, *Laminaria japonica* (Liu RS *et al*., 2002). The level of RIPs in plant tissues is highly variable, ranging from traces to hundreds of milligrams per 100 g. In some plants, RIPs are present in many or even in all tissues examined (roots, leaves, stems, bark, flowers, fruits, seeds, latex, cultured cells), whilst in other plants they are confined to a single tissue. Often different forms were found, sometimes even in the same tissue: for instance, several forms of saporin were found in the seeds, leaves and roots of *Saponaria officinalis* (Ferreras JM *et al*., 1993) and several type 2 non-toxic RIPs were identified in various tissues of *Sambucus* species (Girbés T *et al*., 2003). Type 1 RIPs appeared to be more frequent than type 2 ones, and seemed to be preferentially distributed among plants belonging to some families (e.g. *Caryophyllaceae*, *Cucurbitaceae*, *Euphorbiaceae*, and others). In some plant species both type 1 and type 2 RIPs have been described (de Benito FM *et al*., 1995, 1998; Hao Q *et al*., 2001). Many plant materials were examined for RIP activity, although most screening studies were performed not to study the distribution of RIPs, but to find materials containing a high level of them, and consequently research was focused on plants belonging to families in which RIPs had been found. RIP activity could not be detected in some plants, but even in those cases the presence of a RIP cannot be excluded because (i) initially seeds were more frequently examined, and RIPs present in other tissues could have been missed, and (ii) materials showing activity below a set threshold were not considered, in which RIPs might be present at very low concentration, below detection level. Moreover, (iii) the search for these proteins was based on the effect of crude extracts on protein synthesis, generally by a rabbit reticulocyte lysate, and the possible existence cannot be excluded of RIPs acting on different ribosomes, which could have been missed. Thus, RIPs could be more widely diffused than thought, and possibly even ubiquitous in the plant kingdom. It is noteworthy that the expression of at least some RIPs in plant tissues is enhanced in

senescence and in conditions of stress (Chaudhry B *et al*., 1994; Stirpe F *et al*., 1996; Rippmann JF *et al*., 1997), and after viral infection (Girbés T *et al*., 1996). RIPs are not limited to plants. Shiga and shiga-like toxins were found to be RIPs (Reisbig R *et al*., 1981; Obrig TG, 1997) and an enzymatic activity similar to that of RIPs was detected in animal cells and tissues which, like in plants (Girbés T *et al*., 1996), was higher in virally-infected and stressed cells (Barbieri L *et al*., 2001). Consequently, the distribution of RIPs in nature seems wider than it was originally thought.

Flexner S (1897) described the lesions brought about by ricin and abrin in rabbit and guinea pig. Severe damage was observed in liver, kidney, spleen, lymph nodes and intestine. Remarkably for the time, it was reported that sinusoidal lining was the first damage in the liver. Consistently with these findings, in an electron microscopy study it was observed that in ricin-poisoned rats Kupffer and sinusoidal cells appear to be damaged first, causing formation of thrombi, with subsequent alteration of the hepatocytes (Derenzini M *et al*., 1976). High doses of type 1 RIPs also cause liver necrosis histologically very similar to that brought about by ricin but not identical, as it is shown by the effects on xanthine oxidoreductase. This enzyme was converted from the dehydrogenase into the oxidase form in the liver of ricin-poisoned but not in the liver of saporin-poisoned rats, and leaked out from the liver into blood only in the latter animals (Battelli MG *et al*., 1996). The non-toxic type 2 RIP nigrin b at high doses caused gut derangement, especially of the small intestine, in mice (Girbés T *et al*., 2003). It is noteworthy that when high doses of ricin, modeccin or volkensin are given to rats, the animals die after a time lag of 6–8 h, but no lesions accounting for death are found in parenchymal organs. These toxins are also retrogradely transported along peripheral nerves (Wiley RG and Lappi DA, 1995). Retrograde transport of ricin along nerves has been observed when the toxin was injected outside nerves in several tissues, such as the anterior eye chamber (Dumas M *et al*., 1979), the supramandibular gland (Harper CG *et al*., 1980), the dental pulp (Henry MA *et al*., 1987), the superior lip (Hino M *et al*., 1988) and the lateral rectus muscle (de la Cruz RR *et al*., 1994). These observations indicate that ricin is taken up from tissues by nerve ends. Thus, it is possible that ricin, and presumably other toxins, when administered in large amounts may be taken up by nerve ends and transported to some vital part(s) of the nervous system, where even small lesions could be sufficient to cause death. The signs and pathology of ricin intoxication in man include damage and haemorrhage in various parenchymal organs, especially the spleen and other lymphoid tissues (Balint GA, 1974).

3.2 Biological activities

RIPs damage ribosomes in a less-than-equimolar ratio, indicating a catalytic, i.e. enzymatic, manner (Montanaro L *et al*., 1973). It is noteworthy that some, but not all, RIPs require various cofactors for maximal inhibitory activity on translation (Carnicelli D *et al*., 1992), in the case of gelonin a specific tRNA (Brigotti M *et al*., 1995). The mechanism of the ribosomal damage was discovered by Endo Y *et al*. (1987), who found that ricin cleaved the glycosidic bond of a single adenine residue (A_{4324} in rat liver rRNA). This residue is adjacent to the site of cleavage of rRNA by α-sarcin, in a tetranucleotide $GA_{4324}GA$ in a highly conserved loop at the top of a stem, for this called α-sarcin/ricin loop. This observation was extended to other RIPs (Stirpe F *et al*., 1988), which were officially classified as rRNA N-glycosidases (rRNA N-glycohydrolases, EC 3.2.2.22). RIPs depurinate also non-mammalian ribosomes, from insects (Zhou X *et al*., 2000), plants (Iglesias R *et al*., 1993), yeast (Roberts WK and Selitrennikoff CP, 1986), and bacteria (Girbés T *et al*., 1993). Subsequently, it was found that (i) some RIPs remove more than one adenine residue per ribosome (Barbieri L *et al*., 1994) and (ii) that RIPs remove adenine residues from DNA and other polynucleotides (Barbieri L *et al*., 1997; Nicolas E *et al*., 1998, 2000). The denomination of polynucleotide adenine glycosidase and more recently of adenine polynucleotide glycosylase was proposed for these proteins (Barbieri L *et al*., 2001). Also, it was observed that ricin and other RIPs remove adenine from the poly(A) tail of poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase (activated PARP, Barbieri L *et al*., 2003). The observation that PAP depurinates capped mRNA (Hudak KA *et al*., 2002) is of interest and may have implications for the mechanism of the antiviral activity of this RIP.

Among the biological properties of RIPs there are antiviral, antifungal and insecticidal activity. The antiviral properties of extracts of pokeweed leaves led to the purification of the pokeweed antiviral protein (PAP), which was found to inhibit protein synthesis by the same mechanism as the A chain of ricin (Irvin JD, 1983). Thus, PAP was the first known identified type 1 RIP. Subsequently PAP and other RIPs were found to have antiviral activity against both plant (Stevens WA *et al*., 1981; Lodge JK *et al*., 1993) and animal viruses (Aron GM and Irvin JD, 1980; Foa`-Tomasi L *et al*., 1982), and to have some fungicidal (Vivanco JM *et al*., 1999) and insecticidal activity (Gatehouse AMR *et al*., 1990; Dowd PF *et al*., 1998; Zhou X *et al*., 2000). The subject has been reviewed recently (Wang P and Tumer NE, 2000; Nielsen K and Boston RS, 2001; Van Damme EJM *et al*., 2001). The mechanism of the antiviral activity is still not completely clear. Normally, in their own plants RIPs are segregated from the cytoplasm, being located in protein bodies (e.g. ricin), in the cell wall matrix or in vacuoles (e.g. PAP), in the intracellular space (e.g. saporins). It was thought

that the segregation could be broken by the damage caused by viral infections, so that RIPs could come into contact with plant ribosomes, which would be inactivated, with consequent death of the infected cells and arrest of viral multiplication (Van Damme EJM *et al*., 2001). It was found, however, that PAP-C, a PAP mutant lacking the C-terminus, is not toxic when expressed in transgenic tobacco plants, in which depurination of rRNA was not detected (Tumer NE *et al*., 1997). Moreover, tobacco plants expressing PAP-C are protected from potato virus X (Tumer NE *et al*., 1997) and from the fungal pathogen Rhizoctonia solani (Zoubenko O *et al*., 1997), indicating that ribosome inactivation is not essential for antiviral and antifungal activity. The finding that RIPs depurinate nucleic acids (Barbieri L *et al*., 1997) suggests that RIPs could damage directly viral or virus-induced nucleic acids. A direct effect on intact virus particles is excluded, since the infectivity of TMV was not affected by treatment with PAP (Kumon K *et al*., 1990). These results led to several attempts to improve the resistance of plants to viruses by transfecting them with RIPs. As a matter of fact, PAP protects tobacco and potato plants from viral infection, but high-level expression of PAP is toxic to tobacco (Lodge JK *et al*., 1993) and bentgrass plants (Dai PJ *et al*., 2003). Also ricin A chain, saporin-S6 and a RIP from *Mirabilis expansa* were found to have antifungal activity and to inactivate fungal ribosomes (Park SW *et al*., 2002). The antifungal activity of barley RIPs was increased by the association with glucanase and chitinase, presumably because these enzymes damage the tegument of fungi, thus allowing the entry of RIPs into them (Leah R *et al*., 1991). Transgenic tobacco plants expressing an activated form of the maize RIP showed an enhanced resistance to the insect *Helicoverpa zea* (Dowd PF *et al*., 2003).

All RIPs are strongly immunogenic, and their administration to animals gives rise to formation of antibodies, with cross-reaction only among RIPs from plants belonging to the same family (Strocchi P *et al*., 1992). RIPs are also allergenic. The allergenicity of castor beans is well known from observations in factories producing castor oil (Thorpe SC *et al*., 1988). Ricin administration induced an IgE response, and also enhanced the response against other antigens given at the same time (Thorpe SC *et al*., 1989). Formation of IgE has been observed in mice after administration of several type 1 RIPs, (Zheng SS *et al*., 1991), in laboratory personnel working with RIPs (unpublished observations in our laboratory), in HIV-infected patients treated with trichosanthin (Byers VS *et al*., 1994) and in patients treated with immunotoxins containing ricin or other RIPs. RIPs have immunosuppressive effects, both on humoral and cell-mediated response, in that their administration prevents formation of antibodies and retards graft rejection (Spreafico F *et al*., 1983; Descotes G *et al*., 1985; Benigni F *et al*., 1995). This only if RIPs are given before the antigen, suggesting that they interfere with an early step in the immune response. A possibility is that they act by eliminating antigen-presenting cells, consistently with their high toxicity for macrophages.

Trichosanthin, momordin (Yeung HW *et al*., 1988) and other RIPs (Ng TB *et al*., 1992) have abortifacient activity. Trichosanthin is currently used in China to induce early and mid-term abortion with over 95% success rate and minimal side effects. The protein is highly toxic to trophoblasts and choriocarcinoma-derived cells (Battelli MG *et al*., 1992) and when administered intra-amniotically causes necrosis of syncytiotrophoblastic cells and fragmentation of placental villi. Clumps of disintegrating cells cause blood clotting and circulation impairment leading to large areas of necrosis in the placenta and death of the foetus (Anonymous, 1976).

The DNA-depurinating activity of RIPs led to investigate whether they could cause cell transformation (Barbieri L *et al*., 2003). Indeed, all RIPs tested but gelonin, at concentrations causing minimal toxicity, or even nontoxic, did cause transformation of 3T3 cells. Moreover, RIPs depurinated poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase (activated PARP). Since the latter is involved in DNA repair systems (Satoh MS *et al*., 1994), it is likely that the transforming activity of RIPs results from the combination of the damage they cause both to DNA and the repair system.

3.3 Uptake and routing

Consistently with the lower cytotoxicity of type 1 as compared with type 2 RIPs, the entry process appears to depend largely on the protein structure. Thus, the differences observed in the toxicity of different RIPs were ascribed to the absence or presence of B chains with properties of lectins specific for galactose, N-acetyl galactosamine or N-acetyl neuraminic acid (Van Damme EJM *et al*., 2001), which allow binding to the cell membrane of type 2 RIPs and facilitate their endocytosis process (Olsnes S and Pihl A, 1973a; 1973b). Ricin, however, enters macrophages and liver sinusoidal cells by an alternative mechanism, through the mannose receptors of these cells, which bind the mannose residues present in the ricin molecule. Once bound to cells, the B chain allows and facilitates the entry of the toxins in the cells, where the A chain can exert its enzymatic activity, damaging ribosomes and possibly other structures, with consequent cell damage and death. Indeed, the absence of the lectin moiety considerably limits the entry of type 1 RIPs into cells and justifies their low level of toxicity to cells and consequently to animals. However, the presence of the B chain is not sufficient to confer a high level of cytotoxicity on type 2 RIPs. Type 1 RIPs, though, are very toxic if allowed to enter cells in various ways, by enclosure into carriers that can fuse with cells such as liposomes, erythrocyte ghosts or viral envelopes (Barbieri L *et al*., 1993), by subjecting cells to shock waves (Delius M and Adams G, 1999), by photochemical internalization (Fodstad Ø *et al*., 1999; Selbo PK *et al*., 2002) or by linkage to appropriate carriers capable of binding to cells.

Once inside cells, RIPs follow an intracellular pathway through which the RIP molecules in part go to the Golgi apparatus and from there to the cytoplasm, in part go to the lysosomes, where they are largely degraded, and in part are expelled from the cell (Sandvig and van Deurs, 2002). Differences in each of these destinations and processes could result in different cytotoxicity. Indeed, it was observed that nigrin b, a non-toxic type 2 RIP from *Sambucus nigra* bark (Girbés *et al*., 1993b), enters cells equally well as ricin, but is more rapidly and extensively degraded and excreted from cells (Battelli *et al*., 1997). Consistent with this is the view that the low lysine content of ricin renders this toxin resistant to proteolysis (Deeks *et al*., 2002). Differences exist also among the toxic type 2 RIPs, abrin and volkensin being more toxic to mice than ricin (Barbieri *et al*., 1993). Moreover, all toxic type 2 RIPs are transported retrogradely along peripheral nerves (Wiley and Lappi, 1995), but only modeccin and volkensin are transported retrogradely when injected in the central nervous system (Wiley and Stirpe, 1988). The reasons for these differences are not known, and may reside in the characteristics of the B chains. The endocytosis mechanism of type 1 RIPs is still not well known. The entry of saporin-S6 into many cell types was shown to be mediated by the α2-macroglobulin receptor (Cavallaro U and Soria MR, 1995) as observed for trichosanthin (Chan

WL *et al*., 2000) and Pseudomonas exotoxin A (Kounnas MZ *et al*., 1992). However, the correlation between the level of this receptor and the sensitivity to saporin-S6 is lacking in the case of some cell types, suggesting an α2-macroglobulin receptor-independent way of saporin-S6 endocytosis (Baggaa S *et al*., 2003). Recently, the intracellular routes followed by saporin and ricin to intoxicated cells were compared (Vago R *et al*., 2005). Ricin was confirmed to enter the cytosol after a Golgi-mediated retrograde transport, whereas saporin utilized a Golgi-independent pathway. On the other hand, a recent study (Parikh BA *et al*., 2005) reported that the retro-translocation of PAP from endoplasmic reticulum into cytosol is similar to that utilized by type 2 RIPs to reach their subcellular target. This observation suggests that type 1 RIPs may also be able to follow the cellular route for misfolded proteins without being degraded by the proteasome. The same authors reported that site-directed mutagenesis of PAP expressed by yeast cells abolished cytotoxicity, although not affecting ribosome depurination, thus indicating that the inhibition of protein synthesis is not sufficient for cytotoxicity (Parikh BA *et al*., 2005; Hudak KA *et al*., 2004).

3.4 Cell fate: necrosis *vs* apoptosis

Apoptosis (programmed cell death) and necrosis are currently defined as the two major modes of cell death. In some instances, necrosis is a passive process characterized by the loss of plasma membrane integrity, cell swelling and inflammation due to the release of cellular contents into the periphery. In contrast, apoptosis is an active process generally characterized by morphological cell changes including cell shrinkage associated with cytoplasmic condensation and vacuolation, membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, and loss of cell adhesion (Zimmermann KC *et al*., 2001). Apoptosis is initiated in response to stimuli which activate genetically programmed signaling cascades. Cytoplasmic constituents are not spilled into the extracellular milieu so that inflammation is not a characteristic of apoptotic cell death. Apoptotic bodies are generally internalized and processed by phagocytic cells. A major biochemical feature of apoptosis is the internucleosomal fragmentation of genomic DNA, that appears to be a multi-step process with DNA first cleaved into large fragments (50-200 kb) and subsequently degraded to nucleosomal units of multiples of 180-200 bp. Although many stimuli may activate programmed cell death, most stimuli appear to signal through a common, tightly regulated pathway involving the sequential activation of proteases called caspases. Caspases are cysteine-dependent aspartate-specific proteases that exist as inactive precursors or pro-caspases. In general, caspase activation requires a single proteolytic cleavage of a latent single-chain zymogen to produce a heterodimer. The caspase heterodimers then self pair to form the active tetrameric molecule (Stennicke HR and Salvesen GS, 2000). Programmed cell death takes place through two pathways categorized on whether apoptotic signals originate outside of or within cells. The extrinsic or extracellular pathway of programmed cell death generally requires the ligation of receptors of the tumor necrosis factor receptor (TNFR) superfamily containing cytoplasmic death domains. Caspase 8 is activated following its recruitment to the cytosolic face of the receptor-ligand complex. The intrinsic or mitochondrial pathway involves the disruption of mitochondrial membrane potential, the release of cytochrome c, and the activation of caspase 9. The intrinsic pathway of caspase activation is regulated by members of the Bcl-2 protein family, which include proapoptotic protein BH-3 interacting domain (Bid), Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), and anti-apoptotic proteins such as Bcl-2 and myeloid cell leukemia-1 (Mcl-1). The intrinsic pathway is responsible for apoptosis in response to ionizing radiation and cytotoxic chemotherapeutic drugs. Activation of caspase 8 or caspase 9 by the extrinsic or intrinsic pathways leads to the activation of a common downstream caspase, caspase 3. Caspase 3 activates the DNase that executes the cleavage of DNA. Additional molecular details of caspase activation can be found in recent reviews (Zimmermann KC *et al*., 2001; Kim JS *et al*., 2003).

The cytotoxicity of RIPs is commonly attributed to the inhibition of protein synthesis consequent to ribosomal damage. However, this view was challenged when it was found that ricin induced the expression of Apo2.7 (Bussing A *et al*., 1999) and Bak (Hu R et *al*., 2001), suggesting that *de-novo* protein synthesis occurred in the apoptotic process triggered by the toxin. Furthermore, it was observed that ricin and Shiga toxin cause disruption of DNA in human endothelial (HUVEC) cells, in a manner that appeared before the activation of caspase 3 (Brigotti M *et al*., 2002). This alteration occurred simultaneously to protein synthesis inhibition in the case of ricin, and shortly afterwards in the case of Shiga toxin. Furthermore, saporin caused damage to genomic DNA (Bagga S *et al*., 2003), and it was concluded that both the rRNA glycosidase activity and the DNA internucleosomal fragmentation contributed to cell death. All this suggests that multiple effects of RIPs on nucleic acids may concur to the pathogenesis of cell damage. At the cellular level, both type 2 (ricin, Griffiths GD *et al*., 1987; viscumin, Bussing A, 1996, abrin, Narayanan S *et al*., 2004) and type 1 RIPs (e.g. saporin, Bergamaschi G *et al*., 1996; Bolognesi A *et al*., 1996) cause apoptosis and subsequently, or at higher doses, severe necrosis both in the organs of poisoned animals and in cultured cells (Hughes JN *et al*., 1996). It was reported that various caspases, caspase-like and serine proteases and poly(ADP-ribose) cleavage are involved in the pathogenesis of the cell damage by ricin, and that protein synthesis inhibition may not be the sole cause of ricin-induced apoptosis (Komatsu N *et al*., 1998; Gan YH *et al*., 2000; Hu R *et al*., 2001). At least viscumin (Mannel DN *et al*., 1991) and ricin (Licastro F *et al*., 1993) bring about release from mononucleated cells of cytokines, which undoubtedly contribute to toxicity.

More data are available about the Shiga toxin family (Stxs), the only RIPs belonging to the bacterial kingdom (Cherla RP *et al*., 2003). It has become increasingly clear that Stxs induce apoptosis in some, but not all, cell types. The capacity of the toxins to trigger programmed cell death pathways may contribute to the development of bloody diarrhea and extraintestinal complications. An overall model of apoptosis induction mechanisms by Stxs derived from recently described studies is shown in Figure 32. Apoptosis induction is complex, and may involve extracellular and intracellular signaling pathways. Although Stx-mediated activation of the upstream caspase, caspase 8, has been reported, it is not clear whether caspase 8 is activated by the extrinsic pathway acting through engagement of membrane-associated receptors, or by intrinsic signaling proteins through the ribotoxic stress response. Receptor-ligand complexes directly interacting with caspase 8 following treatment of cells with toxins or purified B-subunits remain to be identified. If apoptosis induction requires retrograde transport, the precise points in the trafficking process initiating apoptotic signals are currently unknown. Studies using HeLa and HMEC-1 cells have suggested that toxin enzymatic activity is necessary to induce apoptosis through a mechanism completely independent of caspase

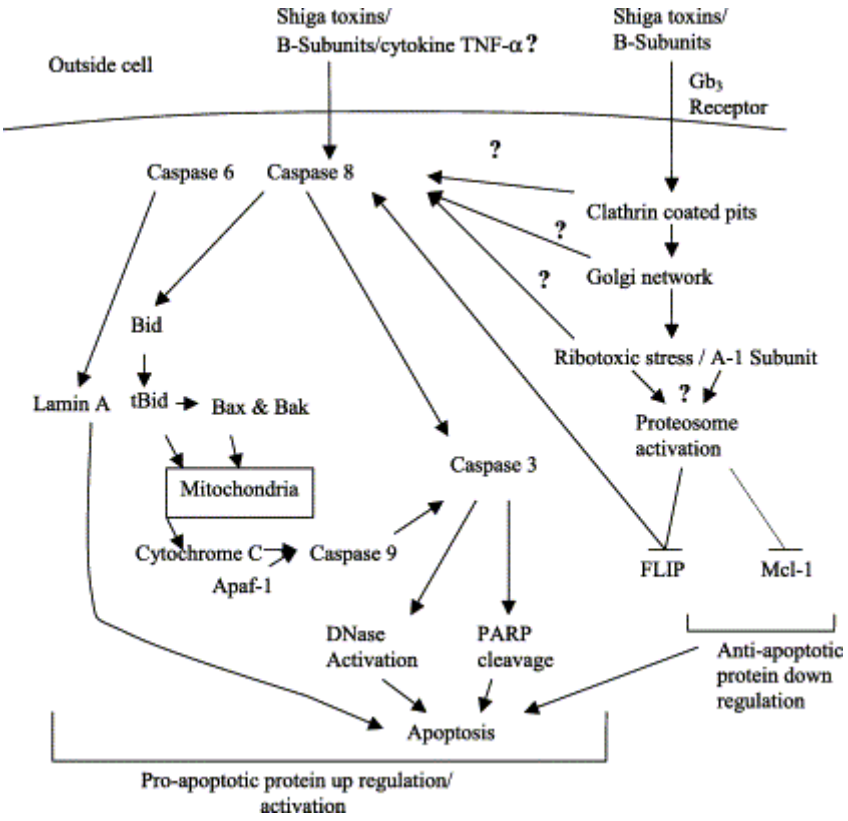


Figure 32. Putative mechanisms of apoptosis triggered by Stxs. Question marks denote steps in programmed cell death pathways requiring further experimental clarification. (Cherla RP *et al*., 2003).

activation. Such a mechanism may involve protein synthesis inhibition and proteasome-mediated degradation of anti-apoptotic proteins. Finally, it should be noted that Stxs may not trigger a uniform apoptotic signaling cascade in all cell types. Thus, the differential signaling events caused by the toxins in different cell types await further clarification.

The mechanism of apoptosis inducted by RIPs might therefore depend on the mechanism of the action of the toxin rather than protein synthesis inhibition, the end result of ribosome inactivation. Further, the picture is made more complex by contradictory reports in the literature with respect to the involvement of p38 MAPK and JNK 1/2 in CHX-induced apoptosis (Yang GH *et al*., 2000; Johnson CR *et al*., 2003). Figure 33 summarizes various signaling cascades and pathways that are triggered by RIPs to induce cell death. It is quite clear from the picture that most of the pathways are funneled into the mitochondria. It is well known that mitochondria play a major role in several stress induced cell death pathways and damage to mitochondria with subsequent loss of mitochondrial membrane potential has been known to be the point of no return in apoptotic cascades. It is logical to assume that RIPs do not activate the cellular apoptosis machinery directly

as the primary activity of these toxins is to inactivate ribosomes and stop protein synthesis in cells. Cells are known to respond to several stress conditions like heat shock, infection by viruses or loss of cellular ATP. Cells, depending upon the extent of stress they are exposed to, either try to overcome the stress by activating various stress response genes or undergo cell death. But how cells decide when to activate the stress negating pathway and when to induce apoptosis is still not known. Mitochondrion is a good candidate for sensing the stress induced by RIPs, namely ribotoxic stress in cells, as it is the origin of intrinsic apoptotic signaling cascade.

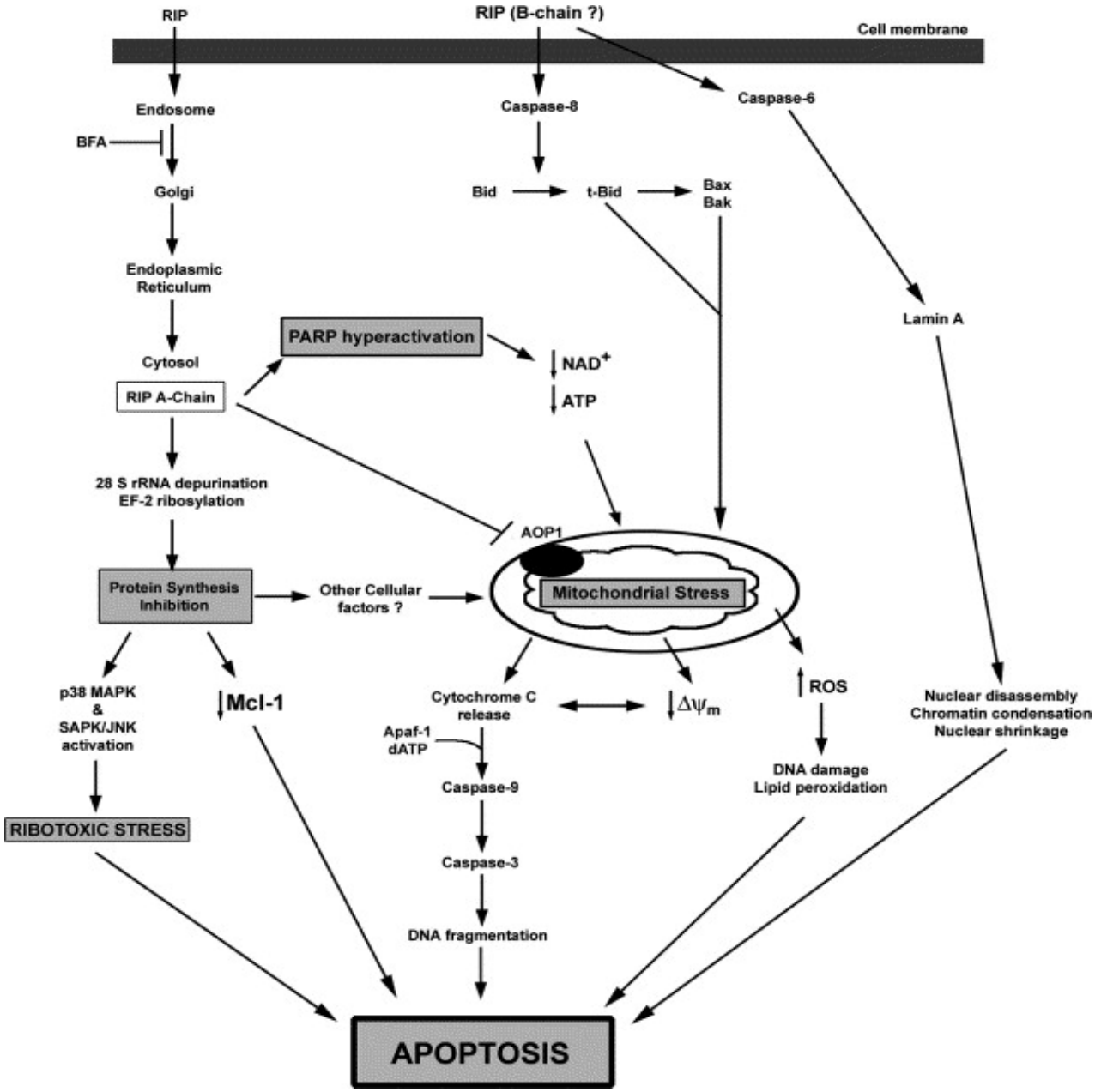


Figure 33. Various pathways of RIP-induced apoptosis. The possible mechanisms and pathways by which RIPs can induce cell death are shown.(Narayanan S *et al.*, 2005).

Figures 32 and 33, even though designed by different authors on different subjects (Stxs family *versus* RIPs in general), share common pathways and general ideas, that are present throughout the literature, even if with many unanswered questions. The concentration of the toxin is supposed to indicate the choice between necrosis and apoptosis, with the first one usually obtained at high concentration. Though the literature indicates that RIPs induce cell death by apoptosis at low concentration, there are considerable differences in the kinetics as well as extent of apoptosis observed for a given concentration of toxin. Though all RIPs induce protein synthesis inhibition, it is possible that the pathway of apoptosis induced by RIPs depends upon the type of cells used as well as the RIP used. RIP structure and concentration might influence the uptake and the routing, therefore changing protein localization and, at the end, cell response and fate.

CHAPTER 4
HYDROGEN SULPHIDE

4.1 Overview of signalling gaseous compounds

The last two decades or so have seen a marked escalation in interest in the biology of endogenous, biologically active gases. With the most significant of these being O_2, cells are indeed constantly enveloped in and thus exposed to a large number of different gases and, consequently, it can be no great surprise that many of those gases will have roles in the regulation of cell function (Figure 34). The turning point in 'gas biology' came with the realization that such gases are generated naturally within mammalian cells; in each case, multiple enzyme isoforms have evolved to generate these signalling molecules. It was a great wonder for scientific community when the endothelium-derived relaxing factor (EDRF) was identified as nitric oxide (NO), a simple inorganic molecule, because all hormones, mediators and neurotransmitters known before were organic compounds. Now there is no doubt that NO plays important regulatory roles in almost all tissues (Stefano GB, 2003). Soon thereafter, the second inorganic gaseous compound, carbon monoxide (CO), was recognized as an endogenously produced mediator and neurotransmitter. CO is synthesized during the catabolism of heme to biliverdin by heme oxygenase (HO). All biological active compounds have many features in common. Apart from being gases at atmospheric pressure, they all are soluble in water to a greater or lesser extent and all are able to freely penetrate cell membranes. Interestingly, NO and CO share at least one common mechanism of action, i.e. they stimulate soluble guanylate cyclase and increase intracellular cGMP concentration, although CO is a much weaker activator than NO (Beltowski J *et al*., 2004; Herman ZS, 1997).

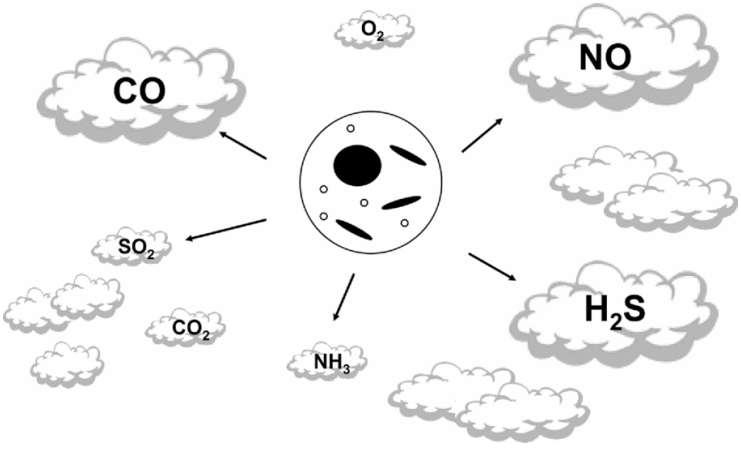


Figure 34. The gaseous environment of mammalian cells. (Li L and Moore PK, 2007).

4.2 Hydrogen sulphide as a new signalling molecule

Recent studies indicate that another "toxic gas", hydrogen sulfide (H_2S), is also produced in substantial amounts by mammalian tissues and exerts many physiological effects suggesting its potential role as a regulatory mediator. H_2S, the colourless gas with a strong odour of rotten eggs, was known for decades only as a toxic environmental pollutant. The main mechanism of its toxicity is a potent inhibition of mitochondrial cytochrome c oxidase. In fact, H_2S is a more potent inhibitor of mitochondrial respiration than cyanide (Reiffenstein RJ *et al*., 1992). Although endogenous H_2S was found in the brain at the end of 1980s (Warenycia MW *et al*., 1989), it was initially suggested to be an artifact since sulfide concentration rapidly increases postmortem in mammalian tissues (Nagata T *et al*., 1990), and may be easily released from so called "sulfane sulfur" (compounds containing sulfur atoms bound only to other sulfur atoms) during tissue preparation (Iciek M *et al*., 2005). That H_2S may operate as an endogenous neurotransmitter was first suggested a decade ago when it was described the enzymatic mechanism of H_2S production in the brain, its biological effects at physiological concentrations and its specific cellular targets (Abe K and Kimura H, 1996). Now H_2S is increasingly recognized as a member of a growing family of "gasotransmitters", together with its two counterparts, NO and CO. However, much less is known about the physiological role of H_2S than about either NO or CO.

Under physiologically relevant conditions, i.e. in aqueous solutions and at pH 7.4, one third of H_2S is undissociated and two thirds dissociate into H^+ and HS^- (hydrosulfide ion), which subsequently may decompose to H^+ and sulfide ion (S^{2-}). However, the latter reaction occurs only at high pH, thus S^{2-} does not occur *in vivo* at substantial amounts. Sodium hydrosulfide (NaHS) is commonly used as an H_2S donor since it dissociates to Na^+ and HS^-; the latter then partially binds H^+ to form undissociated H_2S. Similarly to NO and CO, H_2S is lipophilic and freely permeates plasma membranes, although due to partial dissociation membranes are relatively less permeable to H_2S than to both other gases. H_2S is detectable in serum and most tissues at a concentration of about 50 μM. Its physiological level in the brain is up to three-fold higher than in serum and is in fact close to toxic concentration.

H_2S is produced at significant amounts in most tissues. The highest rate of production was noted in the brain, cardiovascular system, liver and kidney (Doeller JE *et al*., 2005). The only substrate for the generation of endogenous H_2S is L-cysteine, a sulfur-containing amino acid derived from alimentary sources, synthesized from L-methionine through the so-called "*trans*-sulfuration pathway" with homocysteine (Hcy) as an intermediate, or liberated from endogenous proteins. There are two major pathways of cysteine catabolism (Iciek M *et al*., 2005; Stipanuk MH, 2004) (Figure 35). One of them is oxidation of –SH group by cysteine dioxygenase (CDO) to cysteine

sulfinate, which may be decarboxylated to hypotaurine or converted to pyruvate and sulfite, subsequently oxidized to sulfate by sulfite oxidase. The second pathway, referred to as "desulfhydration", is associated with the removal of cysteine sulfur atom without its oxidation and results in H_2S production. This process may be catalyzed by either of the two enzymes of the Hcy *trans*-sulfuration pathway: cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1). Both are pyridoxal 5'-phosphate (vitamin B6)-dependent, but differ in the specific mechanism of H_2S formation. CBS and CSE are widely distributed in tissues, however, CBS is a predominant source of H_2S in the central nervous system whereas CSE is a major H_2S-producing enzyme in the cardiovascular system. In some tissues such as the liver and kidney, both enzymes contribute to H_2S generation. Little is still known about the regulation of these two H_2S-producing enzymes.

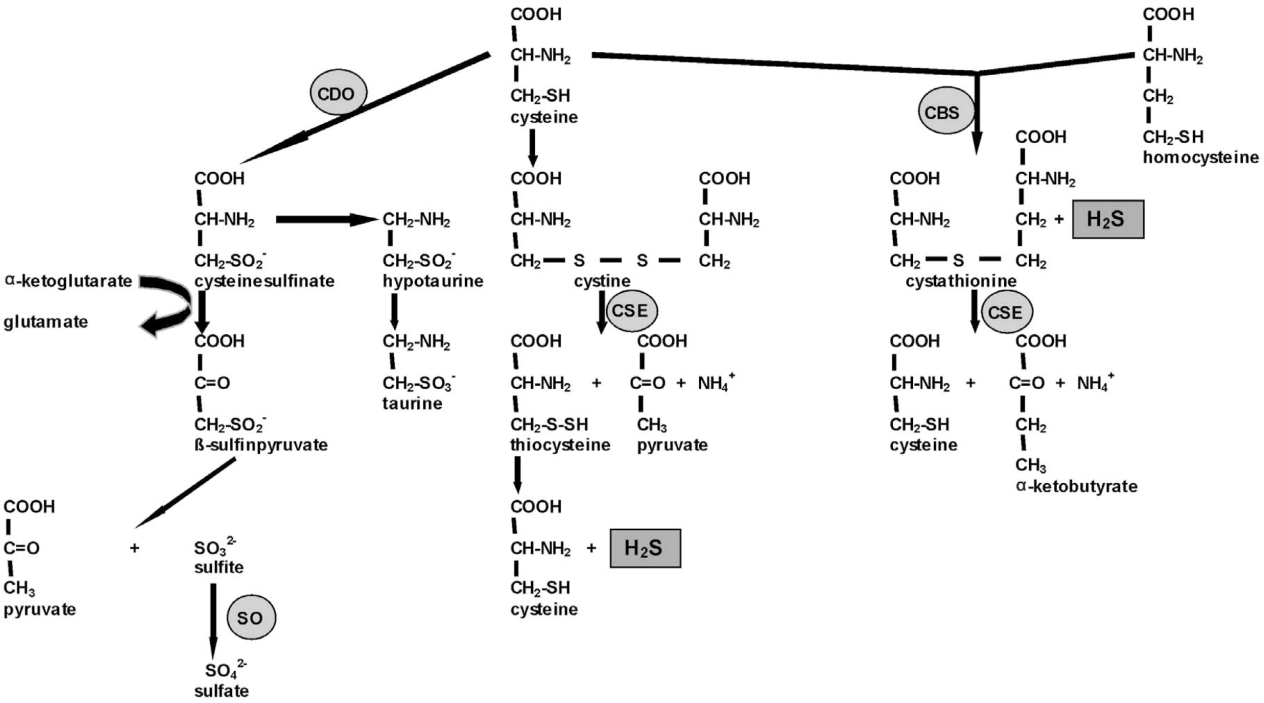


Figure 35. Cysteine metabolism. CBS – cystathionine β-synthase, CDO – cysteine dioxygenase, CSE – cystathionine γ-lyase, SO – sulfite oxidase. (Lowicka E and Beltowski J, 2007).

H_2S is rapidly oxidized, mainly in mitochondria, initially to thiosulfate which is further converted to sulfite and sulfate. Oxidation of H_2S to thiosulfate is probably a nonenzymatic process associated with mitochondrial respiratory electron transport, although superoxide dismutase may also catalyze this reaction (Searcy DG, 1996). The second pathway of H_2S metabolism is the methylation by thiol S-methyltransferase (TSMT) to methanethiol and dimethylsulfide (Furne J *et al*., 2001). This

reaction occurs mainly in the cytosol. Finally, H_2S may bind to methemoglobin to form sulfhemoglobin. Because hemoglobin may also bind NO and CO, it is a common "sink" for all three gasotransmitters.

Signaling mechanisms triggered by H_2S recognized so far are listed in Table 7. H_2S is a strong reducing agent. Therefore, it has been suggested that some of its effects may be mediated by protection of protein thiol groups from oxidation. However, all studies performed to date indicate that effects of H_2S applied at physiological concentration are not reproduced or only partially mimicked by thiol-protecting agents, suggesting that this is not a major mechanism of H_2S action (Abe K and Kimura H, 1996). In many systems, the effect of H_2S is mediated by ATP-sensitive potassium channels (K_{ATP}). H_2S is a highly reactive molecule and may easily react with other compounds, especially with reactive oxygen and nitrogen species (ROS and RNS). It has been demonstrated that H_2S reacts with at least four different ROS, superoxide radical anion (Mitsuhashi H *et al*., 2005), hydrogen peroxide (Geng B *et al*., 2004), peroxynitrite (Whiteman M *et al*., 2004) and hypochlorite (Whiteman M *et al*., 2005). All of them are physiologically relevant ROS or RNS. All these compounds are highly reactive and their interaction with H_2S results in the protection of proteins and lipids from ROS/RNS-mediated damage (Whiteman M *et al*., 2004; 2005). Significance of H_2S reaction with O_2 is ambiguous since the product, sulfite, may have both toxic (Collin M and Thiemermann C, 2005) and antioxidant (Mitsuhashi H *et al*., 2001) properties, most likely depending on its concentration. H_2S also reacts with NO to form a nitrosothiol compound with yet undefined chemical structure (Whiteman M *et al*., 2006). Interestingly, in contrast to other nitrosothiols (R-S-NO) which are considered to be a reservoir of NO and often mimic its activity, the nitrosothiol originating from H_2S and NO is inactive. It has been suggested that H_2S may scavenge the excess of NO produced in the inflammatory state (Whiteman M *et al*., 2006), but may also limit the availability of NO continuously produced at physiological concentrations (Ali MY *et al*., 2006). Additional mechanism through which H_2S may exert antioxidant effect involves stimulation of cysteine transport to the cells and enhancement of glutathione synthesis (Kimura Y and Kimura H, 2004). Moreover, H_2S has been demonstrated to stimulate heme oxygenase expression and CO production, and to have bidirectional effects on the extracellular signal-regulated kinases (ERK) and inducible NO synthase. It is unclear if these effects are primary or result from the stimulation of other targets such as K_{ATP} channels.

Cellular H_2S known effects translate to many disease phenotypes still not well defined. H_2S is involved in the regulation of vascular tone, myocardial contractility, neurotransmission, and insulin secretion. H_2S deficiency was observed in various animal models of arterial and pulmonary hypertension, Alzheimer's disease, gastric mucosal injury and liver cirrhosis. Exogenous H_2S

ameliorates myocardial dysfunction associated with the ischemia/reperfusion injury and reduces the damage of gastric mucosa induced by anti-inflammatory drugs. On the other hand, excessive production of H_2S may contribute to the pathogenesis of inflammatory diseases, septic shock, cerebral stroke and mental retardation in patients with Down syndrome, and reduction of its production may be of potential therapeutic value in these states.

Intracellular signaling mechanisms triggered by H_2S	**Diseases associated with changes in H_2S generation**	**Diseases in which H_2S has been demonstrated to be protective or deleterious**
Stimulation of K_{ATP} channels: blood vessels, myocardium, pancreatic β-cells, neurons, carotid sinus, smooth muscle of the colon Maintaining protein –SH groups in the reduced state Stimulation of adenylate cyclase Reaction with ROS and RNS: O2-, H2O2, ONOO-, ClO-, NO Stimulation of cysteine transport to the cell and GSH synthesis Stimulation and inhibition of ERK Stimulation of HO–CO pathway Stimulation and inhibition of iNOS Increase in intracellular Ca^{2+} Stimulation of TRPV1 channel	**Increased H_2S formation:** Down syndrome, septic shock, NSAID-induced gastric mucosal injury, colitis, caerulein-induced pancreatitis, diabetes mellitus, myocardial ischemia/reperfusion, ischemic preconditioning, COPD, ischemic stroke, febrile seizures **Decreased H_2S formation:** spontaneously hypertensive rats, arterial hypertension induced by NOS blockade, hypoxia-induced pulmonary hypertension, isoproterenol-induced myocardial injury, myocardial ischemia/reperfusion injury, NSAID-induced gastric mucosal injury, liver cirrhosis, Alzheimer's disease	**Protective:** arterial hypertension, pulmonary hypertension, myocardial ischemia/reperfusion injury, erectile dysfunction, gastric mucosal injury, colitis, irritable bowel syndrome, neuronal damage induced by febrile seizures **Deleterious:** septic shock, caerulein-induced pancreatitis, ischemic stroke Only those diseases are listed in which the effect of H_2S donors or inhibitors of H_2S-producing enzymes on the disease course was examined in *in-vivo* models

Table 7. Intracellular signaling and diseases associated with H_2S. For more data and references see Lowicka E and Beltowski J, 2007.

Nevertheless many effects of H_2S are still controversial. For example, H_2S has been demonstrated to either stimulate or inhibit certain intracellular transduction pathways, to stimulate (Deplancke B and Gaskins HR, 2003) or inhibit (Du J *et al*., 2004; Yang G *et al*., 2004a) cell proliferation, to activate (Yang G *et al*., 2004b; Yang G *et al*., 2006) or block (Rinaldi L *et al*., 2006) apoptosis, to be overproduced (Zhu YZ *et al*., 2007) or deficient (Bian JS *et al*., 2006; Geng B *et al*., 2004) in myocardial ischemia, to be pro- (Bhatia M *et al*., 2005) or antiinflammatory (Zanardo RC *et al*., 2006) in the model of hindpaw edema, etc. Only few studies demonstrated alterations in H_2S level in human diseases (Table 7), and in most cases it was done indirectly by measuring H_2S-related compounds such as thiosulfate (Belardinelli MC *et al*., 2001) or sulfhemoglobin (Lyons J *et al*., 2001) rather than H_2S itself. As may be concluded from the data presented above, production of endogenous H_2S is altered in many diseases, at least in experimental studies. In addition, both exogenous and endogenous H_2S has been demonstrated to exert either protective or deleterious effect in many pathologies (Table 7). Thus, the question arises if pharmacological modulation of H_2S level could be of a potential therapeutic in the future.

4.3 Sulphur compounds and radical stress

Functionalized aliphatic thiols (RSHs) are contained in living organisms in considerable amounts. Typical levels of intracellular non-protein thiol (principally glutathione, GSH) are in the order of 5 mM, although values of around 10 mM have been reported (Smith CV *et al*., 1996). The total level of protein SH may approach or exceed that of GSH. For example metallothionein, a protein with a low molecular mass found in the cytosol of eukaryotic cells, is rich in sulfur (23–38% of amino acids are cysteines). Therefore, they represent a significant portion of total cell protein thiols (Dunn MA *et al*., 1987). The thiol group of the main sulfur-containing amino acid, cysteine, is found predominantly in the unionized state under physiological conditions, being the thiol pKa in the range of 8–9. Once formed, the thiolate specie RS– is one of the most reactive functional groups found in proteins. It can react as a nucleophile and attack a disulfide bond, R_1-S-S-R_2, displacing one sulfur atom and forming a new disulfide bond, R-S-S-R_1 or R-S-S-R_2. Such disulfide bond formation is a versatile oxidation that is used biologically in a diversity of processes such as enzyme catalysis, protection against oxidative damage, stabilization of extracellular proteins and regulation of biological activity. This reaction exchanges redox equivalents between different thiol/disulfide pairs and serves to oxidize one thiol (RSH) while reducing another disulfide (R_1-S-S-R_2) (Singh R and Whitesides GM, 1993; Bulaj G *et al*., 1998). Recently, its implication in signal transduction and regulation of redox transcription factors was shown (Bulaj G *et al*., 1998).

Several investigations have focused on the reactivity of thiol groups based on the production of sulfur-centered radical species, RS•. Thiyl radical generation turned out to be important for several biological mechanisms, such as the enzymatic functioning of ribonucleotide reductase and pyruvate formate lyase (Stubbe J, 2003; Buckel W and Golding BT, 1999) . It must be taken into account that different sulfur moieties are present in biomolecules, that is, RSHs, disulfide (RS-SR), thioester [RS-C(O)R'] and thioether functionalities (RSR'), from which pathways can be described for the generation of thiyl radicals.

The most relevant pathway is from thiols, which is well known as the 'repair' reaction. As S-H bond strengths are generally weaker than those characterizing C-H bonds, thiols (RSH) tend to act as radical repair agents by tranferring H to C-centered radical generated from the breakage of a C-H bond, which can occur through enzyme activity, radiation or toxic agents (Halliwell B and Gutteridge JMC, 2001). The repair of radical DNA species shown in equation 1 was mostly investigated in radiation studies of biomolecules (von Sonntag C, 1987). Among others, this reaction provides a rationale for the pharmacological application of thiols as radiation-protecting

substances, certainly in sense of repair reactions instead of primary shielding (Prager A *et al*., 1993; Savoye C *et al*., 1997; Koning AWT *et al*., 1979).

$$DNA\bullet + RSH \rightarrow DNA + RS\bullet \quad (1)$$

An example is given by GSH as the dominant intracellular antioxidant, which exerts its activity in the aqueous phase by enzymatic peroxide reductions as well as by repairing radical species, such as tyrosyl (PhO•) or peroxyl (ROO•) radicals, as shown in equation 2.

$$PhO\bullet \text{ (or } ROO\bullet) + RSH \rightarrow PhOH \text{ (or } ROOH) + RS\bullet \quad (2)$$

It is worth pointing out that the role of thiols as repairing agents is counterbalanced by the formation of thiyl radical species which can damage other biomolecules, such as amino acids (Pogocki D and Schöneich C, 2001), carbohydrates (Nauser T and Schöneich C, 2003) and lipid molecules, as already described in chapter 2**.** That is why thiols can be considered a double-edged sword in the biological environment (Shahid Akhlaq M *et al*., 1987). The reactivity of sulfur-centered radicals is well known in organic chemistry, and there are two processes efficiently carried out by these species: reversible addition to double bonds and H-atom abstraction from activated positions such as bisallylic hydrogen (Shahid Akhlaq M *et al*., 1987; Chatgilialoglu C and Guerra M, 1993; Bertrand MP and Ferreri C, 2001; Schöneich C *et al*., 1992). Only recently these two reactions have been taken into consideration for biologically related mechanisms, and in this context lipids have become an interesting target since their structures have reactive sites, such as C=C double bonds and bis-allylic positions. Recently, Lykakis *et al*. (2007) used a biomimetic model of vesicle suspension which mimics the aqueous and membrane compartments of a cell, and demostrated the potential of sulfhydryl radicals ($HS\bullet/S\bullet^-$) derived from H_2S to access the hydrophobic FA chains and attack the double bonds. The phospholipids produced in this way contained a high proportion of *trans* FA residues. This model (Figure 36) offers some insight into the chemical basis of the biological activity of H_2S, which has not yet been established. All this is important in view of the intriguing role of the sulfhydryl radical induced *cis-trans* coversion of lipids, either as a damaging or a signaling pathway.

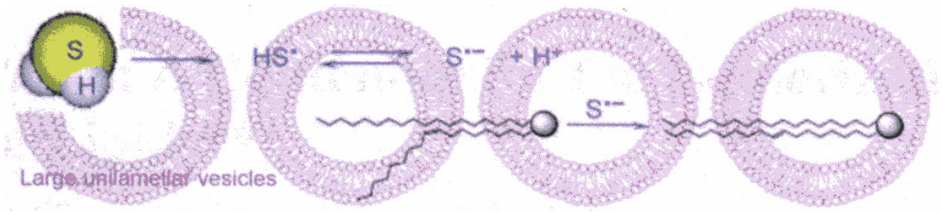


Figure 36. The sulfhydryl radical ($HS\bullet/S\bullet^-$), a contender for the isomerization of double bonds in membrane lipids. (Lykakis *et al*., 2007).

AIM of RESEARCH

Lipids and membranes have strongly emerged as factors with an undeniable influence on cell biology, that have been neglected until recently (van Meer G *et al*., 2008). The connection to medicine is straightforward since alterations of lipid metabolism are associated with human diseases (Maxfield FR and Tabas I, 2005). In the last years lipidomics is standing out as a research area with diverse goals, from mapping the entire spectrum of lipids in organisms to describing the function and the metabolism of individual lipids. This is also connected to the emerging roles of lipids as signaling molecules (Wenk MR, 2005; Watson AD, 2006; Spener F *et al*., 2003). In particular membrane lipidomics evaluates phospholipids and their dynamical changes under metabolic conditions, focusing on the type and quantity of fatty acid residues, which are particularly relevant for the regulation of membrane structure and functions (Dowhan WR and Bogdanov M, 2002; Cevc G, 1993). Fatty acid residues can be analysed after ordinary steps of isolation, derivatization to methyl esters (FAME) and characterization by gas chromatography (Christie WW, 2007). In this context, also the geometry of the double bonds has acquired more and more significance, since the naturally occurring membrane fatty acids have mostly the *cis* configuration, provided enzymatically, whereas the *trans* geometrical isomers can be considered natural only in bacteria (Zhang YM and Rock CO, 2008). In the last years the *cis* to *trans* conversion of unsaturated membrane lipids has been associated to free radical stress mostly deriving from sulfur-containing compounds (Chatgilialoglu C and Ferreri C, 2005; Ferreri C *et al*., 2005; 2005; Zambonin L *et al*., 2006).

The aim of this work was to investigate the fatty acid membrane modifications derived from several stress conditions and to envisage the connections between these changes and the cell response. The types of stress used to perturb cell homeostasis were: lipid supplementation and deprivation, cell intoxication and radical stress.

Lipid supplementation and deprivation are active subjects of research, due to the increasingly important role of lipids in cell functions and signalling, already highlighted in chapters 1 and 2. The effects have been studied by different strategies, for example, supplementing a single or mixed FA to the organism (Wiesenfeld PW *et al*., 2001; Tocher DR and Dick JR, 2001; Menendez JA *et al*., 2004; McKenzie KE *et al*., 1994) or using strains specifically mutated for the expression of desaturase or elongase enzymes (Murakami Y *et al*., 2000; Savonniere S *et al*., 1996). In particular, palmitic acid is an important fatty acid for cell growth (Cook HW and McMaster CR, 2002) but it can also cause apoptosis at higher concentrations (Yang X and Chan C, 2007; Kondoh Y *et al*., 2007; Landau Z *et al*., 2006; Ulloth JE *et al*., 2003; Mu YM *et al*., 2001); however, any information of the correspondent membrane lipidome changes is available to understand these contrasting roles.

Therefore, our investigation was first focused on the reorganization of the lipid bilayer after serum deprivation or palmitic acid supplementation using a neuroblastoma cell line (NB100).

As already described in chapter 3, ribosome-inactivating proteins (RIPs), are potent toxins able to target ribosomes and damage them in an irreversible manner, acting enzymatically in a less-than-equimolar ratio; thus the inhibition of protein synthesis seems to be the main action caused by these toxins (Stirpe F, 2004). However, there are other actions, still uncertain, exerted inside the cell like DNA, PARP and mRNA depurination. It is probable that RIPs do not activate the cellular apoptosis machinery directly, as their primary activity seems to be ribosomes inactivation and protein synthesis termination in cells (Stirpe F, 2004). Cells are known to respond to several stress conditions and, depending upon the extent of stress they are exposed to, either try to overcome the stress by activating various stress response genes or undergo cell death. But how do cells decide when to activate the stress-overcoming pathways, or when to induce apoptosis, is still not known, and nothing is known about what happens in the plasma membrane after RIPs intoxication. Moreover, the concentration of the toxin is supposed to indicate the choice between necrosis and apoptosis, with the first one usually obtained at high concentrations. Though the literature indicates that RIPs induce cell death by apoptosis at low concentration, there are considerable differences in the kinetics as well as the extent of apoptosis observed for a given concentration of toxin (Polito *et al*., 2008; Narayanan S *et al*., 2005). It is also possible that the pathway of apoptosis induced by RIPs depends upon both the type of cells used and the RIP used. RIP structure and concentration might influence the uptake and the routing, therefore changing protein localization and, at the end, cell response and fate (Polito *et al*., 2008; Narayanan S *et al*., 2005). Here the best known type I and type II RIPs, namely saporin and ricin, were used to induce a cytoplasmic stress. In these conditions, changes in the fatty acid content of the plasma membrane can make part of the cell reaction in order to respond to the insult, or to trigger its own apoptosis.

Functionalized aliphatic thiols (RSHs) are contained in living organisms in considerable amounts as they partecipate to the antioxidant network. However, recent investigations focused on the reactivity of thiol groups based on the production of sulfur-centered radical species, RS• (thiyl radicals) as the result of the repair reaction (RSH → RS•) (Halliwell B and Gutteridge JMC, 2001; von Sonntag C, 1987; Prager A *et al*., 1993; Savoye C *et al*., 1997; Koning AWT *et al*., 1979). The reactivity of sulfur-centered radicals is well known in organic chemistry, but only recently these radical species have been taken into consideration for biologically related mechanisms (Pogocki D and Schoneich C, 2001; Nauser T and Schoneich C, 2003), and in this context lipids have become an interesting target since their structures have reactive sites, such as C=C double bonds and bis-allylic positions (Chatgilialoglu C *et al*., 2002; 2005; Chatgilialoglu C and ferreri C, 2005; Ferreri C and

Chatgilialoglu C, 2005). As highlighted in chapter 4, hydrogen sulfide (H_2S) is the simplest thiol produced in substantial amount by mammalian tissues (50 mM in serum and in most tissues) and it exerts many physiological effects suggesting its potential role as a regulatory mediator (Lowicka E and Beltowski J, 2007). Recently, Lykakis IN *et al*. (2007) used a biomimetic model of vescicle suspension which mimics the aqueous and membrane compartments of a cell, and demostrated the potential of sulfhydryl radicals ($HS^{\bullet}/S^{\bullet -}$) derived from H_2S to penetrate the hydrophobic fatty acid chains, to attack double bonds and to produce a high proportion of *trans* fatty acid residues. This model offered some insight into the chemical basis of the biological activity of H_2S, which has not yet been established. All this is important in view of the intriguing role of the sulfhydryl radical-induced *cis-trans* conversion of lipids, either as a damaging or a signaling pathway. Because of the lack of informations still present about live cells, it would have been important to check the H_2S behavior in cells membrane lipids. H_2S was therefore used here to generate a thiyl radical-derived stress, mimicking a diffuse alteration of the radicals/antioxidants ratio in the cytoplasm of the cell. Since thiyl radicals represent the most relevant radical species able to target lipids double bonds and to obtain a *cis-trans* isomerization, the follow-up of fatty acid composition of human neuroblastoma cell membranes (NB-100) is reported with a parallel evaluation of cell viability and apoptosis.

In the present work, the follow-up of fatty acid composition of human neuroblastoma cell membranes (NB-100) is reported, first under deprivation of fetal calf serum (FCS), which affects the essential fatty acid content, mainly polyunsaturated fats, and then under palmitic acid supplementation at different concentrations. Parallel evaluations of cell viability and apoptosis were also carried out. The aim was to explore when and to what extent membrane fatty acid changes occur in these two cases, and to gather information on whether the correspondent lipid reorganization could synergize with phospholipase activation and signalling pathways, for example the release of free arachidonic acid (Cao Y *et al*., 2000; Ghosh J and Myers CE, 1998; Matsuyama M *et al*., 2005; Penzo D *et al*., 2004) connected with the apoptotic fate. Based on the hypothesis of a membrane lipid partecipation to the cells events, other two conditions were studied with NB-100 cell lines: RIPs intoxication and H_2S-derived radical stress. Fatty acid composition of cell membranes was followed in parallel with other biological parameters like cell viability, caspases activity, protein synthesis. Alteration of membrane homeostasis was thus explored as the cause or the consequence of cell response to a perturbation applied at a certain time somewhere in the cellular space; cell fate was followed in connection with membrane fatty acid changes.

To summarize, the aim of this research was to demonstrate that the homeostasis of cell membrane fatty acids is critical for influencing the cell fate. It is logical to connect the changes in the homeostatic percentage of fatty acids present in membranes with the activity of proteic biosensors

embedded in the double layer, that could directly detect membrane changes, resulting in multiple pathways of cell responses. Depending on the extent of cell stress and the ability of reorganization, this could lead the cells to fast and resolving responses, or to death through triggering the apoptotic cascade.

More generally, this work intends to increase attention on the application of lipidomics to the follow-up of the fatty acid status and changes, influencing membrane reorganization that can contribute to prime biochemical cascades. The goal was to highlight the role of membrane fatty acid changes in membranes depending upon the cellular conditions, considering not only the type of fatty acid involved and its quantity, but also the timing of the changes, in order to envisage strategies for reverting cell fate.

MATERIALS AND METHODS

Reagents and instruments

RPMI 1640, fetal calf serum, L-Glutamine, antibiotics, trypan blue, palmitic acid, n-hexane, sodium sulphide (H2S), 2,2'- Azobis(2-methylpropion-amidine) dihydrochloride (AAPH), chloroform and methanol were purchased from Sigma-Aldrich, San Louis, MO. Sodium sulphide (MW = 78.04) was weighted and melted in ethanol. The needed dilution in RPMI was then obtained, with a final ethanol concentration less then 1%. The type 1 RIP saporin-S6, otherwise named saporin, and the type 2 RIP ricin were purified from the seeds of *Saponaria officinalis* (Barbieri L *et al*., 1987; Ferreras JM *et al*., 1993) and *Ricinus communis* (Nicolson GL *et al*., 1974), respectively, as previously described. Flasks and plates were from Falcon, BD Biosciences, NJ. Trypsin/EDTA was from BioWhittaker Europe, Verviers, Belgium. CellTiter 96 Aqueous One Solution Cell Proliferation Assay was from Promega Corporation, Madison, WI. DAPI-Antifade was from Resnova SRL, Genzano di Roma, Italy. Other reagents used were from Sigma-Aldrich and Carlo Erba, Milano, Italy. Absorbance at 490 nm was measured by a microtiter plate reader Multiskan EX (ThermoLabSystems, Basingstoke, UK). Phase contrast microscopy was carried out with a Wilovert Standard PH 20 (HUND, Wetzlar, Germany) and a digital camera from Motic Microscopes, China. Fluorescence microscopy was performed with a Nikon Eclipse E600 fluorescence microscope equipped with a Nikon-dedicated digital camera, DXM1200F (Nikon, Tokyo, Japan).

Phospholipid extraction and fatty acid analysis

Cells (3×10^6) were pelleted by centrifugation at 14,000 × *g* for 40 min at 4°C and phospholipid extraction from cell membranes was performed as described in the literature (Ferreri C *et al.*, 2005a). The phospholipid fraction was treated with 0.5 M KOH/MeOH for 10 min at room temperature, and the corresponding fatty acid methyl esters (FAME) were formed, extracted with *n*-hexane, and examined by GC analyses. Geometrical *trans* fatty acids were recognized by comparison with standard references obtained by synthesis, as already described (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004).

Cell Culture

NB-100 cells, derived from a human neuroblastoma, were cultured at 37 °C in humidified atmosphere at 5% CO_2 in complete medium (RPMI 1640 supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-Glutamine, 100 units/mL Penicillin, 0.1 mg/mL Streptomicin, 0.9% NaCl) (Sigma-Aldrich). Cultures were maintained in log phase of growth with a viability >95%. Cells were checked for the absence of Mycoplasma infection.

To subculture or to seed cells for experiments, the medium was removed and the cell monolayer was washed with PBS Ca^{2+}/Mg^{2+} free. After five minutes of incubation with Trypsin/EDTA (200 mg/L EDTA, 500 mg/L Trypsin; 1 mL per 25 cm^2 flasks) (Sigma-Aldrich), cells were harvested and centrifuged at 500×g for 5 minutes at room temperature. The pellet was re-suspended in complete medium and the required number of cells was transferred in flasks or plates.

Experiments with/without FCS

Growth curves of NB-100 cells cultured in RPMI with or without FCS were investigated. Cells, 5×10^4/flask (25 cm^2), were seeded in 3 mL of medium. Every 24 hours the number of cells was determined by Trypan Blue dye exclusion. To analyse the effect of deprivation of FCS on membrane fatty acids, 1×10^6 cells were seeded in 25 cm^2 flasks, in 3 mL of complete medium with/without FCS. After 24 hours, cells were harvested with a cell scraper, centrifuged and washed twice with PBS. Fatty acid analysis of cell membranes was performed as above described. Data are mean of at least three experiments carried out in triplicate.

Experiments with palmitic acid. Viability tests.

Cells were checked for viability and adjusted to 2.5×10^4 cells/mL in complete medium, then 100 µL of cell suspension were loaded in a 96-well microtiter plate. After 24 hours, palmitic acid was dissolved in ethanol, diluted in complete medium and immediately added to cells (final concentration of ethanol <1%) (Ferreri C *et al.*, 2005a). In continuous incubation experiments, cells were exposed to 50 or 150 µM Palmitic acid for times ranging from 2h to 48h. In pulse and chase experiments cells were treated with palmitic acid at concentration ranging from 50 to 150 µM, for 1 or 2 hours, and then incubated for further 48 h in complete medium.

Viability was checked after the indicated times by adding 20 µL/well of CellTiter 96 Aqueous One Solution Cell Proliferation Assay. The absorbance at 490 nm was measured after one hour at 37°C.

To analyze the effect of palmitic acid on membrane fatty acids, 1×10^6 cells were seeded in 25 cm^2 flasks in 3 mL of complete medium. After 24 hours of incubation, medium supplemented with 150 µM palmitic acid was added. At different times, ranging from 0.5 to 24 hours, membranes were collected and analysed for fatty acid composition as above described.

All the other cell viability assays were evaluated with the same colorimetric assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Cells were seeded in 96-well microtiter plates in 100 µL/well of RPMI complete medium. After the treatment, 20 µl of the kit solution were added and, after 1 more hour of incubation, the absorbance at 492 nm was measured.

Western blot analysis of $cPLA_2$

Cells (3×10^6 /20 mL) were seeded in 75 cm^2 flasks and, after 24 hours, medium supplemented with 150 µM palmitic acid was added. At different times of incubation, ranging from 15 to 180 min, cells were harvested with a cell scraper, collected by centrifugation at 300 x *g* for 5 minutes and washed twice in PBS. Cell pellets were lysed by adding 100 µl of Cell Lytic-M (Sigma-Aldrich) supplemented with Protease Inhibitor Cocktail (1:100), Phosphatase Inhibitor Cocktail 1 (1:100) and sodium-orthovanadate (1:500) (Sigma-Aldrich). After 45 min at 0°C, vortexing every 5 min, insoluble material (nuclear pellet plus membranes) was removed by centrifugation at 12,000×*g* for 20 min at 4°C. Protein supernatant (cell lysate) was collected and stored a -80°C. Protein content was quantified by spectrophotometer and at 80 µg/lane of protein were separated by SDS-PAGE (10% gel) and blotted to Immobilon (polyvinylidene difluoride, PVDF) membrane (Millipore). Non-specific antibody binding sites were blocked by incubation with blocking buffer, TRIS buffered saline, 0.1% Tween 20 (TBS/T) with 5% w/v non-fat dry milk, for 1 hour at room temperature. After 5 washes with TBS/T, membranes were incubated overnight at 4°C with anti-phospho-$cPLA_2^{Ser\ 505}$ mAb (Cell Signaling Technology, Inc.; Beverly, MA) diluted in TBS/T with 5% bovine serum albumin, according to the manufacturer's instructions, as described.[37] After 5 washes with TBS/T, membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated anti-rabbit antibody (Sigma-Aldrich) diluted in blocking buffer. After further 5 washes, proteins were detected by incubating the membrane with Immobilon Western detection reagent (Millipore). The anti-phospho-$cPLA_2$ antibody was then stripped, 30 min in 25 mM glycine-HCl pH 2, 1% SDS (w/v), and, after blocking with non-fat milk, the membrane was reincubated with an antibody that recognises total $cPLA_2$ (Cell Signaling) to account for equal loading. Proteins were detected as above.

The level of expression of different proteins was analysed by using the public domain software Image J (a Java image processing program inspired by NIH Image). This program can calculate area and pixel value statistics of user defined selections. Briefly, X-ray film was scanned and saved as 8-bit greyscale JPEG file. The percentage of measurable pixels in the image was set by using the adjust image threshold command. The number of square pixels in the section selected (the protein band) was then counted by measuring the area in the binary or threshold image.

Cell protein synthesis

Cells (4×10^3/well) were seeded in 24-well microtiter plates in 250 µl of complete medium in the absence (control cultures) or in the presence of 10^{-6} M saporin or 10^{-12} M ricin. l-[4,5-^{3}H]leucine (74 kBq/well; GE Healthcare, Buckinghamshire, UK) was added at the same time of RIP for

exposure up to 4 h, or was pulsed for 6 h when samples were incubated for 4, 8, 16, 24 h. Cells were then incubated for 30 minutes at 4°C with 20% trichloroacetic acid (TCA); after three washes with TCA 5%, 200 µL/well of KOH 1N were added and incubation for 10 minutes at 37°C was performed. Cells were then harvested with a semiautomatic cell harvester onto glass-fibre diskettes and then incorporated radioactivity was determined by a β-counter (Beckman Coulter, Fullerton, CA, USA), with Ready-Gel scintillation liquid (Beckman Coulter).

Caspase-3/7, -8 and -9 activities

The caspase-3/7, -8 and -9 activities were assessed by the luminescent assays Caspase-Glo™ 3/7, Caspase-Glo™ 8 and Caspase-Glo™ 9 (Promega). Cells (0.4×10^3/well) were seeded in 96-well microtiter plates in 100 µl of complete medium containing 10^{-6} M saporin or 10^{-12} M ricin. After incubation for the indicated times, 50 µl/well of RPMI were added. Caspase-Glo™ 3/7, Caspase-Glo™ 8 or Caspase-Glo™ 9 (50 µl/well) were added and the luminescence was measured by Fluoroskan Ascent FL (Labsystem, Helsinki, Finland) following manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted using the XLSTAT-Pro software, version 6.1.9 (Addinsoft 2003). Results are given as means ± SD. Data from the Western blot analysis of $cPLA_2$ were analyzed by ANOVA tests with Dunnet's correction for multiple comparison.

RESULTS

Fetal calf serum fatty acid content

Fatty acids are fundamental blocks for cell growth and, as described in the introduction, essential fatty acids linoleic acid and α-linolenic acid are need from external sources in order to pursue n-6 and n-3 PUFA families. Essential fatty acids necessary to the cell growth are provided by Fetal Calf Serum (FCS), which is routinely added to cell cultures. Therefore, the fatty acid components of the FCS batches used in the experiments represent the "diet" to which cells are submitted. Before starting our experiments, various batches of FCS were therefore examined for their fatty acid content; analysis by gas chromatography were carried out as previously described (Ferreri C *et al.*, 2005a). In Table 8 the Fatty Acid Methyl Esters (FAME) content of five different FCS batches is reported to change considerably. This influenced the mean composition of cell membranes, as shown by comparing controls of the following experiments. On the basis of this observation, a careful follow-up of the experiments was necessary, in order to have good reproducibility of the starting cultures.

FAME[a]	1	2	3	4	5
16:0	30.0	31.3	31.1	35.2	34.6
16:1	5.7	3.0	3.6	7.4	7.3
18:0	13.7	14.2	13.4	8.7	9.7
9*t*-18:1	0.1	0	0	0.2	0.1
9*c*-18:1	20.5	27.1	26.6	29.4	30
11*c*-18:1	5.5	5.3	4.9	8.2	8.1
18:2	4.8	7.7	8.5	1.7	1.4
20:3	1.9	1.5	1.6	1.0	0.7
20:4	5.4	5.6	5.7	2.2	2.1
***trans*-20:4**[b]	0.5	0.2	0.5	0.3	0.3
SFA	43.7	45.5	44.5	43.9	44.3
MUFA	31.7	35.4	35.1	45	45.4
PUFA[#]	24.6	19.1	20.4	21.1	10.3
SFA/MUFA	1.38	1.28	1.27	0.97	0.97
Total *trans*	0.6	0.2	0.5	0.5	0.4

[a]FAME are obtained from membrane phospholipid extraction, derivatization, and GC analysis. [b]Evaluated with reference compounds (monotrans arachidonic acid isomers) obtained following references (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004). [#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA).

Table 8. Major fatty acids and *trans* fatty acids isomers in different batches of FCS used in the experiments.

Cell lines

Membrane fatty acid composition is not evaluated at the beginning of each molecular biology experiment. This standardization has not yet been considered in the general protocol, although the contribution of the membrane status could be an important issue to evaluate before proceeding to other metabolic assays. Five different human cell lines and their specific fatty acid compositions are listed in Table 9. From these data, Neuroblastoma cells (NB-100) were selected for the project because we checked the total absence of *trans* fatty acids; as human cells does not have the set of enzymes necessary for *trans* fatty acid production, their absence was thought to be a sign of unaltered characteristics. Moreover, NB-100 cells were preferred to Jurkat mainly for their capacity to grow adherent to the flask surface.

FAME[a]	**Jurkat** (Leukemia T cells)	**NB-100** (Neuroblastoma)	**Hela** (Uterine Carcinoma)	**RAJI** (Burkitt's Linfoma)	**JM** (Monocytes)
16:0	30.9	27.1	23.8	24.6	26.6
16:1	6.2	6.3	14.2	14.6	12.3
18:0	17.3	12.6	14.2	14.6	12.3
9*t*-18:1	0	0	0.3	0	0
9*c*-18:1	28.9	36.6	34.6	29.5	30.7
11*c*-18:1	7.1	9.4	6.3	7.5	8.7
18:2	0.6	3.2	2.8	5.6	4.5
20:3	2.3	0.2	0.2	0.9	0.2
20:4	4.7	4.2	3.6	4.4	2.0
***trans*-20:4[b]**	0	0	0.8	0.1	0.3
SFA	48.2	39.7	38	39.2	38.9
MUFA	42.2	52.3	55.1	51.6	51.7
PUFA[#]	9.6	8	6.9	9.2	9.4
SFA/MUFA	1.14	0.76	0.69	0.76	0.75
Total *trans*	0	0	0.8	0.1	0.5

[a]FAME are obtained from membrane phospholipid extraction, derivatization, and GC analysis. [b]Evaluated with reference compounds (monotrans arachidonic acid isomers) obtained following references (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004). [#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA)

Table 9. Major fatty acids and *trans* fatty acid isomers in different human cell lines.

Deprivation of fetal calf serum

Membrane phospholipid changes were evaluated in human neuroblastoma-derived NB-100 cells cultured for 24 hours in presence or absence of FCS, as described in Materials and Methods. Membrane isolation, phospholipid derivatization and analysis by gas chromatography were carried out as previously described (Ferreri C *et al.*, 2005a). The fatty acid determination included the recognition of geometrical *trans* isomers, by comparison with a *trans* lipid library obtained by commercially available references (such as elaidic acid, 9t-18:1) and by thiyl radical-catalyzed reaction of arachidonic acid, as already described (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004). Results are reported in table 10 for four independent experiments and compared with control cell lines under standard conditions.

FAME[a]	**GROUP A** [b] **(n=4)**	**GROUP B** [b] **(n=4)**
16:0	36.1 ± 2.3	43.7 ± 1.3 [c]
16:1	4.7 ± 0.5	4.5 ± 0.2
18:0	18.9 ± 1.9	22.0 ± 0.7 [c , e]
9*t*-18:1	0.7 ± 0.0	0.9 ± 0.1 [c]
9*c*-18:1	25.0 ± 1.9	18.5 ± 1.2 [d]
11*c*-18:1	6.9 ± 0.5	5.3 ± 0.4 [d]
18:2	2.4 ± 0.4	1.5 ± 0.4 [d , f]
20:3	0.8 ± 0.2	0.7 ± 0.2 [g]
20:4	3.5 ± 0.5	1.9 ± 0.1 [d]
trans*-20:4	0.36 ± 0.30	0.42 ± 0.37
SFA	55.0 ± 4.1	65.7 ± 1.8 [c]
MUFA	36.6 ± 2.8	28.3 ± 1.7 [d]
PUFA[#]	6.7 ± 1.0	4.1 ± 0.7 [d]
SFA/MUFA	1.5 ± 0.2	2.3 ± 0.2 [c]
Total *trans*	1.06 ± 0.3	1.32 ± 0.4

[a]FAME are obtained from membrane phospholipid extraction, derivatization, and GC analysis. [b]Values are mean ± SD and are expressed as percentage of the peak areas detected in the GC chromatogram. [c]Values higher than control group (GROUP A) p=0.03. [d]Values lower than control group (GROUP A) p=0.03. [e]Value correlated with oleic acid (r=-0.99 p=0.005) and with vaccenic acid (r=-0.96 p=0.04). [f]Value correlated with arachidonic acid (r=0.97 p=0.03). [g]Value correlated with arachidonic acid (r=0.976 p=0.02). ∗Geometrical mono-*trans* arachidonic acid isomers, evaluated with reference compounds according to references (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004). [#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA).

Table 10. Membrane phospholipid fatty acids of NB-100 cells: GROUP A is the control (with FCS) and GROUP B is incubated without FCS for 24 hours.

After 24 hours, the membrane fatty acid composition showed several statistically significant changes compared to the control group:

- An increase of the saturated components, palmitic (16:0) and stearic (18:0) acids (P=0.03);
- A decrease of the monounsaturated components, oleic (9c-18:1) and vaccenic (11c-18:1) acids (P=0.03) which are inversely correlated with stearic acid (P=0.005 and P=0,04, respectively);
- The decrease of n-6 fatty acids, linoleic (9c,12c-18:2) and arachidonic (5c,8c,11c,14c-20:4) acids (P=0.03);
- The presence of two types of geometrical *trans* isomers, 9t-18:1 and geometrical *trans* isomers-20:4, the latter being the sum of the four mono-*trans* geometrical isomers of arachidonic acid (Ferreri C *et al.*, 2005a; Penzo D *et al.*, 2004). It is worth noting that the presence of elaidic acid was significant compared to control cells (P=0.03), and it cannot be confused with a dietary contribution since FCS is absent.

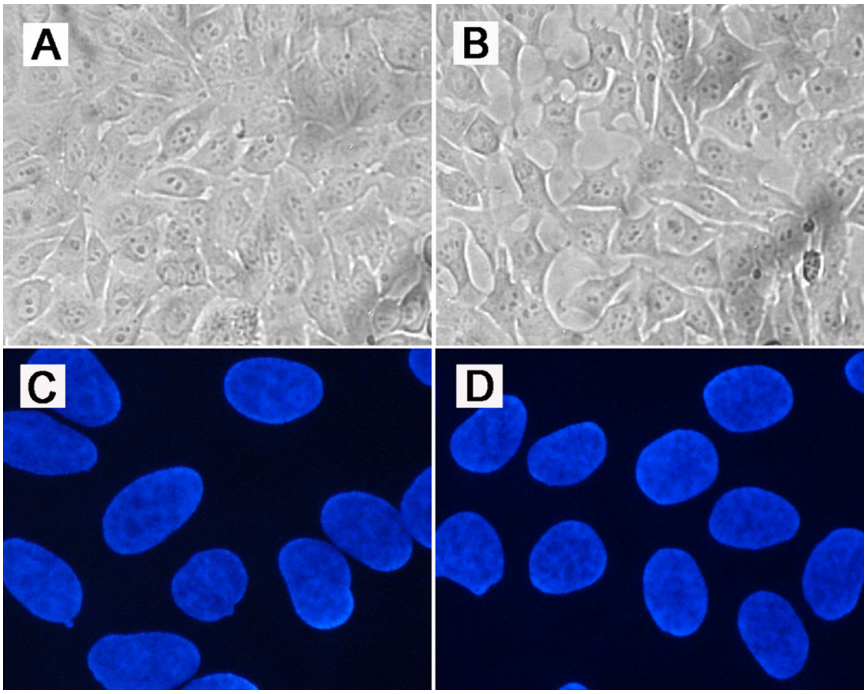


Figure 37. NB-100 cells morphology with and without FCS. NB-100 cells morphology assessed by phase contrast microscopy (A, B, ×200 magnification) and fluorescence microscopy (C, D, ×600 magnification objective). Control cultures grown in complete medium for 24 hours are shown in (A) and (C). Cells incubated in RPMI without FCS for 24 hours are shown in (B) and (D).

To analyze the effect of FCS deprivation on the growth, NB-100 cells were followed up by phase contrast microscopy during the first five days of cultivation with or without FCS. Cultures grown for 24 hours in medium with or without FCS are shown in Figures 37A and 37B, respectively. Cells cultured in absence of FCS appeared less packed. Therefore, growth curves and duplication times of NB-100 cells were calculated for cultures grown in complete medium ($y=48714e^{0,041x}$; $R^2=0,998$ and 17.943 hours) or in absence of FCS ($y= 50000e^{0,0245x}$; $R^2=0,986$ and 26.661 hours).
Apoptosis was monitored by fluorescence microscopy using DAPI nuclear staining. Figure 37 illustrates nuclear morphology of control cells compared with cells grown in RPMI without FCS (Figures 37C and 37D, respectively). No typical sign of early or late apoptosis was evident. These data suggest that deprivation of FCS in NB-100 cells is able to cause a decrease in the cellular duplication rate but not lethal damages.

Palmitic acid supplementation

Palmitic acid apoptotic effects have been reported in several papers, also recently, without providing information on lipidome changes (Yang X and Chan C, 2007; Kondoh Y *et al.,* 2007; Landau Z *et al.*, 2006; Ulloth JE *et al.*, 2003; Mu YM *et al.*, 2001). Our attention focused on palmitic acid, also because during FCS deprivation this saturated fatty acid was found to increase, therefore suggesting a comparison with a real supplementation. Concentrations <100 μM are reported to have no effect on cell viability (Nano JL *et al.*, 2003).
Figure 38 shows the detection of the NB-100 cell viability. In figure 38A (left graph) the viability of NB-100 cells in the presence of 50 μM (■) and 150 μM (□) palmitic acid was evaluated in a continuous incubation. Palmitic acid at 150 μM caused a strong reduction of viability. Viability was statistically lower than control cells already after 8 hours and was reduced to less than 25% after 48 hours. On the other hand, palmitic acid at concentration of 50 μM caused only a weak reduction of cell viability, not statistically significant. In order to better understand the fate of cells exposed for a short time (1-2 hours) to palmitic acid at various concentrations (50, 75, 100, 125 and 150 μM), pulse and chase experiments were performed. Figure 38A (right graph) shows the living cell percentage after 1 (●) or 2 hours (○) of exposure to palmitic acid, followed by washing and incubation in complete medium for additional 48 hours. It is worth noting that 2 hours, but not 1 hour, exposure at 150 μM palmitic acid is enough to produce a cell viability reduction of about 50%.

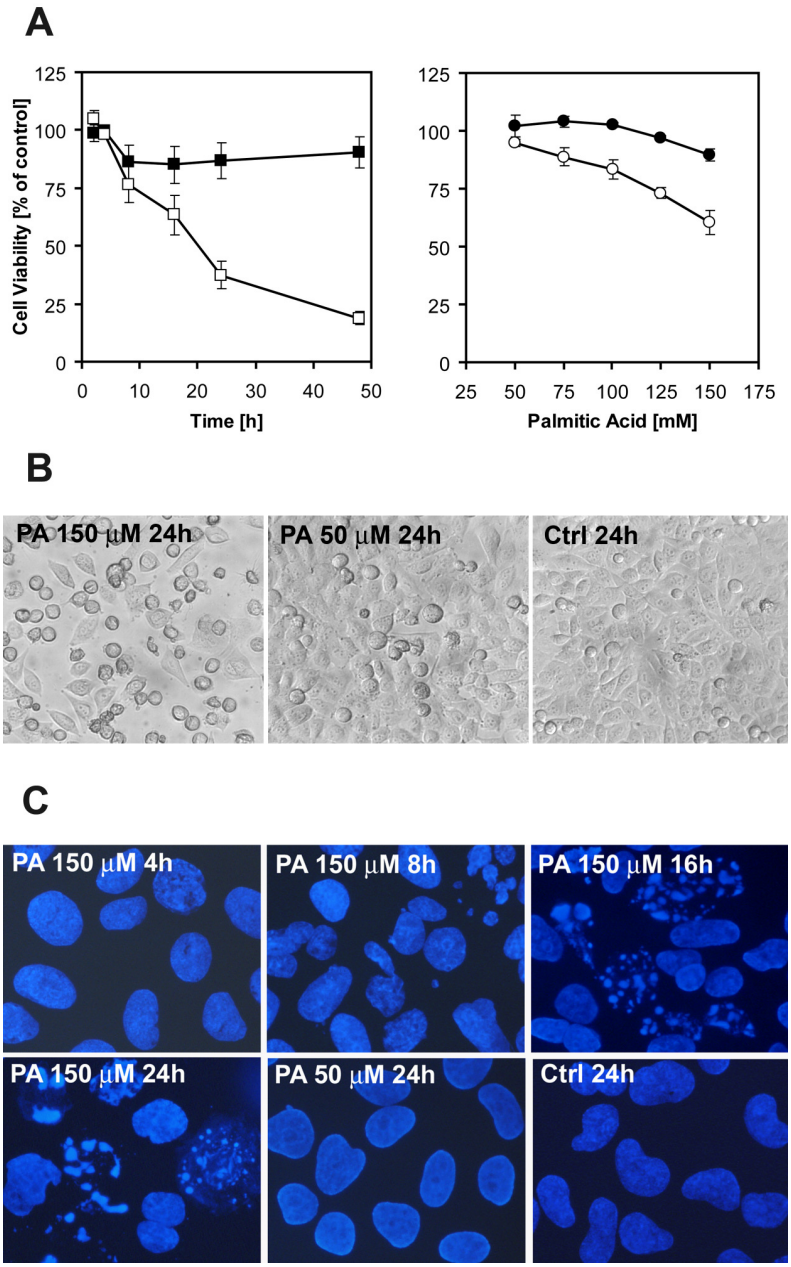


Figure 38. Effect of palmitic acid on NB-100 cells viability and morphology. (A) Effect of palmitic acid on cell viability - Left graphic: cells were incubated in complete medium supplemented with 50 µM (■) or 150 µM (□) palmitic acid. Right graphic: cells were incubated in complete medium supplemented with palmitic acid at various concentrations for 1 hour (●) or for 2 hours (○) and then incubated in complete medium for 48 hours after wash. Cell viability was determined by MTS assay. Values are means ± SD of four determinations. (B) NB-100 cells morphology assessed by phase contrast microscopy. Control cultures grown in the absence of palmitic acid are shown in comparison with cells treated for 24 hours with 50 µM and 150 µM palmitic acid. Magnification, 200×. (C) Nuclei of NB-100 cells stained with DAPI and assessed by fluorescence microscopy (×600 magnification objective). Cells were incubated in complete medium supplemented with 50 µM (24 hours) and 150 µM palmitic acid (4, 8, 16, 24 hours). Control cultures grown in the absence of palmitic acid (24 hours) are also shown.

Based on these indications, membrane fatty acid changes were strictly monitored at early stages of palmitic acid supplementation, carrying out analyses every 30 minutes after addition of 50 and 150 μM concentrations up to a three hours period. Control cells were followed up for the same time. Table 11 shows the results at the highest dose.

FAME[a]	Control[b] (n=6)	PA 30'[b] (n=4)	PA 1h[b] (n=4)	PA 1h30'[b] (n=4)	PA 2h[b] (n=4)	PA 2h30'[b] (n=4)	PA 3h[b] (n=6)
16:0	26.7 ± 1.9	32.2 ± 0.2 [c]	38.7 ± 1.6 [c]	40.2 ± 0.3 [c]	43.1 ± 0.2 [c]	44.8 ± 0.7 [c]	45 ± 1.4 [e]
16:1	6.6 ± 0.8	5.4 ± 0.1	5.1 ± 0 [d]	4.7 ± 0.1 [d]	4.7 ± 0 [d]	5 ± 0 [d]	6.4 ± 0.9
18:0	14.1 ± 1.3	10.1 ± 0.4 [d]	9.3 ± 1.4 [d]	10.7 ± 0.5 [d]	10.2 ± 0.2 [d]	8.6 ± 0.6 [d]	10.7 ± 1.4 [f, l]
9*t*-18:1	0.4 ± 0.4	0.1 ± 0 [d]	0.2 ± 0 [d]	0.2 ± 0 [d]	0.2 ± 0 [d]	0.2 ± 0 [d]	0.3 ± 0.2
9*c*-18:1	32.2 ± 2.1	31.4 ± 01	28.3 ± 0.3 [i]	26.7 ± 0.1 [d]	24.9 ± 0.2 [d]	24.6 ± 0.1 [d]	22.4 ± 1.4 [f]
11*c*-18:1	8.1 ± 1	6.1 ± 0.2 [i]	5.3 ± 0.2 [d]	5 ± 0.1 [d]	5 ± 0.1 [d]	4.3 ± 0.1 [d]	5.3 ± 0.7 [f]
18:2	2.3 ± 0.1	4.4 ± 0.2 [c]	4.3 ± 0.4 [c]	4 ± 0.3 [c]	3.4 ± 0.1 [c]	5 ± 0.5 [c]	2.7 ± 1.4
20:3	0.8 ± 0.3	1.1 ± 00	1.3 ± 0.1	1.1 ± 0	1.3 ± 0	1.4 ± 0.2 [h]	0.8 ± 0.4
20:4	4.4 ± 0.2	5.1 ± 0.1 [c]	4.4 ± 0	3.7 ± 0.1 [d]	3.6 ± 0.1 [d]	3.1 ± 0.1 [d]	3.4 ± 0.2 [g]
trans*-0:4	0.5 ± 0.2	0.1 ± 0 [d]	0 [d]	0.5 ± 0	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
SFA	40.8 ± 3.2	42.3 ± 0.2	48 ± 0.2 [c]	50.9 ± 0.2 [d]	53.3 ± 0 [c]	53.4 ± 0.1 [c]	55.7 ± 1.9 [e]
MUFA	47.2 ± 3.8	43.1 ± 0.1	38.9 ± 0.4 [d]	36.6 ± 0.1 [d]	34.7 ± 0.1 [d]	34.1 ± 0.3 [d]	34.3 ± 0.9 [f]
PUFA[#]	10.4 ± 0.5	13.9 ± 0.4 [c]	12.3 ± 0.4 [c]	11.2 ± 0.3 [h]	10.6 ± 0.2	11.5 ± 0.3 [h]	9 ± 1.5
SFA/MUFA	0.9 ± 0.1	1 ± 0	1.2 ± 0 [c]	1.4 ± 0 [c]	1.5 ± 0 [c]	1.6 ± 0 [c]	1.6 ± 0.1 [e]

[a]FAME are obtained from total lipid extraction, derivatization, and GC analysis. [b]These values are mean ± SD and are expressed as percentage of the peak areas as detected in the GC chromatogram. [c]Values higher than untreated control (p=0.001). [d]Values lower than untreated control (p=0.001). [e]Values higher than untreated control (p=0.002). [f]Values lower than untreated control (p=0.002). [g]Value lower than untreated control (p=0.02). [h]Values higher than untreated control (p=0.04). [i]Values lower than untreated control (p=0.04). [l]Value correlated with arachidonic acid (r=0.987, p=0). * Evaluated with reference compounds (monotrans arachidonic acid isomers) obtained following references (Ferreri C *et al.*, 2005a; Ferreri C *et al.*, 2004). [#] This value includes n-3 FA (EPA and DHA).

Table 11. Membrane phospholipid fatty acids of NB-100 cells treated for the indicated times with 150 μM palmitic acid and compared to controls grown in the same conditions without palmitic acid.

Palmitic acid is incorporated in membrane phospholipids since the first 30 minutes and at increasing percentages during the incubation time. In the meantime, other significant changes occurred:

- Palmitoleic acid (16:1) temporarily decreased (from 1 to 2.5 hours) in a significant manner compared to controls (P=0.001).
- Stearic acid (18:0) significantly diminished along the 3 hours period (P=0.001,0.002).
- In the same period also the monounsaturated fatty acids, oleic (9cis-18:1) and vaccenic acids (11cis-18:1), diminished significantly (P=0.002).

- In the first 30 minutes arachidonic acid (20:4) increased significantly compared to controls (P=0.001), then started to diminish along the incubation time (P<0.05). Considering the precursors of arachidonic acid (20:4) in the n-6 pathway - linoleic (18:2) and eicosatrienoic (20:3) acids - their percentages did not vary. It is also worth noting that the diminution of arachidonic acid (20:4) at 3 hours incubation correlated with that of stearic acid (18:0) (r=0.987; P<0.05).
- The total polyunsaturated fatty acid value was not consistently affected during the monitoring. It is worth underlining that an oxidative damage to polyunsaturated fatty acids should have indiscriminately involved all polyunsaturated members (cfr., 18:2, 20:3 and 20:4 in Table 10) (Gutteridge JMC, 1995; Girotti AW, 1998).

In Figure 39, the graph summarizes the effects of 150 µM palmitic acid on NB-100 membrane fatty acids during a 3 hours period. While palmitic acid level reached 170% of controls, stearic acid (18:0) decreased by a 40% of the starting content after 30 minutes; the increase of saturated fats sum is 30% higher than controls, but 40% lower than the palmitic acid increase. The decrease of monounsaturated fatty acids is also highlighted, as well as that of arachidonic acid (20:4), that started decreasing after 90 minutes and reached a 30% diminution after 3 hours in comparison to controls.

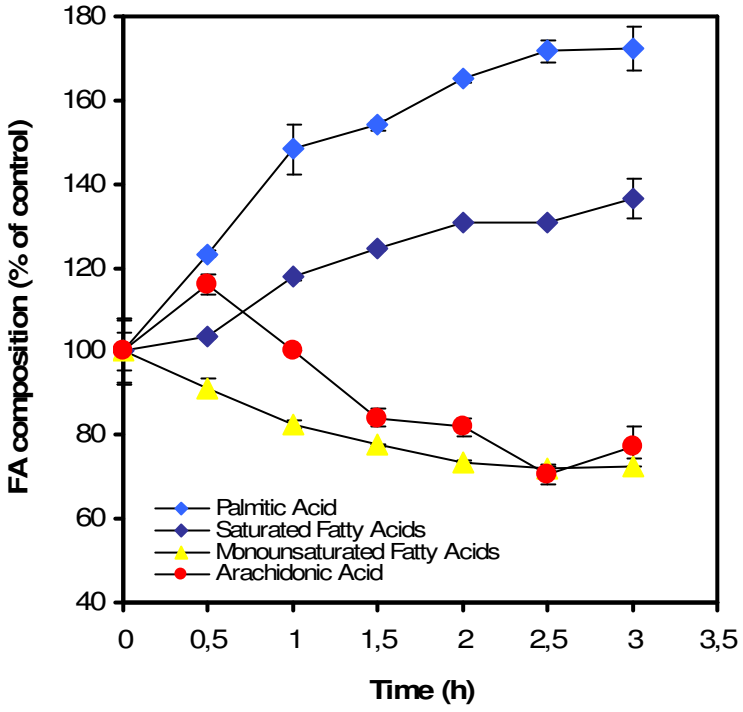


Figure 39. Changes of fatty acid percentages in NB-100 cell membrane phospholipids after continuous incubation with complete medium supplemented with 150 µM palmitic acid. Values are means ± SD of four determinations.

Membrane fatty acid compositions in the presence of 50 μM and 150 μM palmitic acid were also examined at 8 and 24 hours (data not shown), confirming the progression of fatty acid changes discussed so far. At 150 μM palmitic acid, a 40% diminution of arachidonic acid (20:4) was reached after 24 hours, compared to the start or to controls. Again, comparing with other polyunsaturated fatty acid levels, this did not correspond to a diminution due to an oxidative free radical process. Analogously, no change of the level of *trans* fatty acid isomers was observed.

As far as the morphology of palmitic acid-treated cells is concerned, it was assessed by phase contrast microscopy. The cell morphology after a 24 hours treatment with 50 μM and 150 μM palmitic acid is shown in figure 38B, in comparison with control cultures. Reduction of cell viability and apoptotic changes are clearly visible in cells treated with 150 μM palmitic acid.

Apoptosis was monitored by fluorescence microscopy using DAPI nuclear staining. Figure 38C shows the nuclear morphology of control cells compared with cells treated for different times with 50 μM and 150 μM palmitic acid. At 150 μM palmitic acid, early apoptosis processes started at 8 hours with nuclear blebs eterochromatin organized in fine clumps and apoptotic cells characterized by compacting and margination of nuclear chromatin. After 16 and 24 hours, apoptotic cells in early karyorrhexis containing numerous micronucleations and late apoptotic cells characterized by peripheral nuclear fragmentation were also visible. In case of 50 μM palmitic acid, any effect was detectable by incubation up to 24 hours.

cPLA$_2$ activation

The fatty acid monitoring during 150 μM palmitic acid supplementation evidenced that, among polyunsaturated fatty acids, there was a specific diminution of arachidonic acid (20:4). This effect could suggest its liberation as unesterified fatty acid, an event connected to the activation of phospholipase enzymes. In particular, PLA$_2$ enzymes are known to be central regulators of stimulus-coupled cellular arachidonic acid mobilization (Balsinde J *et al*., 2002; Kudo I and Murakami M, 2002), exhibiting a significant selectivity toward phospholipids bearing arachidonic acid moieties at the *sn*-2 position. Cytosolic PLA$_2$ (c-PLA$_2$) activation can be monitored by a specific antibody detecting the phosphorylation at the Ser^{505} residue (Tavolari S *et al.,* 2008). Figure 40B shows the detection of the c-PLA$_2$ phosphorylation during 150 μM palmitic acid supplementation by western blot analyses. In this cell line c-PLA$_2$ was always present, whereas its activated form markedly increased in a time-dependent manner within 60 min of treatment compared to controls. C-PLA$_2$ activation was also compared to FCS deprived cells (Figure 40A).

Under the latter condition we recall that all membrane essential fatty acids diminished, however this did not produce enzyme activation over 24 hours.

The complete evaluation of the pathways connected to arachidonic acid release is beyond the scope of this work. Instead the focus was on fatty acid changes due to culturing conditions, that can affect membrane assembly; with these data it was demonstrated that some culture conditions can produce membrane perturbation with the activation of biochemical pathways, such as the phospholipase activation, and the release of signalling lipids from membranes.

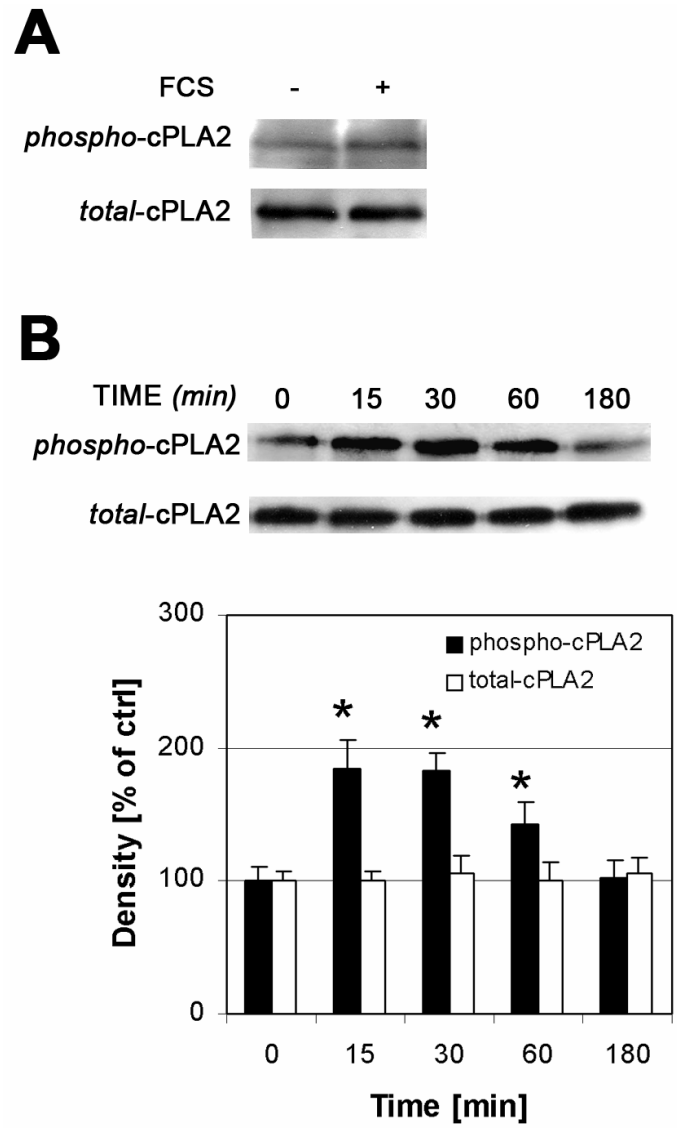


Figure 40. c-PLA_2 activation under FCS deprivation and palmitic acid supplementation. Western blot analyses of the effects of FCS deprivation (A) and palmitic acid treatment (B) on c-PLA_2 protein expression and its phosphorylated form in NB-100 cells. Cells were examined after serum deprivation for 24 hours, or after treatment for 15-180 min with 150 µM palmitic acid. Cell lysates were resolved by SDS/PAGE. Proteins were blotted and detected with monoclonal antibodies against c-PLA_2 or phospho-cPLA_2. The bar graph represents the band intensity values obtained by the Image J analysis, expressed as percentage of the corresponding control (Time 0). Results are mean ± SD of at least three independent experiments. Representative gels are shown.

RIPs intoxication

Ribosome inactivating proteins (RIPs) target ribosomes in an irreversible manner, thus inhibiting protein synthesis. Although many actions already shown *in vitro*, like DNA, PARP and mRNA depurination, are still uncertain inside the cell, it is logical to assume that RIPs do not activate the cellular apoptosis machinery directly as the primary activity of these toxins is to inactivate ribosomes and to stop protein synthesis (Stirpe F, 2004). But how cells decide when to activate the stress negating pathway and when to induce apoptosis is still not known, and nothing is known on what happens in the plasma membrane after RIPs intoxication. Although the literature indicates that RIPs induce cell death by apoptosis at low concentration, there are considerable differences in the kinetics as well as extent of apoptosis observed for a given concentration of toxin. It is also possible that the pathway of apoptosis induced by RIPs depends upon the type of cells used as well as the RIP used, with RIP structure and concentration influencing the uptake and routing, therefore changing protein localization and, at the end, cell response and fate (Polito L *et al*., 2008).

It seemed reasonable to pursue a fatty acid analysis of cells intoxicated with these proteins, because there are no data availble on membrane reconfiguration following a RIP treatment; coupling this new study with previous data led us to obtain a more complete view of membrane dinamics. The choice for this work was for saporin and ricin. Saporin and ricin are the best known and most studied RIPs, and lot of literature has been published since their discovery. However, there are still noumerous questions about these toxins that need an answer; not much is known about their mechanisms of entering cells, their routing and their actions other than inactivate ribosomes, especially for saporin. No studies are available about RIPs actions on membranes and on fatty acid reorganization. The investigation of fatty acid membrane changes after RIP intoxication was therefore obtained, with many significant results to use as a starting point for future researches.

At the beginning a cell viability test was conducted on NB-100 cells; graphs in figure 41 put in relation the amount of toxin with the resultant cell viability at different times. These data have to be considered a main overview of toxin-cell realationship, i.e. a starting point from which useful concentrations and times can be extrapolated for the following experiments. Concentrations of 10^{-6} M for saporin and of 10^{-12} M for ricin were therefore selected, with time ranging from 4 to 24 hours for protein synthesis inhibition, caspase activation and fatty acid follow up. These concentrations caused very similar viability curves (see top graphs of figure 42) and in particular an 80% of cell death after 48 hours.

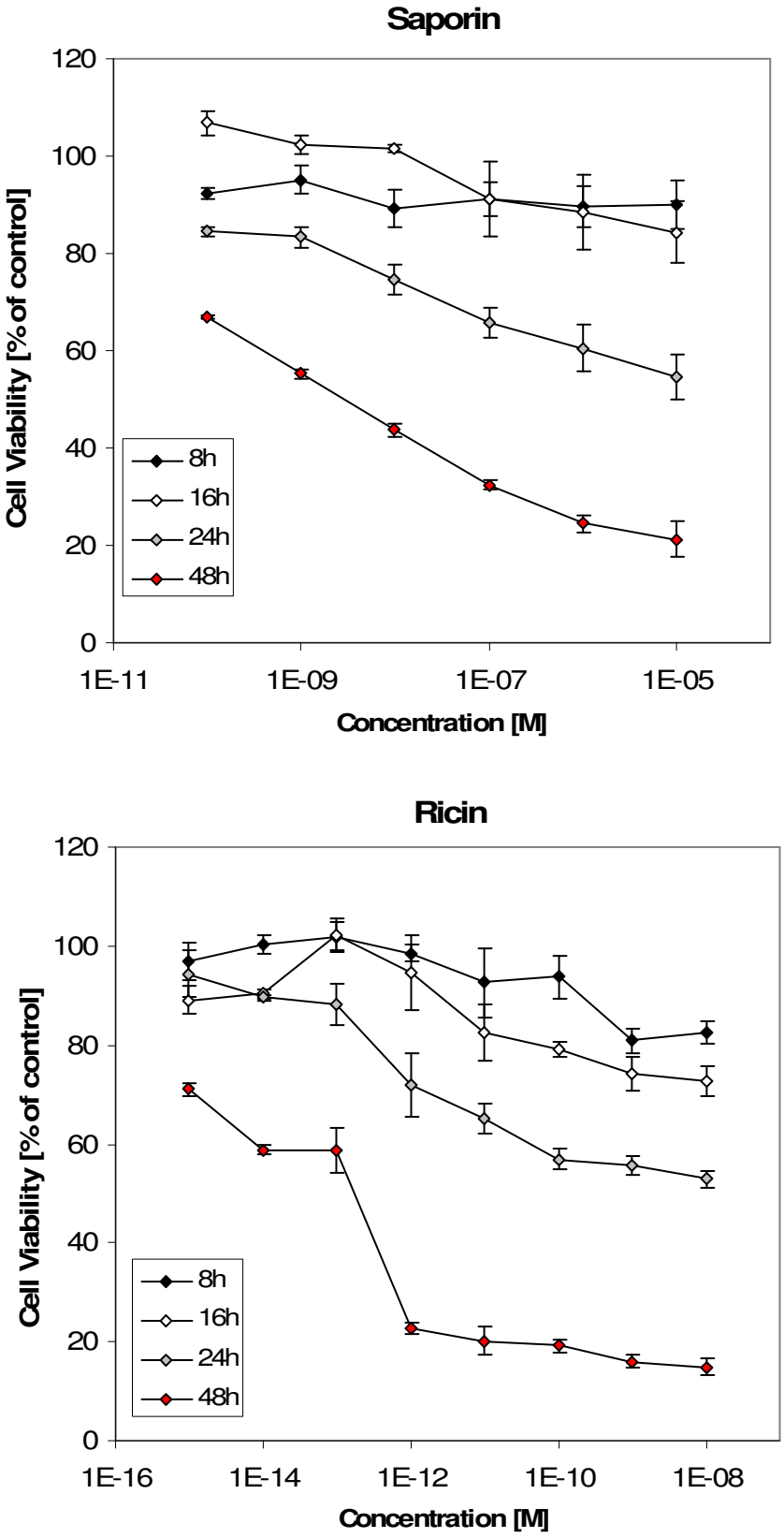


Figure 41. Viability curves for NB-100 cells. Top graphic: saporin. Bottom graphic: ricin. Cells were incubated in complete medium and treated with toxins at various concentrations for different amount of time. Cell viability was determined by MTS assay. Values are means ± SD of four determinations.

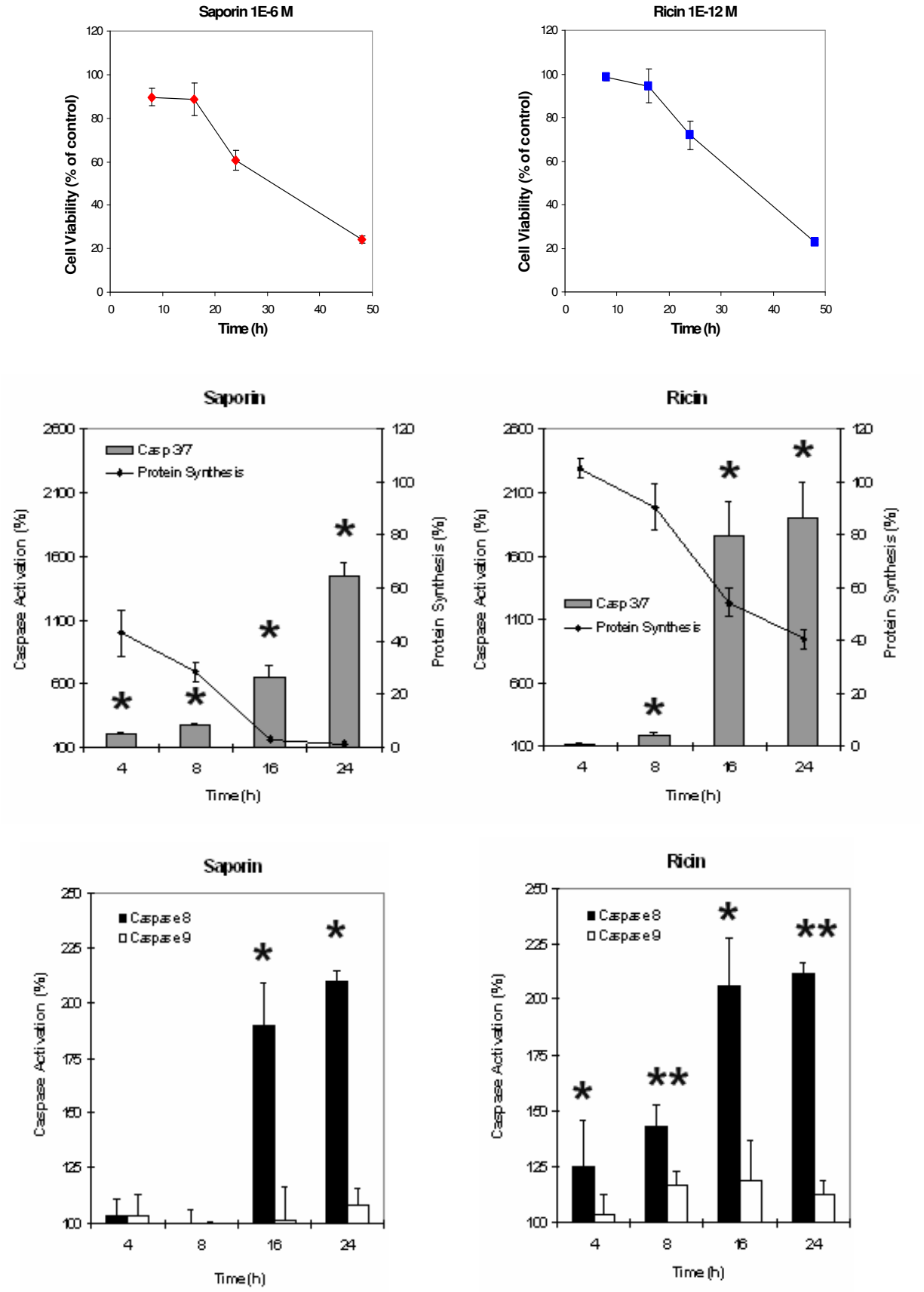


Figure 42. Effect of saporin [10^{-6} M] and ricin [10^{-12} M] on caspases activation and protein synthesis of NB-100 cells. Top graphs: viability curves. Center graphs: caspases 3/7 activation and protein synthesis. Bottom graphs: caspases 8 and 9 activation. Values are means (% of controls) ± SD of four determinations.

Following the viability curves, four new parallel experiments were carried out. Three of them were conducted to detect caspases activation in NB-100 cells after RIPs intoxication with saporin 10^{-6} M and ricin 10^{-12} M. Indeed, caspases 8, 9 and 3/7 were evaluated at the different timepoints of 4, 8, 16 and 24 hours. Because the primary activity of these toxins is to inactivate ribosomes, the inhibition of protein synthesis was evaluated in a parallel experiment using the same RIPs concentrations and the same timepoints. In figure 42 saporin-induced caspases activation and evaluation of protein synthesis are shown in the left column (center and bottom graphs), while ricin effects are reported in the right column. Values are illustrated as a percentage of untreated cells. Caspases activation seems to be slightly different between saporin- and ricin-treated cells; however, common timing of expression appeared to be present in both the type of caspases expressed and the time of the expression. In particular, caspase 3/7 expression at 16 hours after the beginning of the intoxication seems to be the point of no return, leading cells to apoptosis. It is worth pointing out that protein synthesis is the primary relevant biological effect that appears after RIPs intoxication. With ricin the inhibition started between 4 and 8 hours, reaching a 50 % at 16 hours and 60 % at 24 hours. From this point of view, saporin seemed to be more active, reaching a loss of protein synthesis of 50% after 4 hours and a complete stop already at 16 hours.

After having evaluated viability, caspases activation and protein synthesis, our attention focused on membranes. Because of the lack of informations toward membrane changes after RIPs intoxication, a complete fatty acid analysis was carried out. NB-100 cells were treated with saporin 10^{-6} M and ricin 10^{-12} M for 4, 8, 16 and 24 hours; following the intoxication, cells were collected and fatty acid analysis was conducted as already described in Materials and Methods. Results for saporin- and ricin-intoxicated cells are listed in Table 12 and 13 respectively. For a better understanding of data, the most relevant changes in fatty acids are reported in Figure 43 and can be grouped in some major points of discussion:

- Saturated fatty acids, in particular palmitic acid, showed a completely opposite behaviour between the two RIPs; while with saporine they tended to increase slightly, with ricin they increased of a 10% in the first 4 hours, with a subsequent fall of 20% and a stationary phase until the 24 hours.
- Monounsaturated fatty acid, mainly oleic acid, behaved in the opposite way as saturated ones. While with saporin there was a slightly decrease, ricin caused a decrease of 10% at 4 hours, followed by a noteworthy rise after 8 hours, with a following stationary phase at 115% of controls until the 24 hours.

FAME[a]	Control[b] (n=4)	Saporin 4h[b] (n=3)	Saporin 8h[b] (n=3)	Saporin 16h[b] (n=3)	Saporin 24h[b] (n=3)
16:0	29,5 ± 2,1	28,9 ± 0,4	29,4 ± 0,9	30,3 ± 0,7	31,1 ± 0,1
16:1	5,7 ± 0,6	5,4 ± 0,1	5,3 ± 0,0	3,8 ± 0,1 [d]	4,1 ± 0,0 [d]
18:0	12,0 ± 0,2	11,5 ± 0,0	11,8 ± 0,5	13,2 ± 0,2 [c]	13,0 ± 0,1 [c]
9*t*-18:1	0,2 ± 0,1	0,4 ± 0,0 [c]	0,4 ± 0,0 [c]	0,3 ± 0,0	0,4 ± 0,0 [c]
9*c*-18:1	31,0 ± 0,7	30,8 ± 0,1	30,7 ± 0,5	29,4 ± 0,2 [d]	29,9 ± 0,2 [d]
11*c*-18:1	7,8 ± 0,5	7,6 ± 0,1	7,6 ± 0,1	7,5 ± 0,1	7,8 ± 0,1
18:2	3,6 ± 0,4	3,9 ± 0,1	4,1 ± 0,1	3,9 ± 0,1	3,8 ± 0,0
20:3	0,9 ± 0,0	0,8 ± 0,1	0,7 ± 0,0 [d]	0,6 ± 0,0 [d]	0,6 ± 0,0 [d]
trans*-0:4	0,3 ± 0,6	0,1 ± 0,0	0,1 ± 0,0	0,1 ± 0,0	0,2 ± 0,0
20:4	4,6 ± 0,4	5,4 ± 0,1 [c]	4,9 ± 0,1	5,5 ± 0,1 [c]	4,7 ± 0,0
SFA	41,6 ± 2,2	40,3 ± 0,4	41,2 ± 0,4	43,5 ± 0,5 [c]	44,0 ± 0,2 [c]
MUFA	44,4 ± 1,7	43,7 ± 0,1	43,6 ± 0,7	40,7 ± 0,4 [d]	41,8 ± 0,3 [d]
PUFA[#]	13,6 ± 0,6	15,0 ± 0,3	14,3 ± 0,2	15,0 ± 0,1 [c]	13,3 ± 0,1
SFA/MUFA	0,9 ± 0,1	0,9 ± 0,0	0,9 ± 0,0	1,1 ± 0,0 [c]	1,1 ± 0,0 [c]
Total *trans*	0,7 ± 0,6	0,9 ± 0,0	0,9 ± 0,1	0,7 ± 0,0	0,8 ± 0,1

FAME[a]	Control[b] (n=4)	Ricin 4h[b] (n=3)	Ricin 8h[b] (n=3)	Ricin 16h[b] (n=3)	Ricin 24h[b] (n=3)
16:0	29,5 ± 2,1	33,2 ± 0,1 [c]	25,4 ± 0,6 [d]	25,5 ± 0,5 [d]	25,6 ± 0,8 [d]
16:1	5,7 ± 0,6	4,9 ± 0,0 [d]	6,2 ± 0,0	5,2 ± 0,1	6,2 ± 0,1
18:0	12,0 ± 0,2	11,4 ± 0,1	11,8 ± 0,1	12,1 ± 0,1	11,7 ± 0,2
9*t*-18:1	0,2 ± 0,1	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,0
9*c*-18:1	31,0 ± 0,7	29,5 ± 0,0 [d]	32,6 ± 0,6	33,2 ± 0,1 [c]	35,0 ± 0,5 [c]
11*c*-18:1	7,8 ± 0,5	6,5 ± 0,0	8,6 ± 0,2 [c]	8,4 ± 0,1 [c]	9,1 ± 0,1 [c]
18:2	3,6 ± 0,4	3,9 ± 0,1	3,2 ± 0,0	3,2 ± 0,2 [d]	2,9 ± 0,2 [d]
20:3	0,9 ± 0,0	0,9 ± 0,0	0,9 ± 0,0	0,8 ± 0,1	0,7 ± 0,0 [d]
trans*-0:4	0,3 ± 0,6	0,2 ± 0,0	0,2 ± 0,1	0,2 ± 0,1	0,2 ± 0,0
20:4	4,6 ± 0,4	4,7 ± 0,1	5,6 ± 0,1 [c]	5,8 ± 0,0 [c]	4,2 ± 0,1 [d]
SFA	41,6 ± 2,2	44,5 ± 0,1	37,2 ± 0,5 [d]	37,6 ± 0,3 [d]	37,2 ± 0,6 [d]
MUFA	44,4 ± 1,7	40,9 ± 0,0 [d]	47,4 ± 0,7 [c]	46,7 ± 0,2 [c]	50,4 ± 0,6 [c]
PUFA[#]	13,6 ± 0,6	13,7 ± 0,1	14,5 ± 0,0	14,9 ± 0,2	11,6 ± 0,0 [d]
SFA/MUFA	0,9 ± 0,1	1,1 ± 0,0 [c]	0,8 ± 0,0	0,8 ± 0,0	0,7 ± 0,0 [d]
Total *trans*	0,7 ± 0,6	0,8 ± 0,0	0,8 ± 0,2	0,8 ± 0,1	0,9 ± 0,1

[a]FAME are obtained from total lipid extraction, derivatization, and GC analysis. [b]These values are mean ± SD and are expressed as percentage of the peak areas as detected in the GC chromatogram. [c] Values higher than untreated controls (p=0.001). [d] Values lower than untreated controls (p=0.001). [#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA).

Table 12-13. Membrane phospholipid fatty acids of NB-100 cells treated for the indicated times with saporin 10^{-6} M or ricin 10^{-12} M and compared to controls grown in the same conditions without intoxication.

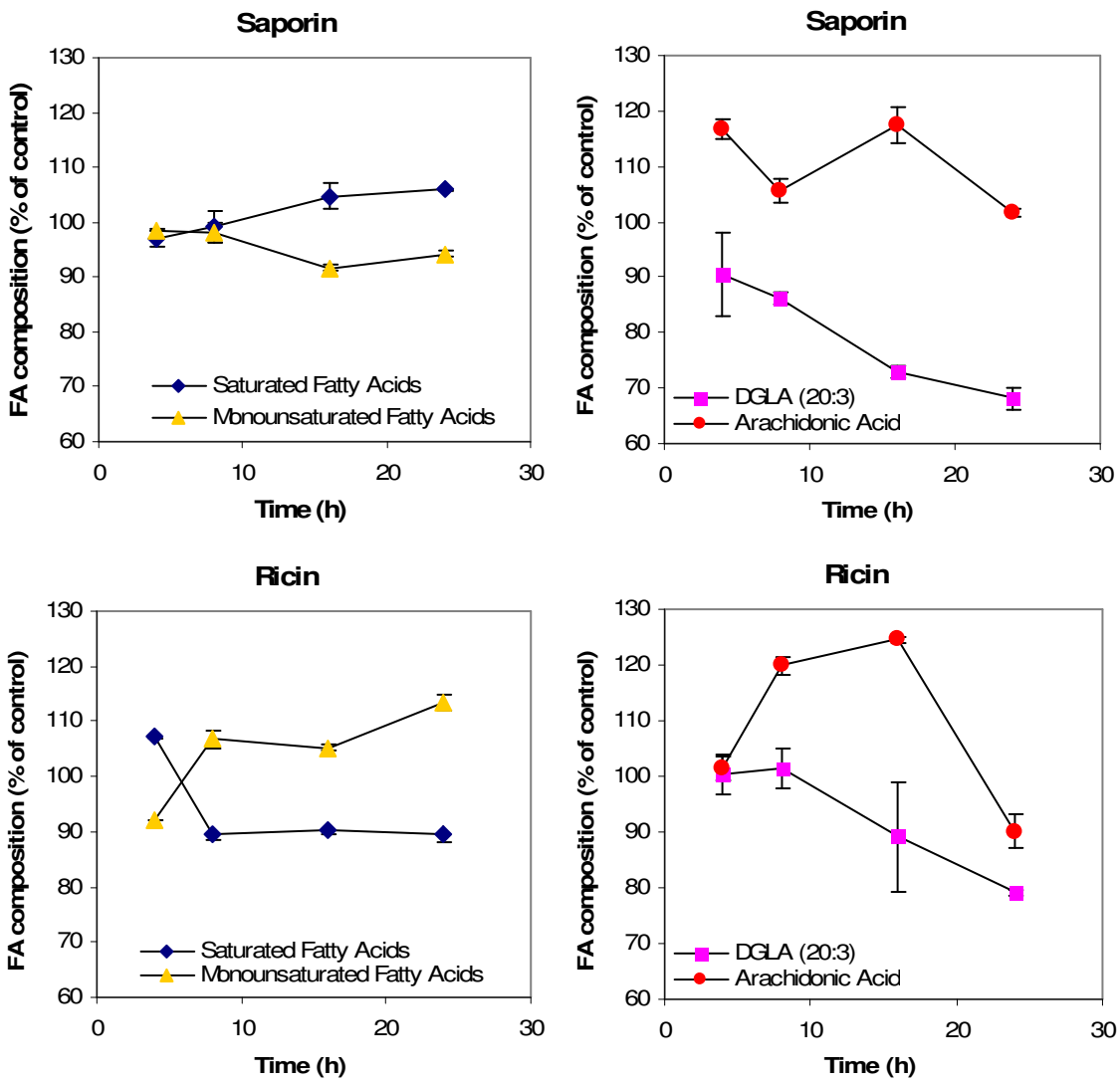


Figure 43. Membrane fatty acid changes of NB-100 cells incubated for a defined amount of time with saporin 10^{-6} M and ricin 10^{-12} M. Values are means ± SD of three determinations.

- n-6 pathway of synthesis was highly altered. In both cases 20:3, namely DGLA, was subjected to a big decrease, quantified in the order of 20-30%. On the other hand, arachidonid acid behaved differently between the two proteins: with saporin its percentage oscillated between 100% and 120% of controls, with a loss of 20% between 16 and 24 hours, while with ricin it reaches 125% of controls after 16 hours and then fall to 90% between 16 and 24 hours.
- There were no significant statistical differences neither in *trans*-fats categories nor in the total amount of *trans* fatty acids.

FAME[a]	Control[b] (n=4)	Saporin 10^{-5} M [b] (n=3)	Saporin 10^{-6} M [b] (n=3)	Saporin 10^{-7} M [b] (n=3)	Ricin 10^{-11} M [b] (n=3)	Ricin 10^{-12} M [b] (n=3)	Ricin 10^{-13} M [c] (n=1)
16:0	31,3 ± 2,1	35,1 ± 2,9	33,9 ± 1,0	33,1 ± 2,8	31,8 ± 3,5	30,3 ± 1,4	28,4 ± 0,0
16:1	5,0 ± 0,6	3,5 ± 0,4 [e]	3,9 ± 0,2 [e]	4,2 ± 0,1 [e]	4,3 ± 0,1 [e]	5,6 ± 0,1	5,5 ± 0,0
18:0	11,5 ± 0,2	11,5 ± 1,0	10,7 ± 0,4	12,3 ± 0,0	12,5 ± 2,3	10,0 ± 0,0	12,2 ± 0,0
9*t*-18:1	0,3 ± 0,1	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,0	0,4 ± 0,0
9*c*-18:1	29,0 ± 0,7	27,5 ± 1,2	28,6 ± 0,1	28,7 ± 1,2	28,3 ± 0,3	31,1 ± 0,3 [d]	31,1 ± 0,0 [d]
11*c*-18:1	6,6 ± 0,5	6,2 ± 0,8	6,7 ± 0,2	6,6 ± 0,6	7,1 ± 0,3	7,5 ± 0,6 [d]	7,9 ± 0,0 [d]
18:2	3,3 ± 0,4	5,1 ± 0,8 [d]	5,3 ± 0,0 [d]	4,5 ± 1,0 [d]	3,3 ± 0,2	3,4 ± 0,2	3,3 ± 0,0
20:3	1,0 ± 0,1	0,5 ± 0,0 [e]	0,6 ± 0,0 [e]	0,6 ± 0,0 [e]	0,7 ± 0,0 [e]	0,7 ± 0,1 [e]	0,8 ± 0,0 [e]
trans*-0:4	0,0 ± 0,0	0,3 ± 0,2	0,0 ± 0,0	0,0 ± 0,0	0,1 ± 0,2	0,3 ± 0,0	0,0 ± 0,0
20:4	5,5 ± 0,4	4,9 ± 0,5	5,5 ± 0,0	5,6 ± 0,3	5,9 ± 0,5	6,2 ± 0,2 [d]	6,2 ± 0,0 [d]
SFA	42,8 ± 2,0	46,6 ± 2,0	44,6 ± 0,6	45,4 ± 2,7	44,4 ± 1,2	40,3 ± 1,4	40,6 ± 0,0
MUFA	40,6 ± 1,7	37,2 ± 2,4	39,2 ± 0,6	39,6 ± 1,7	39,7 ± 0,8	44,2 ± 0,8 [d]	44,5 ± 0,0 [d]
PUFA[#]	15,6 ± 0,7	14,7 ± 0,2	15,3 ± 0,0	14,6 ± 1,0	15,0 ± 0,7	14,4 ± 0,8	14,3 ± 0,0
SFA/MUFA	1,1 ± 0,1	1,3 ± 0,1	1,1 ± 0,0	1,1 ± 0,1	1,1 ± 0,1	0,9 ± 0,0	0,9 ± 0,0
Total *trans*	1,0 ± 0,0	1,4 ± 0,2 [d]	0,8 ± 0,0	0,5 ± 0,0	1,0 ± 0,2	1,0 ± 0,2	0,6 ± 0,0

[a]FAME are obtained from total lipid extraction, derivatization, and GC analysis. [b]These values are mean ± SD and are expressed as percentage of the peak areas as detected in the GC chromatogram. [c]These values are without SD and are expressed as percentage of the peak areas as detected in the GC chromatogram. [d] Values higher than untreated controls (p=0.001). [e] Values lower than untreated controls (p=0.001).[#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA).

Table 14. Membrane phospholipid fatty acids of NB-100 cells treated for 16 hours with saporin or ricin at the indicated concentrations and compared to controls grown in the same conditions without intoxication.

To complete the whole picture of membrane dynamics after RIPs intoxication, a new fatty acid analysis based on different concentrations of toxins was carried out. Three different concentrations were chosen for both saporin (10^{-5} M, 10^{-6} M, 10^{-7} M) and ricin (10^{-11} M, 10^{-12} M, 10^{-13} M), and the unique timepoint was selected to be 16 hours. Results are listed in Table 14 and in Figure 44 for both saporin and ricin. Patterns of fatty acid changes similar to the previous experiments were obtained:

- Increase in the total saturated fatty acid content with the increase in toxin concentration
- Decrease in the total monounsaturated fatty acid content with the increase in toxin concentration
- Alteration of the n-6 pathway, with a loss of DGLA of about 30-40 % and a raise or release of arachidonic acid for low or high concentrations respectively
- Increase in the total *trans* fatty acids with the increase of RIPs concentration

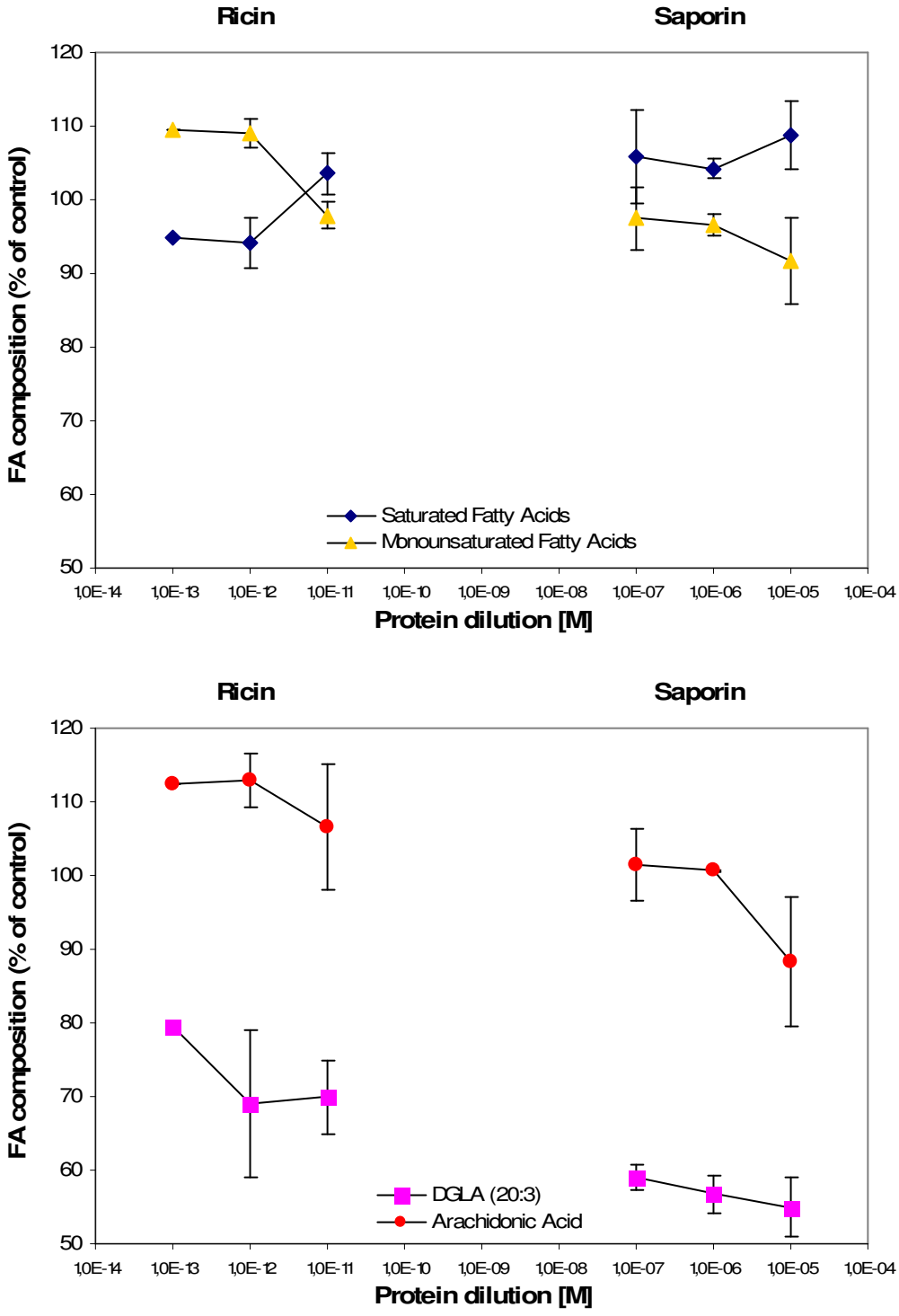


Figure 44. Effect of different concentrations of saporin and ricin on membrane fatty acid composition. NB-100 cells were incubated for 16 hours with toxins and then fatty acid analysis was conducted. Values are means ± SD of three determinations.

To note, pilot experiments were conducted on NB-100 cells supplemented with oleic acid (data not shown), and further analyses are already programmed. Palmitic and oleic acid 150 μM were added to the complete medium at the same time of RIPs. Different outcomes resulted between saporin and ricin in the two conditions. The intoxication with both RIPs in the presence of palmitic acid led to a synergic effect, with more apoptosis as a result. On the other hand, while cells were not protected from saporin, oleic acid caused a 10% delay of cell death for cells intoxicated with ricin. Even if these are preliminary data, the interesting results can be easily linked to the previous experiments concerning alterations of saturated and monounsaturated groups of fatty acids. Discussion of relations and implications will be presented in the following part.

Radical Stress

H_2S is known to be an important mediator of neuronal cells, with a concentration in the brain reaching more than 0.1 mM (Doeller JE *et al*., 2005). Several studies have recently reported the reactivity of thiol grups based on the production of sulfur-centered radical species, RS•. In fact, thiols tend to act as radical repair agents, although this action is counterbalanced by the formation of thiyl radical species which can damage other biomolecules, such as aminoacids, carbohydrates and in particular lipids. Data have recently been published on H_2S and its thiol-based isomerization of fatty acid double bonds in liposomes (Lykakis IN *et al*., 2007). Therefore, it would have been important to check the H_2S behaviour in cells, with a particular concern on the membrane lipids and stress conditions. So, for our purposes we decided to use H_2S-treated NB-100 cells. Moreover, we used H_2S in the presence of 2,2'- Azobis(2-methylpropion-amidine) dihydrochloride (AAPH), a well known radical initiator also for biological experiments. AAPH is an hydrophilic azoinitiator that at 37°C is able to generate the initiating radicals shown in equation (1). These radicals are able to extract hydrogen from thiols and to generate thiyl radicals, as explained in the equation (2).

(1) $R\text{-}N{=}N\text{-}R \rightarrow$ at 37°C $\rightarrow 2R\bullet + N_2$

(2) $R\bullet + H_2S \rightarrow RH + HS\bullet$

Two parallel studies were developed, one with cells grown in RPMI supplemented with FCS, the other with cells grown in a FCS-free medium for at least 24 hours before the treatment. For each study three cell lines were used: one was the control, one line was treated with 0.1 mM H_2S and the third line with 0.1 mM H_2S in the presence of 50 mM AAPH. Cells were harvested 20 minutes after the beginning of the experiment, and membrane fatty acids were isolated as already described in Materials and Methods. It is well established in literature that H_2S, being a gas, evaporates from the culture medium in no more than 20 minutes. It was therefore evaluated to consider only short timepoints, to envisage some instant changes occurring in membranes. It is however worth pointing out that later timepoints were also studied (data not shown), but they did not show any significant data, due to the volatility of the reagent, as explained above. Results are listed in table 15, where it is worth noting that the main difference of lipid compositions concerns fatty acids between controls grown in the presence and absence of FCS. This had previously been discussed as a consequence of essential fatty acid deficit.

FAME[a]	Control [b] (n=4)	H_2S 0.1 mM [b] (n=4)	H_2S 0.1 mM AAPH 50 mM [b] (n=4)	Control [b] (n=4)	H_2S 0.1 mM [b] (n=4)	H_2S 0.1 mM AAPH 50 mM [b] (n=4)
FCS	+	+	+	-	-	-
16:0	36,4 ± 0,6	34,7 ± 1,2	35,5 ± 1,1	41,8 ± 1,8	40,0 ± 1,4	41,3 ± 3,5
16:1	5,1 ± 1,5	4,2 ± 0,3	4,1 ± 0,5	4,5 ± 0,2	5,1 ± 0,2	4,6 ± 0,8
18:0	13,3 ± 3,7	15,8 ± 3,7	15,8 ± 4,4	18,2 ± 6,4	16,6 ± 5,8	15,6 ± 4,6
9*t*-18:1	1,0 ± 0,2	1,0 ± 0,5	1,2 ± 0,6	1,2 ± 0,9	1,0 ± 0,5	1,2 ± 0,5
9*c*-18:1	29,9 ± 3,2	28,2 ± 5,2	28,2 ± 6,2	22,5 ± 7,0	24,7 ± 3,2	24,2 ± 2,7
11*t*-18:1	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
11*c*-18:1	6,6 ± 1,3	5,8 ± 0,6	5,7 ± 0,5	5,5 ± 0,7	6,4 ± 0,6	6,0 ± 0,2
18:2	2,3 ± 0,4	2,9 ± 0,1	2,6 ± 0,1	1,5 ± 0,4	1,3 ± 0,2	2,0 ± 0,3
***trans*-18:2**	1,6 ± 0,8	1,8 ± 1,0	1,9 ± 0,6	1,3 ± 0,5	0,9 ± 0,3	1,2 ± 0,0
20:3	0,4 ± 0,4	0,6 ± 0,7	0,5 ± 0,6	0,7 ± 0,2	0,7 ± 0,1	0,8 ± 0,2
20:4	3,3 ± 0,7	3,8 ± 0,3	3,5 ± 0,1	1,8 ± 0,2	1,8 ± 0,4	1,6 ± 0,5
***trans*-20:4**	**0,2 ± 0,1**	**0,5 ± 0,1**[c]	**0,5 ± 0,2**[c]	**0,1 ± 0,2**	**0,7 ± 0,0**[cd]	**0,9 ± 0,2**[cd]
SFA	49,7 ± 4,2	50,5 ± 4,8	51,3 ± 5,5	60,0 ± 8,1	56,6 ± 4,4	56,8 ± 1,0
MUFA	41,6 ± 4,2	38,2 ± 4,2	37,9 ± 5,2	32,5 ± 7,6	36,1 ± 3,7	34,7 ± 1,7
PUFA[#]	8,8 ± 1,4	11,4 ± 0,6	10,9 ± 0,3	7,5 ± 0,6	7,2 ± 0,8	8,5 ± 0,7
SFA/MUFA	1,2 ± 0,2	1,3 ± 0,3	1,4 ± 0,3	1,9 ± 0,6	1,6 ± 0,3	1,6 ± 0,1
total *trans*	1,2 ± 0,3	1,4 ± 0,7	1,6 ± 0,8	1,3 ± 1,0	1,7 ± 0,5	2,0 ± 0,3

[a] FAME are obtained from total lipid extraction, derivatization, and GC analysis. [b] These values are mean ± SD and are expressed as percentage of the peak areas as detected in the GC chromatogram. [c] Values higher than untreated control (p=0.001).[d] Values higher than + FCS respective data. [#] This value includes n-3 FA (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA).

Table 15. Membrane phospholipid fatty acids of NB-100 cells cultivated with or without FCS supplementation and treated for 20 minutes with H_2S 0.1 mM or with H_2S 0.1 mM + AAPH 50 mM.

Another interesting data concern the level of *trans* isomers of membrane lipids. Figure 45 shows the arachidonic acid isomers, which have been described as biomarkers of radical stress also in cell cultures (Ferreri C *et al*., 2002; 2003; 2004). It is clearly shown that H_2S-treated cells have a statistically very significant increase of *trans*-20:4 isomers and this increment is higher in cells cultivated without FCS. This could be expected since the lack of FCS brings as a consequence the absence of antioxidants and radical-scavenging molecules in general, such as albumin. Figure 45 also shows that the radical initiator AAPH did not affect significantly the level of *trans* fatty acid isomers in the presence of FCS, whereas in the absence of it there was a slight increase in the formation of these isomers.

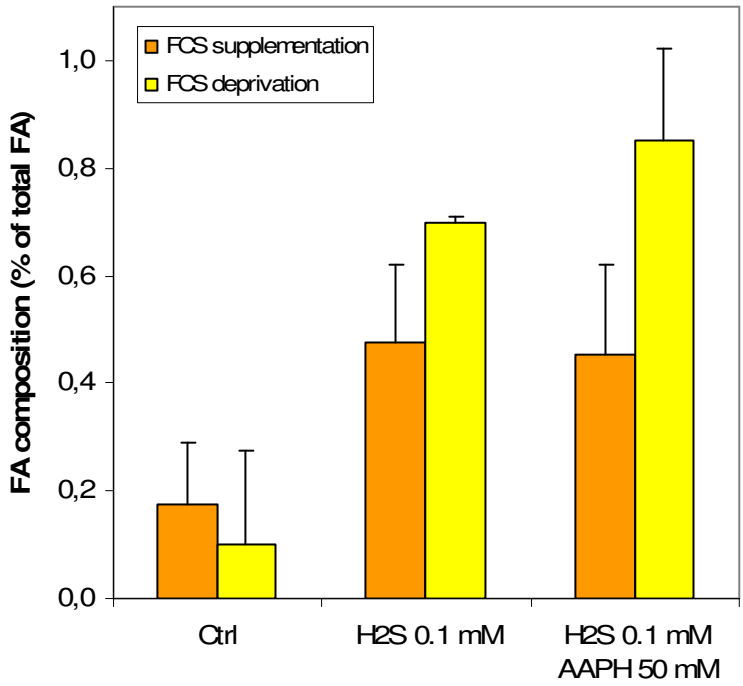


Figure 45. Isomers of arachidonic acid present in membrane fatty acids after the treatment with H_2S-derived radical stress. Data are reported from the previous table.

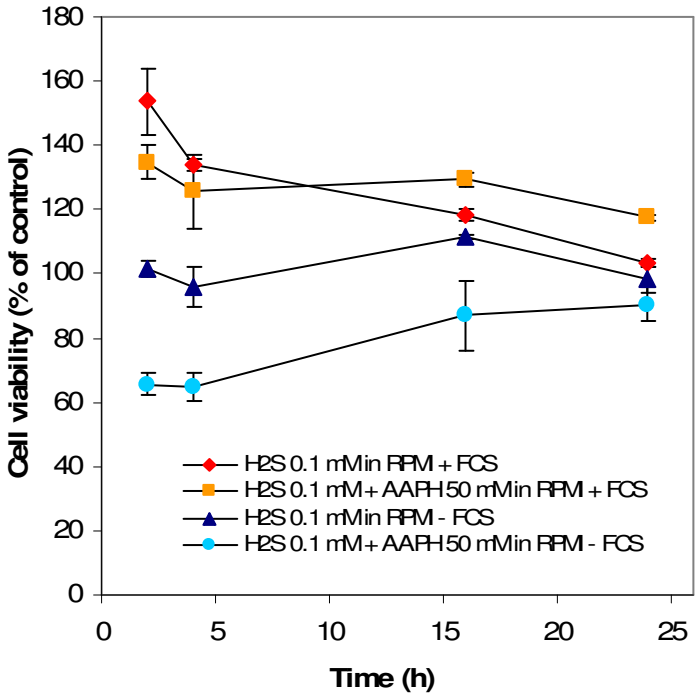


Figure 46. Cell viability of NB-100 cells treated with H_2S 0.1 mM or with H_2S 0.1 mM + AAPH 50 mM in RPMI with or without FCS supplementation. The 20 minutes treatment was followed by incubation in complete medium. Values are means ± SD of three determinations.

It is worth pointing out that cell viability was not diminished by H_2S treatment. In figure 46 it is clearly shown that H_2S is eventually stimulating cell growth when the experiment is carried out in RPMI supplemented with FCS. While no effects on cell viability were seen with H_2S treatment in the absence of FCS, a decrease was noted with the H_2S+AAPH treatment; in this case, the high amount of radical species induced by AAPH in a antioxidant-free envinronment increased cell stress with the consequent drop of cell viability.

DISCUSSION

In the last decades cell biology developed widely. Genomics was the first to be uncovered, with DNA secrets being unveiled since Watson and Crick's discovery in 1953. The decription of genes and whole genomes happened in the last two decades, with the parallel raise of the huge field of proteomics. Indeed, all cellular activities and functions were thought to be dependent from the genome-proteome; thus, only processes linked to protein synthesis and regulation were truly investigated. However, with the better understanding of cellular processes and the developing of new analytical tecniques, a vast pool of new molecules was discovered. The sequencing of the human genome, the development of gene arrays, and the availability of soft-ionization mass spectrometry techniques have led the way for high-throughput genomics and proteomics (Domon B and R Aebersold, 2006; Sickmann A *et al*., 2003). Although the human genome comprises just over 25000 genes, the human proteome, considering a multitude of post-translational modifications, is composed of millions of different proteins. The end product of genetic and protein expression is the metabolome, the total complement of metabolites within a cell or an organism. Although genomics, transcriptomics, and proteomics are fundamental to the functional integrity of the organism, its metabolites reflect the most downstream effects of gene and protein regulation and thus may provide vital information regarding the biological state of the system (Goodacre R *et al*., 2004). Metabolomics represents a paradigm shift from looking at individual metabolites to examining complete metabolic networks in an entire cell or organism. Thousands of metabolites can be accurately measured and high-throughput technology started to spawn data in amounts that flood our databases. However, data generation does not make science. It became soon clear that all data published until that moment have to be rediscussed under the light of the new molecules, with a lot still to be discovered in the complex universe of the cell. In particular it was quite impressive the discovery that human cells produce more than one thousand different lipid molecules. Therefore it became clear that a whole part of cell biology was left behind: the era of lipidomics was began.

The cellular lipidome comprises over 1000 different lipids, most of them looking similar due to their polar head and hydrophobic tails. Still, cells recognize lipids with exquisite specificity. The functionality of lipids is determined by their local concentration, which varies between organelles, between the two leaflets of the lipid bilayer and even within the lateral plane of the membrane. To incorporate functions, cellular lipidomics must not only determine which lipids are present, but also the concentration of each lipid at each specific intracellular location in time and the lipid's interaction partners. Moreover, cellular lipidomics must include the enzymes of lipid metabolism and transport, their specificity, localization and regulation. Finally, it requires a deep understanding of the physical properties of lipids and membranes, especially lipid–lipid and lipid–protein interactions. In the context of a cell, the complex relationships between metabolites can only be

understood by viewing them as an integrated system. Cellular lipidomics provides a framework for understanding and manipulating the vital role of lipids, especially in membrane transport and sorting and in cell signaling. Lipidomics is linked via glycolipids to the field of glycomics, and via the lipid second messengers to the field of signalomics. Notably, in the end all precursors for lipid synthesis and products of lipid degradation are water-soluble metabolites, solidly embedding lipidomics in metabolomics.

Lipidomics is more than just the complete characterization of all lipids in a particular cell type. It is the comprehensive understanding of the influence of lipids on a biological system with respect to cell signaling, membrane architecture, transcriptional and translational modulation, cell-cell and cell-protein interactions, and response to environmental changes over time. There is an immense combinatorial structural diversity among lipids, the importance of which is not entirely clear. The possibility that each individual lipid has been conserved throughout the evolution for a particular purpose is intriguing. In the recent classification of lipids, meant to serve as an international basis for data storage, lipids are loosely defined as biological substances generally hydrophobic in nature and in many cases soluble in organic solvents (Fahy E *et al*., 2005). In practice, the organization of lipids in cells is determined by the bulk lipid classes, and one can consider the behavior and function of the hundreds of minor lipids as superimposed on the dynamic organization of the major ones. Which are the major lipids in animal cells? While triacylglycerols and cholesteryl esters fill the core of lipid droplets in the cytosol and of lipoproteins being secreted or endocytosed (van Meer G, 2001), the bulk of the cellular lipids is organized in membranes.

Membrane lipidomics evaluates in particular phospholipids and their dynamical changes under metabolic conditions, focusing on the type and the quantity of fatty acid residues, which are particularly relevant for the regulation of membrane structure and functions (Dowhan WR and Bogdanov M, 2002; Cevc G, 1993). Fatty acid residues can be analysed after ordinary steps of isolation, derivatization to methyl esters (FAME) and characterization by gas chromatography (Christie WW, 2007). In this context, also the geometry of the double bonds has acquired more and more significance, since the naturally occurring membrane fatty acids have mostly the *cis* configuration, provided enzymatically, whereas the *trans* geometrical isomers can be considered natural only in bacteria (Zhang YM and Rock CO, 2008).

Bearing in mind all this, it is noteworthy to point out that lipidomics analysis is still not used to characterize cell cultures, despite of its importance in influencing many cellular behaviours. Therefore it is high probable that the same cell line can respond in a different way to a treatment, depending also on its membrane composition. This work intends to increase attention on the application of lipidomics to the follow-up of the fatty acid status and changes of cell cultures,

influencing membrane reorganization that can contribute to prime biochemical cascades. The goal was to highlight the role of membrane fatty acid changes in membranes, depending upon the cellular conditions, considering not only the type of fatty acid involved and its quantity, but also the timing of the changes, in order to envisage strategies for reverting cell fate.

The first step in our approach was the characterization of fatty acid composition of fetal calf serum (FCS). As clearly appear from Table 8, every batch tested differed noteworthy from the others. Since FCS derives from a natural source, the variability can be expected, but this observation highlights once more the importance of cell culture standardization, also regarding the fatty acid sources. This standardization has not yet been considered in the general protocol, although the contribution of the membrane status can be considered an important issue before proceeding to other metabolic assays. FCS is the main "diet" for cells, as it is the only source of fatty acids present in cell cultures. Without any doubt, cells prefer fatty acids ready to use instead of synthetizing them through the waste of energy. Thus, the main composition of cell membranes can basically vary with a simple change in the FCS batch used, and with their membranes also many properties, some of them still not well understood. On the basis of this observation, a careful follow-up of the experiments was necessary, in order to have good reproducibility of the starting cultures.

In order to pursue a set of experiments, the first thing to choose was a cell line to work with. Therefore, a screening of different cell lines used in biology laboratories was carried out. Five different human cell lines and their specific fatty acid compositions are listed in table 9. From these data, Neuroblastoma cells (NB-100) were selected for the project because of the total absence of *trans* fatty acids; as human cells does not have the set of enzymes necessary for *trans* production, their absence was thought to be a sign of unaltered characteristics. Moreover, NB-100 cells were preferred to Jurkat mainly for their capacity to grow adherent to the flask surface. However, fatty acid composition of NB-100 cells resulted to be always slightly different during our experiments, even with the following of a really strict cultivation protocol. The main difference was the appearance of *trans* fatty acids in NB-100 membranes. Nevertheless, the origin of these *trans* fats can be traced to be exogenous, mainly because FCS presented always a baseline level ot *trans* fats; therefore, it can be excluded their origin from lipid isomerization due to radical stress. However, for a more precise way of interpreting data, from this point onward it is better to relay the fatty acid composition in each experiment to its respective control, even if differences between experiments resulted to be not very significant.

Lipid supplementation and deprivation are active subjects of research, due to the increasingly important role of lipids in cell functions and signalling, already highlighted in Chapters 1 and 2. The effects have been studied by different strategies, for example, supplementing a single or mixed fatty

acid to the organism (Wiesenfeld PW *et al*., 2001; Tocher DR and Dick JR, 2001; Menendez JA *et al*., 2004; McKenzie KE *et al*., 1994) or using strains specifically mutated for the expression of desaturase or elongase enzymes (Murakami Y *et al*., 2000; Savonniere S *et al*., 1996). In particular, palmitic acid is an important fatty acid for cell growth (Cook HW and McMaster CR, 2002) but it can also cause apoptosis at higher concentrations (Yang X and Chan C, 2007; Kondoh Y *et al*., 2007; Landau Z *et al*., 2006; Ulloth JE *et al*., 2003; Mu YM *et al*., 2001); however, any information of the correspondent membrane lipidome changes is available in helping to understand these contrasting roles. Therefore, our investigation was first focused on the reorganization of the NB-100 lipid bilayer after serum deprivation or palmitic acid supplementation.

The monitoring of lipidome changes of NB-100 was performed under serum deprivation, as well as in the presence of various concentrations (50, 75, 100, 125 and 150 μM) of palmitic acid, a saturated fatty acid which is important for cell growth (Cook HW and McMaster CR, 2002), but it is also known for its apoptotic effects (Yang X and Chan C, 2007; Kondoh Y *et al*., 2007; Landau Z *et al*., 2006; Ulloth JE *et al*., 2003; Mu YM *et al*., 2001). Particularly, fatty acid residues of membrane phospholipids were monitored, including the possibility of a change of the natural geometry of the *cis*-unsaturated fatty acid double bonds, with the formation of geometrical *trans* isomers. This is an endogenous process that can be correlated to free radical stress (Chatgilialoglu C and Ferreri C, 2005; Zambonin L *et al*., 2006), whose involvement as cell signalling in human metabolism has still to be defined (Mozaffarian D, 2006).

The first usefulness of lipidome monitoring was to evidence differences between the control cells of our two sets of experiments (Tables 10 and 11). These changes depended from FCS, the “essential lipid diet” given to cells, whose composition can vary as already explained previously, thus influencing the cell membrane composition during cultivation. On this basis, it seems reasonable to suggest the use of lipidomics as a metrology tool, for the standardization of cell cultures, characterizing the starting status and subsequent changes of the membrane fatty acid content due to the growth conditions.

NB-100 cells were monitored for the changes of the membrane fatty acid content in parallel with measurements of duplication time, viability, determination of morphological characteristics and apoptosis (Figures 37 and 38). Under FCS deprivation, saturated fatty acids, such as palmitic and stearic acids, increased accompanied by the decrease of all polyunsaturated components (Table 10). This can be expected because FCS is the only essential fatty acid source. Under this condition it is remarkable the significant increase of the geometrical *trans* isomers of oleic acid, namely elaidic acid (9t-18:1; P=0.03). During starvation this isomer cannot be related to dietary lipids, whereas it can be explained as the product of a free radical-catalysed isomerisation of the corresponding *cis*

isomer (oleic acid), a process linked to cellular stress conditions (Ferreri C *et al*., 2005; 2005b). In serum-deprived cells other signs of oxidative stress, such as formation of malondialdehyde and decreased glutathione levels, have been reported (Charles I *et al*., 2005; Lindenboim L *et al*., 1995; Goyeneche A *et al*., 2006). Fatty acid changes are in the direction of a more rigid packing of membranes, influencing their properties such as permeability and fluidity (Dowhan WR and Bogdanov M, 2002; Cevc G, 1993). This condition, together with the absence of growth factors, could therefore produce the observed 35% diminution of the duplication time. After 24 hours, our NB-100 cultures did not show signs of apoptosis, as detected by DAPI staining (Figure 37), thus indicating that the membrane transformations were not yet translated into apoptotic signals. We are aware that the absence of apoptosis in NB-100 cells under FCS deprivation cannot be generalized to other cell systems. In fact, several studies have been performed on the effects of FCS deprivation in cell lines that brings to apoptosis (Charles I *et al*., 2005; Lindenboim L *et al*., 1995; Goyeneche A *et al*., 2006; Higuchi A *et al*., 2006), but it is also reported that neuroblastoma cell lines in delipidated serum display only morphological differentiations (Monard D *et al*., 1977). As previously mentioned, many of these reports lack of the analysis of membrane fatty acids, although it can be easily imagined that the absence of lipid supplementation turns out to produce membrane changes. The lipidomic monitoring can always furnish an important piece of information in order to understand the membrane contribution, to be used as a key of comparison among different conditions and further to understand the corresponding biological outcome.

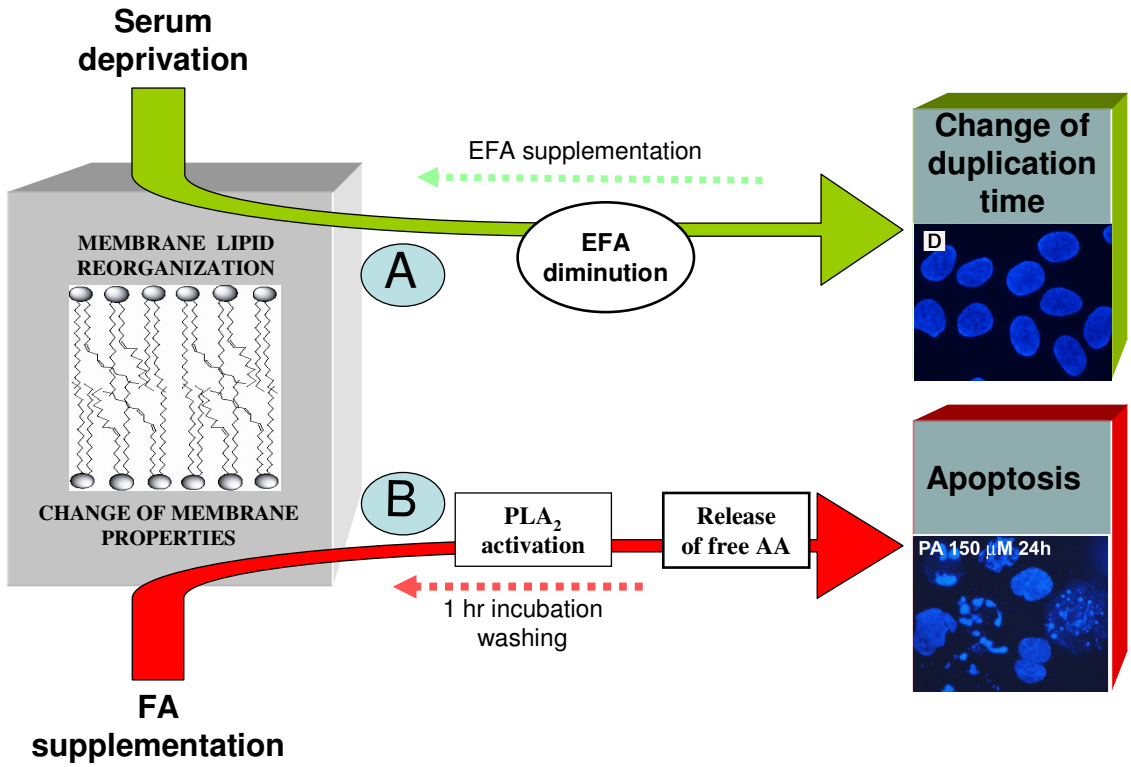
Having seen the enrichment of palmitic acid in membranes during FCS deprivation and the resistance to apoptosis of these cells, we were interested to follow-up the specific supplementation of this fatty acid. When palmitic acid was directly supplemented at four different concentrations (50, 100, 125 and 150 μM), it caused its own increase in membrane phospholipids. Cell viability was evaluated after supplementation followed by washing after 1 or 2 hours incubation, and re-suspension in complete medium under standard conditions for additional 48 hours. As shown in Figure 38A, cell viability started to be affected after 2 hours incubation with palmitic acid, even when a 75 μM concentration was used. With 150 μM palmitic acid after 1 hour incubation followed by washing and further 48 hours incubation the viability was only slightly affected. Matching this status with the membrane fatty acid content (Table 11), it can be seen that the incorporation of palmitic acid already occurred in phospholipids in the first hour, with diminution of stearic acid and monounsaturated fatty acids, but without affecting the polyunsaturated content. After 1.5 hours incubation with 150 μM palmitic acid, the increase of palmitic acid is accompanied by the diminution of arachidonic acid whereas linoleic acid, the arachidonic acid precursor, remained essentially unchanged during the whole period. In Figure 39 the graphic evidences that at 1.5 hours,

when the palmitic incorporation is about 40% of the controls, the loss of arachidonic acid is almost 20%, similar to the loss of stearic and oleic acids.

Other effects are reported to be associated to the supplementation or liberation of fatty acids in the medium, such as secretion of inflammatory mediators (Lindenboim L *et al*., 1995; Goyeneche A *et al*., 2006), or alteration of permeability, with synergistic effect with calcium levels, enzyme activation and lipid signalling, as previously mentioned (Menendez JA, 2004; Murakami Y *et al*., 2000; Cook HW and McMaster CR, 2002; Kondoh Y *et al*., 2007; Landau Z *et al*., 2006; Ulloth JE *et al*., 2003; Mu YM et al., 2001; Matsuyama M *et al*., 2005). The scope of this work was to focus on the lipidomics, because the membrane fatty acid changes were not as well documented as other signalling pathways. In particular, the aim was to discern the membrane lipid reorganization between the one occurring during lipid deprivation and that occurring during a specific lipid supplementation. Focusing on palmitic acid, this fatty acid increased in membranes both in case of FCS deprivation and in case of palmitic acid supplementation. However, the dynamic of membrane changes monitored by lipidomics allowed a fundamental difference to be envisaged. In the FCS-deprivation, essential fatty acids are not present in the medium and the lipid reorganization occurring in membranes mainly depends on biosynthesis, that provides saturated and monounsaturated fatty acids. In fact, both saturated fatty acids, palmitic and stearic acids, increased in membranes. At the same time, the diminution of the n-6 series is expected and involved all the members (e.g., linoleic, 20:3 and arachidonic acids). Under such conditions, the diminution of arachidonic acid from membranes likely derives from the lack of its precursor. On the other hand, in the case of palmitic acid supplementation, only this saturated fatty acid increased, whereas stearic acid diminished. Essential fatty acids were regularly provided by FCS, but arachidonic acid progressively diminished, up to 30% in 3 hours and 40% in 24 hours (data not shown), and correlates significantly with stearic acid diminution ($r=0.987$; $P<0.05$).

The fatty acid monitoring during 150 μM palmitic acid supplementation evidenced that, among polyunsaturated fatty acids, there was a specific diminution of arachidonic acid (20:4). This effect could suggest its liberation as unesterified fatty acid, an event connected to the activation of phospholipase enzymes. In particular, PLA_2 enzymes are known to be central regulators of stimulus-coupled cellular arachidonic acid mobilization (Balsinde J *et al*., 2002; Kudo I and Murakami M, 2002), exhibiting a significant selectivity toward phospholipids bearing arachidonic acid moieties at the sn-2 position. Cytoplasmic PLA_2 (c-PLA_2) activation was monitored by a specific antibody detecting the phosphorylation at the Ser^{505} residue (Tavolari S *et al*., 2008). Figure 40B shows the detection of the c-PLA_2 phosphorylation during 150 μM palmitic acid supplementation by western blot analyses. In this cell line c-PLA_2 was always present, whereas its

activated form markedly increased in a time-dependent manner within 60 min of treatment compared to controls, a timing that matches exactly the release of arachidonic acid from membranes (Table 11 and Figure 39). c-PLA_2 activation was also compared to FCS deprived cells (Figure 40A). Under the latter condition we recall that all membrane EFA diminished, however this did not produce enzyme activation over 24 hours. The complete evaluation of the pathways connected to arachidonic acid release is beyond the scope of this thesis. Instead the focus was on fatty acid changes due to culturing conditions that can affect membrane assembly. With these data it was demonstrated that some culture conditions can produce membrane perturbation with the activation of biochemical pathways, such as the phospholipase activation and the release of signalling lipids from membranes.



Scheme 1. Membrane response and cell fate connected to serum deprivation (path A) and fatty acid supplementation (path B). Serum deprivation (path A) and fatty acid supplementation (path B) affect fatty acid composition of cell membrane phospholipids, and prime a membrane reorganization which affects membrane properties. Path A: Deficit of essential fatty acids (EFA) produces the decreasing of duplication time that is reversible by restoring the complete medium. Path B: membrane lipidome changes are coupled with the c-PLA_2 activation and arachidonic acid release from membranes, thus producing a signalling for the apoptotic process. Path B is reversible for 1 hour palmitic acid exposure followed by washing and restoring the complete medium.

The distinct scenarios based on the results of lipidome monitoring can be summarized by the Scheme 1: during the FCS-deprivation (Path A), essential fatty acids were lacking and the lipid metabolism could only proceed with saturated and monounsaturated components. This condition resulted in membrane lipid reorganization, with change of the membrane properties that did not cause phospholipase activation, but only reduction of duplication time, recovered by restoring the proper medium. On the other hand, in the case of palmitic acid supplementation (Path B), this saturated fatty acid was immediately incorporated in membranes, affecting the lipid packing as well. This occurred in the first hour together with the c-PLA_2 enzyme activation, which caused the release of arachidonic acid, an apoptotic signal. The one-hour time interval was indeed identified as the limit time of exposure to palmitic acid, during which membrane changes could be recovered by restoring the complete medium. This scenario can explain previous reports on the addition of linoleic and arachidonic acids that rescued from apoptosis cell cultures treated with palmitic acid (Mu YM *et al*., 2001; Beeharry N *et al*., 2004).

Ribosome-inactivating proteins (RIPs) are plant toxins that were exploited in this work as a cellular stress, because of their capacity to target ribosomes and damage them in an irreversible manner, acting enzymatically in a less-than-equimolar ratio. Morover, *in vitro* experiments showed other interesting actions exerted by these proteins, like DNA, PARP and mRNA depurination. The choice for this work was for saporin and ricin, that are among the best known and most studied RIPs, and lot of literature has been published since their discovery. However, there are still noumerous questions about these toxins that need an answer; not much is known about their mechanisms of entering the cell, their routing and their actions other than inactivate ribosomes, especially for saporin. Saporin, belonging to type I group, is a single A toxic chain, while ricin is a type II RIP, composed of a toxic A chain linked to a B chain with lectin properties. The B chain confers to ricin the capacity of binding galactose residues on the cell membrane, thus allowing a more efficient internalization of the toxin. Besides the different internalization, these two proteins differ also in their intracellular routing, and probably in localization and modality of action other than inactivate ribosomes. At the beginning, through a cell viability test conducted on NB-100 cells, concentrations of 10^{-6} M for saporin and of 10^{-12} M for ricin were selected for the following experiments. These concentrations caused very similar viability curves (see top graphs of Figure 42) and in particular an 80% cell death after 48 hours.

Figure 42 shows the inhibition of protein synthesis and the caspases activation for NB-100 cells intoxicated with saporin and ricin at concentrations reported above. The timing of protein synthesis inhibition was quite different for the two RIPs, with saporin causing a faster inhibition. This difference could possibly reflect a faster process of endocytosis for saporin as compared to ricin.

This result was opposit to the one shown elsewhere for L540 human Hodkin's lymphoma-derived cell line (Polito L *et al*., 2009), and highlights once more the variability that caracterize intoxication by RIPs. In NB-100 cells the routing could be completely different for both RIPs as compared to L540 cells, with saporin somehow reaching ribosomes faster than ricin, thus resulting more active on NB-100 cell ribosomes.

It is known that ricin induces apoptosis through caspase activation (Battelli MG, 2004; Narayanan S *et al*., 2005), whilst saporin has only been described to induce some apoptotic features, such as chromatin fragmentation, apoptotic bodies and hypodiploid cells (Bergamaschi G *et al*., 1996; Bolognesi A *et al*., 1996). Recently, caspase-dependent apoptosis in U937 cells has been reported to be induced by saporin through mitochondrial cascade, independently of translation inhibition (Sikriwal D *et al*., 2008). Polito L *et al*. (2009) suggested that more than one death mechanism was elicited by both ricin and saporin in L540 cells, with different timing and strength. Nevertheless, the pathway(s) followed and the factors involved are still not clear.

In our experiments, both ricin and saporin were able to induce a time-related activation of caspases. Saporin-induced caspases 3/7 were activated already at 4 hours, with a following exponential increase until 24 hours. This fast activation could be due to the rapid inactivation of protein synthesis described above, but links between these two processes are still far away to be discovered. Saporin appeared also to be slower than ricin in activating the extrinsic pathway. The activation of caspase-8 induced by saporin only at 16 hours could be related to some actions exerted on the membrane, and could stimulate the exponential increase of caspases 3/7 starting from 16 hours. According to Cavallaro U and Soria MR (1995), saporin may enter into cells after a low affinity binding to the urokinase and α2-macroglobulin receptors, which modulate the endocytosis of ligands. Cell surface binding ability could be the mechanism by which also this type 1 RIP may activate the extrinsic caspase pathway; however, if this is the case, it would have been expected a faster activation of caspase 8. Moreover, the binding of saporin to the cell membrane might not be essential for its cytotoxicity, in accordance with the reported irrelevance of the expression of the α2-macroglobulin receptor on eukaryotic cells (Bagga S *et al*., 2003); in fact, the inhibition of caspase-8 activity in human leukaemia cells was not able to inhibit the apoptosis caused by the type 2 RIP *Viscum album* agglutinin (Lavastre V *et al*., 2005). Our data together with those already reported suggest that the activation of the extrinsic pathway may not be essential in saporin-induced apoptosis, but can contribute to increase the amount of apoptotic signals.

Shifting our analysis towards ricin-intoxicated cells, it can be noted the elevate expression of caspases 3/7 at 16 hours. In spite of the over stimulation of caspases 3/7, ricin produced the same level of cell death as compared to saporin.. The modality of cell surface binding and the endocytic

pathway have been intensely studied in the case of ricin (Sandvig K and van Deurs B, 2005) and it always appeared to be more efficient than saporin in activating the extrinsic pathway. The usually strong activation of caspase-8 induced by ricin is thought be related to the lectin property of the B chain as already suggested (Hasegawa N *et al*., 2000), possibly by linking molecules on the cell surface. The significative amount of caspase-8 induced by ricin already at 4 hours could possibly be responsible for the downstream higher caspase-3/7 activity, thus working in a synergic way with the inhibition of protein synthesis.

The mitochondrial-driven intrinsic apoptosis pathway appeared not to be activated by ricin or saporin, which is highlighted from the non-expression of caspase-9 activity. It was thought that apoptosis could have been induced through the intrinsic pathway by various cell injuries including inhibition of protein synthesis as well as DNA damage, both of which may have been caused by either type 1 or type 2 RIPs, but in NB-100 cells seemed not to be true.

It should be stressed that caspase-8 had a statistically significant activation over the normal range at different times by both RIPs. This was already noticed for L540 cells (Polito L *et al*., 2009), where the cytotoxic consequence of intoxication with saporin and ricin (DNA fragmentation and loss of cell viability) showed similar intensities but different temporal patterns. In the present study, the timecourse of protein synthesis inhibition induced by saporin did not correlate with the levels of cell death, thus suggesting the occurrence of RIP intracellular targets apart from rRNA. This hypothesis is in agreement with the reported irrelevance of protein synthesis inhibitory activity in the induction of apoptosis that has been observed with saporin mutants (Sikriwal D *et al*., 2008). On the other hand, the timecourse of protein synthesis inhibition induced by ricin well correlate with the levels of cell death, thus suggesting for this RIP protein synthesis inhibition as the main damage for NB-100 cells. This hypothesis is in contrast with the reported irrelevance of protein synthesis inhibitory activity in the induction of apoptosis that has been observed with ricin mutants (Li XP *et al*., 2007). In any case, the differences between ricin and saporin in caspase-8 and caspase-3/7 activation appeared not to influence the level and the kinetics of cell killing. This finding is compatible with the reported redundancy of RIP-induced apoptosis (Komatsu N *et al*., 1998), in which a single stimulus can simultaneously activate more than one apoptotic pathway, although not all having the same relevance in inducing cell death. Alternative types of cell death, distinct from necrosis and requiring gene expression, have been described and classified as programmed death (Sperandio S *et al*., 2000; Formigli L *et al*., 2000; Jaattela M and Tschopp J, 2003; Kroemer G and Martin SJ, 2005). These forms of cell death are independent of caspase activation and also appear different from apoptosis according to both morphologic and biochemical criteria. Thus, RIP-induced damage

could occur as a consequence of a multi-directional pathway, apoptosis being the most typical followed.

Despite the enormous literature about RIPs, there are still a few points that remain obscure, as already explained above. However, any study is available on membrane reconfiguration following a RIP treatment. Certainly, membrane is not the main target of RIPs, but, with several open questions still unanswered, it could be suggested that membrane lipid bylayers could play an important role. It seemed therefore reasonable to pursue a fatty acid analysis of cell intoxicated with these proteins; coupling this new study with previous data led us to obtain a more complete view of RIPs dinamics inside the cell. Two sets of experiment were carried out: the follow-up of intoxicated NB-100 cells for a definite interval of time and the investigation of cells loaded with different concentrations of toxins. Results reported in Tables 12, 13 and 14 enclose a lot of ideas that will be discussed hereinafter. As highlighted in Figures 43 and 44, a completely opposite behaviour of the two RIPs can be noticed, concerning the total amount of saturated and monounsaturated fatty acids. It is worth recalling that saturated fats can influence membrane assembly and properties. On the other hand, monounsaturated fatty acids contribute positively to the lipid packing, counteracting the rigidity of membranes produced by saturated fatty acids. Saporin induced a slight increase of total saturated fatty acids, mainly palmitic acid, and a slight decrease of total monounsaturated fatty acids, mainly oleic acid, as it is clearly shown in the top graph of Figure 43. This can be interpreted as a cell response to envinronmental changes; therefore, protein toxicity can induce a membrane change in the direction of the decrease of the membrane exchanges with the outside. However, as we have previously seen, an increase of saturated fats could also trigger apoptosis. On the other hand, the behaviour of cell membranes intoxicated with ricin appears to be in different directions. At 4 hours saturated and monounsaturated fats behaved as in saporin-intoxicated cells, with a raise in membrane packing. After 8 hours an inversion of behaviour is observed, leading to an increase of 25% of unsaturated components with a contemporary loss of 20% of saturated acyl chains. This suggests that the two toxins induce different responses of lipid metabolism. Further investigation of related enzyme expressions (Δ^9 desaturase) will be carried out in order to monitor the possible metabolic pathways.

Bearing in mind the different behaviour between the two RIPs about the total content of saturated and monounsaturated fats, we decided to use the toxins in the presence of 150 μM saturated (palmitic acid) or monounsaturated (oleic acid) fatty acid (data not shown). Palmitic acid acted in a synergic way with both RIPs, causing a more pronounced level of cell death. This could be explained by the fact that both palmitic acid supplementation, previously demonstrated, and toxin incubation cause the release of arachidonic acid from membranes, an apoptotic signal. The addition

of oleic acid did not influence the viability curve of cells intoxicated with saporin; on the contrary, cells incubated with ricin in the presence of oleic acid ameliorate their survival of about 10% after 24 hours, compared to the corresponding control cells with the sole ricin. It could tentatively explained that the increase of oleic acid in membranes is a cell response in order to face with the intoxication. Further investigation will be carried out in order to envisage pathways of protein activation correlated to the oleic acid synthesis.

Tables 12, 13 and 14 and Figures 43 and 44 show that in intoxicated cells there is an alteration of the n-6 pathway. In each pool of cells treated with RIPs, DGLA was significantly lower than controls. In figure 43 it is clearly evident that DGLA decrease in proportion to the time, reaching a loss of 20-30% at 24 hours for both saporin and ricin, and it is not dose-dependent (Figure 44). It is worth recalling that DGLA is the precursor of arachidonic acid and the costant loss of DGLA over time could be due to its transformation (desaturation) to arachidonic acid. On the other hand, DGLA can be also trasformed into antiinflammatory messengers, and this can be a pathway to contrast the inflammated phenotype induced by RIPs intoxication. Moreover, changes occurred to arachidonic acid residues in membranes. In ricin-intoxicated cells there is an increase of this fatty acid of about 20% until 16 hours, and a release 30% between 16 and 24 hours. The timing of arachidonic acid release from membranes matched perfectly caspases 3/7 activation. Is shoud be noted that caspases 3/7 are already highly expressed at 16 hours (Figure 42); thus, caspases activation happened slightly before arachidonic acid release, suggesting the connection between these two pro-apoptotic events. On the other hand, saporin induced a fluctuation of arachidonic acid percentage in membranes, and the 10% loss that happened at 8 hours could instead occurr before caspases 3/7 activation (Figures 42 and 43). The two toxins appear to be different for the membrane involvement, suggesting for saporin a synergy between membrane lipid reorganization and apoptotic signalling. More investigation is needed, in particular on the differences of protein expression (lipid biosynthesis) between the two toxins.

Since several factors affecting cell machinery could be responsible for cell death, a better knowledge of death mechanisms induced by RIPs could be strategically important for their therapeutic use. RIPs have been utilized in biology and in medicine, mostly linked to antibodies or other carriers to prepare “immunotoxins” or other conjugates (Bolognesi A and Polito L, 2004). These hybrid molecules are selectively toxic to target cells and have been mainly used for the experimental therapy of haematological neoplasias (Polito L *et al*., 2004; Tazzari PL *et al*., 2001; Bolognesi A *et al*., 2005; Bolognesi A and Polito L, 2004). The possibility of modulating cell killing of neoplastic cells through different pathways adds new possibilities to develop combined synergic immunochemotherapy.

The geometry of lipid double bonds has recently acquired more and more significance, since the naturally occurring membrane fatty acids have mostly the *cis* configuration, provided enzymatically, whereas the *trans* geometrical isomers can be considered natural only in bacteria (Zhang YM and Rock CO, 2008). Few studies are available about *trans* fatty acid actions on cells (Wei Y *et al*., 2007; Kondoh *et al*., 2007), coupling the presence on *trans* fats in the medium to cells apoptosis. However, there are no informations reported about the lipidomics of cell membranes, and any connection linking the presence of *trans* fats to apoptosis is discussed. Therefore we decided to investigate in a deeper way this new fiels of radical stress, trying to use an endogenous radical-producing way, in order to stay closer to what happens in a real cell envinronment. Literature highlights that in the last years the *cis* to *trans* conversion of unsaturated membrane lipids has been associated to free radical stress mostly deriving from sulfur-containing compounds (Chatgilialoglu C and Ferreri C, 2005; Ferreri C *et al*., 2005; 2005; Zambonin L *et al*., 2006). Functionalized aliphatic thiols (RSHs) are contained in living organisms in considerable amounts as they partecipate to the antioxidant network. However, recent investigations focused on the reactivity of thiol groups based on the production of sulfur-centered radical species, RS• (thiyl radicals) as the result of the repair reaction (RSH → RS•) (Halliwell B and Gutteridge JMC, 2001; von Sonntag C, 1987; Prager A *et al*., 1993; Savoye C *et al*., 1997; Koning AWT *et al*., 1979). The reactivity of sulfur-centered radicals is well known in organic chemistry, but only recently these radical species have been taken into consideration for biologically related mechanisms (Pogocki D and Schoneich C, 2001; Nauser T and Schoneich C, 2003), and in this context lipids have become an interesting target since their structures have reactive sites, such as C=C double bonds and bis-allylic positions (Chatgilialoglu C *et al*., 2002; 2005; Chatgilialoglu C and Ferreri C, 2005; Ferreri C and Chatgilialoglu C, 2005). As highlighted in Chapter 4, hydrogen sulfide (H_2S) is the simplest thiol produced in substantial amount by mammalian tissues and it is an important mediator of neuronal cells, with a concentration in the brain reaching more than 100 μM (Doeller JE *et al*., 2005). It exerts many physiological effects suggesting its potential role as a regulatory mediator (Lowicka E and Beltowski J, 2007). Recently, Lykakis IN *et al*. (2007) used a biomimetic model of vescicle suspension which mimics the aqueous and membrane compartments of a cell, and demostrated the potential of sulfhydryl radicals (HS•/S•-) derived from H_2S to penetrate the hydrophobic fatty acid chains, to attack double bonds and to produce a high proportion of *trans* fatty acid residues. This model offered some insight into the chemical basis of the biological activity of H_2S, which has not yet been established. All this is important in view of the intriguing role of the sulfhydryl radical-induced *cis-trans* conversion of lipids, either as a damaging or a signaling pathway. H_2S was therefore used here to generate a thiyl radical-derived stress,

mimicking a diffuse alteration of the radicals/antioxidants ratio in the cytoplasm of the cell. Since thiyl radicals represent the most relevant radical species able to target lipids double bonds and to obtain a *cis-trans* isomerization, the follow-up of fatty acid composition NB-100 cells was reported with a parallel evaluation of cell viability. Table 15 highlights once more the main difference of lipid compositions between controls grown in the presence or absence of FCS, that had previously been discussed as a consequence of essential fatty acid deficit. The main interesting data concern the level of *trans* isomers of membrane lipids. Figure 45 shows the arachidonic acid isomers, which have been described as biomarkers of radical stress also in cell cultures (Ferreri C *et al*., 2002; 2003; 2004). The lipid isomerization is due to radical species able to diffuse into cell membranes and to convert the cis double bonds of membrane lipids into their correspondet *trans* geometrical isomers, as already described for H_2S in liposomes (Lykakis IN *et al*., 2007). In particular, these free radicals diffuse in the membrane bilayer and, due to the supramolecolar organization of the phospholipids, the double bonds closer to the aqueous phase are the most susceptible to the radical attack. Previous reports showed that arachidonic acid is the most sensitive fatty acid for free radical attack and the library of *trans* isomers of this fatty acid is available for recognition in biological samples. The increment of *trans*-20:4 isomers in H_2S-treated cells is statistically very significant and it is higher in cells cultivated without FCS. This could be expected since the lack of FCS brings as consequence the absence of antioxidants and radical-scavenging molecules in general, such as albumin. Figure 45 also shows that the radical initiator AAPH did not affect significantly the level of *trans* fatty acid isomers in the presence of FCS, whereas in the absence of it there was a slight increase in the formation of these isomers. It is worth pointing out that cell viability was not diminished by H_2S treatment. In Figure 46 it is clearly shown that H_2S is eventually stimulating cell growth when the experiment is carried out in RPMI supplemented with FCS, due to its important role as mediator of neuronal cells (Eto K *et al*., 2002; Nagai Y et al., 2004; Kimura Y *et al*., 2006). While no effects on cell viability were seen with H_2S treatment in the absence of FCS, a decrease was noted with the H_2S+AAPH treatment; in this case, the high amount of radical species induced by AAPH in antioxidant-free envinronment increased cell stress with the consequent drop of cell viability. From these data it emerges that endogenous-produced *trans* fatty acids are not toxic or apoptotic for cells, the main reason being their very low level compared to the whole pool of fatty acids. In previous works reporting apoptosis (Wei Y *et al*., 2007; Kondoh Y *et al*., 2007), cells were cultivated in a higher concentration of *trans* fats; moreover, they derived from exogenous sources. Thus, the main action exerted by endogenous-produces *trans* fatty acids could be only the decrease of membrane fluidity due to the loss of the spatial width of a *cis* double bond. However, other major effects of these endogenous-produced *trans* fatty acids could be present and still obscure. *Trans*

lipids can be seen as signalling molecules, activating cell responses at a certain low concentration, whereas at higher amounts (such as the one used for supplementation) they become toxic, also for their activity on cell membranes.

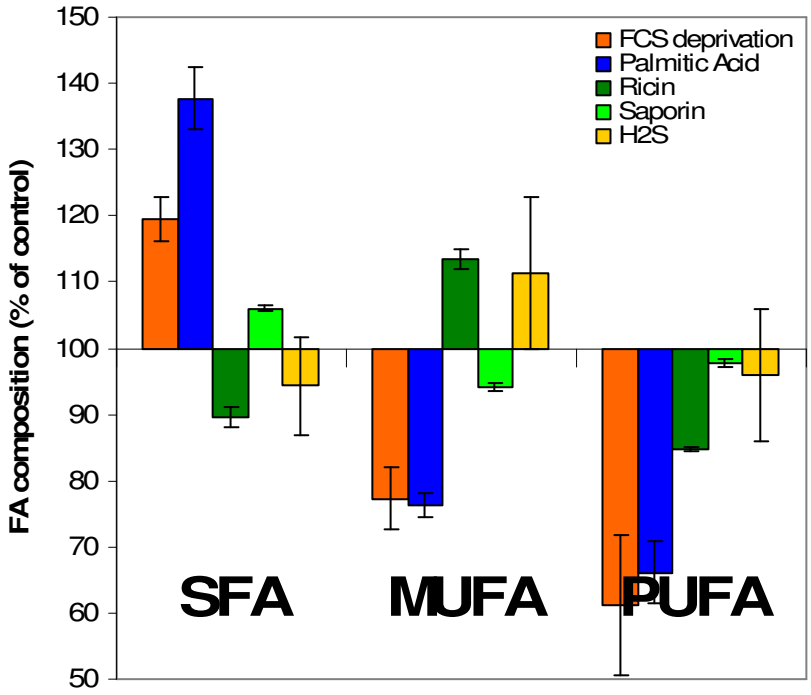


Figure 47. Total amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in the five different conditions tested in this work: FCS deprivation at 24h, palmitic acid 150 µM at 24h, ricin 10^{-12} M 24h, saporin 10^{-6} M 24h, H_2S 0.1 mM 20 min. Data have been chosen between experiments illustrated before.

Figures 47 and 48 summarize all the data discussed above and point out the main observations carried out by this work. In figure 47 the behaviour of the main classes of fatty acids are reported, summarizing the results obtained in this work and showing the fluctuation of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fats in NB-100 cells treated with five different stresses. It is important underlining that different stress types could act in a different way on cells. Obviously, fatty acid supplementation and FCS deprivation can act directly on the plasma membrane and instantly influence membrane composition, in that way connecting to the release of biologically active mediators. On the other hand, RIPs intoxication or H_2S-derived radical stress can induce cell defence mechanisms, where changes of the membrane composition can make part of the

response to the stressor. This could explain the different extent of alterations observed in the five conditions: small alterations of cell membrane lipidome for RIPs intoxication or H_2S-derived radical stress, whereas fatty acid supplementation and FCS deprivation caused a more pronounced change of the fatty acid status.

It is worth pointing out that the total amount of PUFA tends to decrease in each situation of stress, especially when a reorganization of the membrane is involved. Thus, the high concentration of palmitic acid caused its inclusion in the phospholipids acyl chains, with a big loss of total PUFA, whereas the lack of FCS produced a huge decrease in polyunsaturated fats, due to the absence of essential fatty acids in the medium. On the other hand, SFA and MUFA vary considerably, and the increase of one class always corresponds to the decrease of the other. From figure 47 is also clear that processes in which membranes are not directly involved (ricin, saporin and H_2S stresses), are not subjected to consistent variations in the total amount of SFA and MUFA. In fact, only in the case of FCS deprivation and palmitic acid supplementation differences appeared in these categories compared to controls. In the palmitic acid supplementation, its incorporation in membranes occurs**,** with a contemporary decrease of stearic acid, but with a resulting net gain for total SFA; as a consequence, MUFA diminished by a 30% after 24 hours. On the contrary, with FCS deprivation, the most probable reason for the decrease in MUFA (mainly oleic acid) could be a reduced enzymatic activity (desaturase Δ^9), due to the lack of cofactors (NADH, vitamins) that are usually present in FCS. Therefore, the net result was the contemporary increase of SFA. The two toxins have an opposite effect on the SFA and MUFA families (Figure 47), whereas ricin caused a higher loss of PUFA than saporin. It can be observed that the combination of toxicity with membrane reorganization is more direct in the case of saporin (since it resembles those reported for fatty acid supplementation or deprivation) than in the case of ricin, whereas ricin seems to have a relationship with the apoptotic signalling of arachidonic acid. It can be hypothesized a role of the two different protein structure and their interaction with membranes, but this needs further investigations.

In figure 48 arachidonic acid and its *trans*-isomers are reported. FCS deprivation causes a general lack of essential fatty acids but not a specific release of arachidonic acid from membranes and in this condition cells do not undergo apoptosis. On the other hand, palmitic acid supplementation caused its release from membranes through c-PLA_2, that leads cells to programmed cell death. Instead, the presence of *trans* isomers is present in a very high amount in H_2S exposed cells, due to the increased formation of thiyl radicals, the isomerising agent for the arachidonic double bonds. Moreover, there is a slight increase also in FCS deprivation that could derive from the lack of FCS antioxidants. The decrease of the other 20:4 isomer values could be interpreted as a dilution of pre-existent *trans* fatty acids due to cell growth.

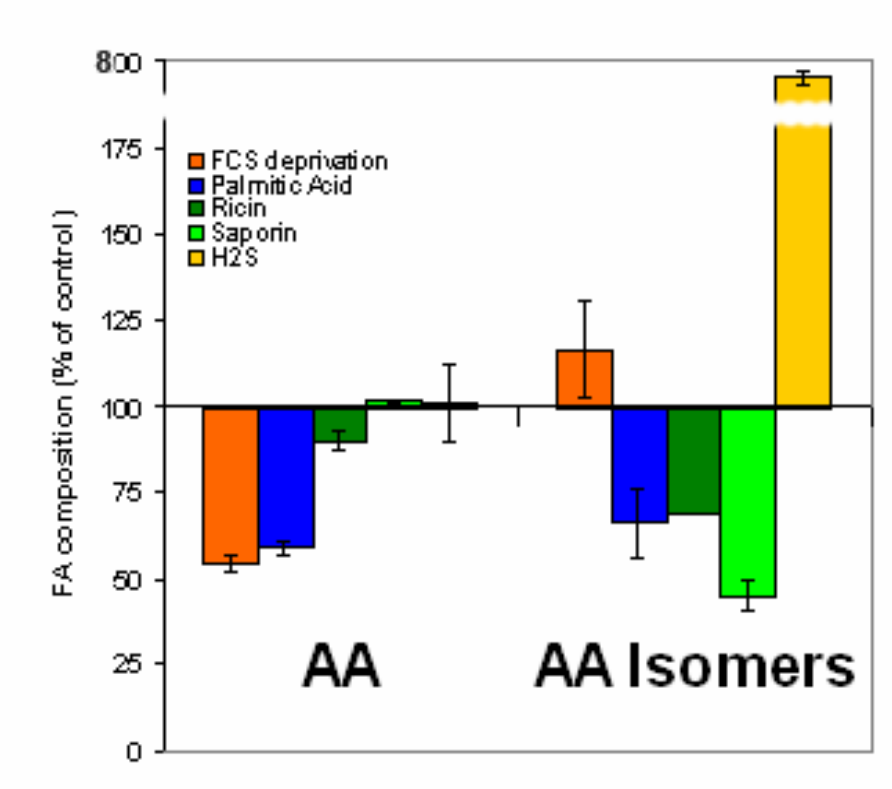


Figure 48. Total amount of arachidonic acid and its *trans*-isomers in the five different conditions tested in this work: FCS deprivation at 24h, palmitic acid 150 µM at 24h, ricin 10^{-12} M 24h, saporin 10^{-6} M 24h, H_2S 0.1 mM 20 min. Data have been chosen between experiments illustrated before.

In conclusion, membrane lipidome monitoring is a meaningful tool in cell biology experiments for the characterization of cell cultures and the examination of fatty acid reorganization during an experiment. The results of this work gives further molecular evidences that dietary fatty acids can give different effects, depending not only from the dose but also from the duration of their supplementation, involving directly the membrane balance and indirectly biochemical pathways of cell signalling. These results confirm the association of membrane lipid status to culture conditions, and indicate in the understanding of fatty acid reorganization and in the synergy with signalling pathways methods to envisage strategies of intervention in the fate of cell cultures. More generally, this work intends to increase attention on the application of lipidomics to follow-up the fatty acid status and changes influencing membrane reorganization that can contribute to prime biochemical cascades.

It is clear that in the last years lipidomics has become a big branch of cell biology, with no less importance in comparison to genomics and proteomics. However, as for them, there are still numerous questions without an answer, and the picture that emerges from lipidomics is becoming

more and more complex. A particularly interesting outstanding question is how do the sensors of membrane viscosity operate at the molecular level. These sensors are either coupled to transcription factors or are enzymes that have activities that are intrinsically regulated by the biophysical properties of the membrane. Advances in genomics and lipidomics will facilitate the future biomedical investigation of this and many other questions concerning membrane homeostasis. Moreover, many effective antibiotics function by disrupting the membrane, and lipid synthesis is a validated target for new drugs against the expanding repertoire of bacteria that are resistant to current therapies (Zhang YM *et al*., 2006). Also, understanding membrane homeostasis is crucial for the construction of 'designer' organisms that are engineered to either produce complex organic molecules or degrade selected environmental pollutants. Although studies of membranes can now benefit from the large-scale detailed analyses of lipid molecular species, there is currently a paucity of data regarding key points, including: complete lipid compositional analysis for each organelle, the lipid compositions of each bilayer leaflet, the coupling of phase behaviours of the two leaflets, and the influence of proteins on lipid phase behaviours. To overcome the technical and conceptual barriers confronted by the field, a multidisciplinary approach is required, involving chemists, biochemists, cell biologists, physicists and information technologists working together. Such progress will highlight the basic working principles of cells and tissues, but will also allow fundamental insights to be gathered into the pathogenicity of disease. Important human diseases, such as atherosclerosis, infectious diseases, Alzheimer's disease and cancer, all have a lipid component in their epidemiology. A molecular understanding of the contribution of lipids to the disease process will allow the development of novel approaches to prevention, diagnosis and cure. Methods are being developed for dealing with large databases and interpreting the data in the context of the cell as a system, and it will be exciting to see what predictions and new insights these approaches will come up with. Cellular lipidomics or how cells use lipids for their vital functions: a lifting fog and thrilling vista.

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