ROMANIAN ACADEMY
INSTITUTE OF BIOCHEMISTRY

DOCTORAL DISSERTATION SUMMARY

THE ROLE OF THE BALANCE BETWEEN SATURATED *VERSUS* UNSATURATED FATTY ACIDS IN NORMAL CELLULAR MECHANISMS AND IN PATHOLOGY

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INTRODUCTION

Lipids, due to their various fatty acid composition (including the saturated /unsaturated fatty acids ratio), interact with the transmembrane domains of membrane proteins in different ways, influencing their conformational arrangement, determining modulations of their functions. The effects induced by lipid chemistry alteration are manifested both in the normal function of the cells, and in pathological modifications, including cancer. Therefore, in the past decade, the study of the effects of membrane lipid composition has been re-launched from the perspective of the effects on membrane protein function in cell physiology and pathology, including cancer. Moreover, there has been increased interest in the investigation of the therapeutic role of membrane lipids' chemistry modelling.

At the international level, the interest in the study of membrane lipids, within the context of the investigation of membrane molecular organization is increasing. The first article reporting studies on membrane lipids and their modifications in cancer cells, with effects on membrane fluidity, was published in 1974 [1]. Regarding the interest in the therapeutic importance of membrane lipids in cancer, the first article was published in 1987 [2]. The membrane-lipid therapy refers both to fat-soluble drugs, which influence the organization of the lipid bilayer, *i.e.* its physicochemical characteristics, and the membrane organization behaviour of this ultra-structural element, and to the modulation of the chemical composition of bio-membrane lipids, with similar effects. Such approaches are currently focused on the research of:

- the structure-function correlations at the level of membrane lipids;
- the lipid-protein interactions;
- the effects of the structure of lipids and the lipid-protein interactions on cell signalling and pathophysiology.

The studies are somewhat recent, however they approach a large variety of pathologies: cardiovascular pathologies, neurodegenerative pathologies, obesity, other metabolic disorders, inflammations, infectious and autoimmune diseases. As far as cancer is concerned, lipid modifications have been reported, with alterations at the

level of the cell structure and function, including proliferation, or inducing multiple drug resistance.

Starting from the information found in the literature, regarding the diversity and the role of fatty acids from cell lipids, the aim of this paper is to characterize certain cell types, in terms of the fatty acids content and the saturated fatty acids/unsaturated fatty acids ratio, grouped in two main directions:

1. analysis of fatty acids from the lipids of certain (normal and tumoral) cell lines

The first part of the paper aimed to identify the changes in cell behaviour, following the modelling of the saturated fatty acids/unsaturated fatty acids ratio, by the supplementation of the culture medium with polyunsaturated fatty acids.

The second part of the paper analyzed the effect of the treatment with polyunsaturated fatty acids and exposure to UVA radiation on skin cells.

2. analysis of fatty acids from organs, using experimental animal models

The aim was to investigate the types of lipids and fatty acids from mouse organs, in the context ischemic pathology, to identify certain ways to ensure myocardium protection.

Thus, the present doctoral thesis introduces new methodological approaches, studying the role of the balance between saturated fatty acids and unsaturated fatty acids in normal cellular mechanisms and in pathology. The study distinguishes itself by the high number of experiments conducted, allowing for various conclusions to be drawn, revealing details related to the information already known and opening new ways, to be used as a basis for future research.

The doctoral thesis entitled "The Role of the Balance between Saturated Fatty Acids and Unsaturated Fatty Acids in Normal Cellular Mechanisms and in Pathology" is divided into 3 chapters – which, in turn, are made up of several sections – and references. Chapter 1 contains a general section, illustrating the current stage of knowledge in the thesis topic, Chapter 2 presents the study methodology, and Chapter 3 contains the results of the experiments conducted, followed by the related conclusions.

GENERAL SECTION

The main structural component of the cell membrane is the lipid bilayer, the structural function of the membrane lipids organized as a bilayer being essential. The lipid bilayer confers the cell membrane the role of a barrier, this purpose of the membrane lipids being formulated ever since their presence in the structure of the membrane has been demonstrated [3]. Far from having a merely structural role, membrane lipids are also involved in important functions of the ultrastructure, allowing the cell to communicate with the environment, exchanging information and matter.

Fatty acids play a very important part in cellular physiology (membrane transport, cell signalling), both by modulating membrane fluidity, and by their effect on the interactions with the transmembrane parts of integral proteins, or by the action of second messengers, which can be obtained after being released from the structure of the lipid.

Numerous cell functions, such as the localisation and activity of membrane proteins, may be modulated by the saturated/unsaturated fatty acids ratio. Disorders in the saturated/unsaturated fatty acids ratio have been described in numerous human pathologies, however such influence has not been anticipated. This gives rise to the current interest in designing specific therapies at the level of membrane lipids, influencing the organization of lipids (functions and structure) by simultaneously inducing the modulation of the membrane proteins' activity, and of their localisation; this type of adjustment may eventually induce changes in cell signalling and genic expression which may hypothetically serve to the reversibility of the pathological condition [4,5].

The alterations in the three-dimensional structure of membrane lipids, by the modification of their composition in fatty acids, lead to numerous diseases (hypertension, sudden death, cardiac hypertrophy, atherosclerosis, coronary diseases, thromboses), cancer (proliferation and drug resistance), obesity, neuronal diseases (Alzheimer, schizophrenia), respiratory diseases, kidney diseases, inflammations, immune system diseases, infectious diseases, coagulation, fetal injuries due to alcohol [6-10]. Studies on tumoral cell lipids have revealed differences at the level of phospholipids and glycolipids, and one may even speak about a metastatic phenotype.

Such experiments conducted on tumoral cells compared to healthy cells have shown that the phospholipids from the tumoral cells contain a higher level of oleic acid (C18:1) and a lower percentage of arachidonic acid (C20:4) and C22 polyunsaturated acids [11-15].

Cellular activity is significantly influenced by membrane behaviour. Membrane function can be modulated by the properties of the lipid bilayer, and they also depend on the types of fatty acids from the composition of lipids.

Studies regarding supplementation of the culture medium on various cell lines with polyunsaturated fatty acids (PUFA) describe major changes in fatty acid composition in the plasma membrane, as well as in lipid rafts [16]. Also, an increasing number of papers have shown the effect of PUFA (primarily DHA – docosahexaenoic acid and EPA – eicosapentaenoic acid) on the physical properties of the membrane. These studies describe changes in the membrane permeability [17], membrane elasticity [18-20], thus modulating membrane fusion processes [21], vesicle formation [22] and flip-flop mechanisms [23]. PUFA are incorporated in cells, being esterified in position sn-2 in membrane phospholipids, i.e. phosphatidylcolines and phosphatidylethanolamines, and, in the neuronal tissue, in phosphatidylserines [24]. Cellular incorporation of free PUFA, and their insertion in the membrane phospholipids modulates the physical properties of the membrane. They are able to change the mutual accommodation properties of membrane phospholipids at the level of the bilayer, by reducing Van der Waals interactions[25], the degree of disorganization caused in the membrane varying according to the fatty acid used (stearic acid < oleic acid < EPA < DHA). PUFA affect membrane fluidity, influencing membrane permeability for ions by changing the conformation of the carrier proteins [26], modulate the activity of ion channels [27-33] and influence the behaviour of lipid rafts and caveolae. PUFA selectively modulates the sub-cellular localization of signalling proteins with lipid residues attached, controlling their intracellular transport path.

During the past years, the interest in the study of the beneficial effects of PUFA consumption on health has increased continually. Clinical trials have shown that a diet rich in omega 3 fatty acids helps prevent cancer, or stop its advancement.

EXPERIMENTAL PART

The effect of DHA treatment on the fatty acid content from cell lipids

Depending on the type and number of membrane lipids (saturated/unsaturated fatty acids ratio), numerous cell functions are controlled, such as the localization and activity of membrane proteins. Disorders in the saturated/unsaturated fatty acids ratio have been described in numerous human pathologies, in an attempt to design specific therapies at the level of membrane lipids, by influencing the lipid organization (functions and structure), simultaneously inducing the modulation of membrane protein activity, and of their localization. This has given rise to the current interest in the investigation of fatty acids composition of membrane lipids, as a possible therapeutic target.

Therefore, the present study monitored the effect of the administration of *cis*-docosa-4,7,10,13,16,19-hexaenoic (DHA) acid, 100 μM and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), 10 μM as an antioxidant, on MCF-7 cells. For the control cells, the culture medium was supplemented only with 10uM BHT. It was noticed that, at the concentration of 100 μM, the survival rate of the MCF-7 was the highest, compared to other tumoral cell lines investigated. The MCF-7 cell line used does not synthesize caspase 3, therefore the survival of a significantly higher number of cells after the treatment may be explained by the fact that the apoptosis was not induced. Considering these results, MCF-7 line was chosen for further studies on consequences of DHA treatment beyond the cytotoxic effect.

The gas cromatography analysis has revealed that DHA reverts the ratios of saturated fatty acids, and unsaturated fatty acids, respectively, in the treated cells, compared to the control cells. Also, the oleic acid ratio has dropped, the monounsaturated fatty acid has increased in the tumoral cells, while the palmitic acid/saturated fatty acid ratio has increased. Such modifications suggest an effect of the DHA treatment on the stearoyl-CoA dehydrogenase, an enzyme responsible for the synthesis of oleic acid. The results of the study conducted on the levels of expression of SCD1 have shown (both by western blot, and by immunofluorescence) that no significant changes occur, which indicates that the drop in the oleic acid level, following the DHA treatment, is based on other mechanisms than the cellular level reduction for the stearic acid dehydrogenation enzyme.

Subsequent studies have been made, regarding the effect of the modulation of the content of fatty acids from cell lipids in the level of the membranes of the various cell fractions, on MCF-7 cells. The control cells and the cells treated with DHA were collected, homogenized and subject to fractionation, to obtain nuclear, plasmalemmal and cytoplasmic sets. The purity of these fractions was verified by western blot, and the fatty acids content in their membrane lipids was determined by gas cromatography. High purity cellular fractions were obtained. For total lysate, the saturated fatty acids content has dropped, primarily due to the palmitic acid. This variation can also be found in the cellular fractions, although, in the plasmalemmal fraction, the stearic acid increase has a significant contribution to this. Such changes in the content of saturated fatty acids are accompanied by a drop in the content of unsaturated fatty acids, with essential contribution for monounsaturated fatty acids. The effect is due to the oleic acid, although the palmitoleic acid also contributes in the plasmalemmal fraction. Global polyunsaturated fatty acids do not seem to vary significantly, although arachidonic acid obviously drops in all cellular fractions.

DHA treatment has determined a change in cellular morphology, leading, on the one hand, to a dimensional levelling of the nuclei and, on the other hand, to accumulations of lipid inclusions. Real time video-microscopy monitoring has allowed us to show that DHA treatment determines faster adherence and spreading on collagen of the MCF7 cells. Lower effects have been recorded in regards to cell motility, examined both in terms of the migration speed, as well as directionality of movement in basal conditions.

In conclusion, the fatty acids composition of cellular lipids has been modulated by DHA treatment, resulting in modifications in the morphology and behaviour of the MCF7 cells.

The effect of the treatment with unsaturated fatty acids and exposure to UVA radiation on skin cells

Starting from the idea that oxidative stress has adverse effects on the recovery of the epidermis, we investigated the concerted effect of the administration of DHA 50 μM in the culture environment and UVA irradiation on the behaviour normal and dysplastic keratinocytes. First, at a biochemical level, the effect of DHA treatment and UVA exposure on the modifications of the fatty acids content of cell lipids, by gas cromatography, was analyzed. Then the effects of DHA+UVA on the cell

adherence/spreading and proliferation were investigated, in real time, through cell layer impedance measurements. The keratinocytes' capacity to recover the denuded surface, in a wound healing experimental model was also investigated. The results presented in this paper refer to comparative studies on the behaviour of DOK dysplastic keratinocytes and şi HaCaT normal keratinocytes. Experiments were conducted on cells whose culture medium was supplemented with 50 μM DHA and 5 μM BHT, as antioxidant and cells whose culture medium was supplemented only with 5 μM BHT. To investigate the effect of UVA radiation, the cells were irradiated for 30 minutes, in PBS. In order to monitor the dynamics of the modifications produced by UVA in time, the experiments were conducted at various time intervals: immediately after irradiation, respectively at 6, 12 and 24 hours post-irradiation (there was one control sample for each experimental condition).

The gas cromatography analysis of the unsaturated/saturated fatty acids ratio, calculated as the irradiated samples/non-irradiated samples ratio, has revealed a decrease in the ratio of unsaturated fatty acids from the membrane lipids in irradiated cells, compared to non-irradiated cells, especially for the DHA treated cells, both in the case of dysplastic keratinocytes, and normal keratinocytes. The rate of monounsaturated fatty acids (oleic acid) is lower in the case of DHA treated cells, for both cell types (higher effect in the case of the HaCaT cells), both post-irradiation, and for the non-irradiated cells. The amount of polyunsaturated fatty acids is influenced both by the cell exposure to UVA radiation, and by their treatment with docosahexaenoic acid. In the case of DOK cells treated with DHA, the rate of polyunsaturated fatty acids is higher for the irradiated cells, compared to non-irradiated cells. The same trend may be noticed in the case of the HaCaT cells, within less than 24 hours post irradiation. The highest difference between the irradiated and non-irradiated cells was noticed in the case of the samples examined 12 hours post-irradiation. In the case of the cells which were not treated with DHA, for HaCaT, we noticed a lower variation of the polyunsaturated fatty acids, following the UVA exposure for 30 minutes.

Alongside many other cellular phenomena, adherence and spreading are processes which depend on numerous factors, including the membrane behaviour at a physical and chemical level. That is why the changes occurring in the ratio between the types of fatty acids from the membrane lipids may influence the adherence and

spreading capacity of the cells. During the studies conducted in relation to this paper, we investigated the dynamics of the adherence and spreading of keratinocytes through cell layer impedance measurements, with the xCELLigence system.

Using real time video-microscopy investigations on the BioStation IM equipment, we studied the effects of the DHA treatment and UVA exposure on the motility of normal and dysplastic keratinocytes. The analysis of the images obtained has shown that UVA exposure influences the keratinocytes' capacity to recover the cell layer after scratching, in the wound healing experimental model. This effect is obvious in the case of normal keratinocytes. DHA treatment appears to have a positive effect on the irradiated dysplastic keratinocytes, following UVA exposure for 30 minutes, the treated cells having the capacity to cover the denuded surface faster than the un-treated (control) cells.

The study of fatty acids from mouse hearts with receptor deficiency for SDF-1α

The study of the effects of ischemia and reperfusion on the myocardium is still on the map, in terms of the impact on the knowledge regarding cardiovascular pathologies. The interpretation, at a cellular and molecular level, of the phenomena induced by the ischemic events allows the identification of methods to protect and regenerate the myocardium. The Cxcr4/SDF-1α axis mediates the recruitment of progenitor cells in the ischemic area and promotes myocardium regeneration.

Taking into consideration that the SDF-1/CXCR4 interaction is being increasingly exploited for the stem cell therapy after myocardial infarction, in collaboration with the team coordinated by Dr. Elisa Liehn from the "Institute of Molecular Cardiovascular Research" in Aachen, where, in an experimental model of transgenic mouse ($CXCR4^{+/-}$), lipid accumulations in cardiomyocytes with different morphological appearance than those of the control mice, we proposed to investigate the types of fatty acids from the lipid extract of myocardium. The various types of lipids have been separated, by HPLC, from the lipid extract, and then analyzed in our laboratory, for the types of fatty acids.

The purpose of the analysis conducted in regards to the content of fatty acids within the triglyceride fraction was to explain the electron dense appearance of lipid inclusions in the cardiomyocytes of transgenic mice. The hearts of the heterozygote animals have shown a higher accumulation of lipid drops in the cardiomyocytes, as

evidenced by electron microscopy. Moreover, the osmiophilia of these lipid inclusions is significantly higher than that of the inclusions from the cardiomyocytes of normal animals. In the heart of heterozygote mice, lipid drops have a higher content of unsaturated fatty acids (by ~10%), and also a significantly higher diversity of unsaturated fatty acid types (six types, which are not present in the myocardium of normal mice: C16:1, C18:3n6, C20:1, C20:3n6, C22:1n9 and C22:2).

The study of fatty acids from organs of mice treated with phosphatidylserine

Cardiovascular diseases are the main cause of mortality worldwide, and one of their most important clinical manifestations is the ischemic cardiac disease. Phosphatidylserine is a phospholipid which is normally found in the inner leaflet of the cell membrane. The translocation of phosphatidylserines to the outer leaflet of the membrane bilayer is a first sign of the cell entering apoptotic or necrotic processes. The externalization of phosphatidylserines is a phenomenon encountered in myocardial ischemia and reperfusion. The results presented in this section are based on an ongoing study conducted in collaboration with the same team coordinated by Dr. Elisa Liehn from the "Institute of Molecular Cardiovascular Research" in Aachen. The aim of the experiments is to monitor the effect of oral administration of phosphatidylserines on the ischemic myocardium of the mouse. Also, the study investigated the effect of the administration of phosphatidylserines on the cellular lipids from various organs, for the purpose of revealing any possible metabolic changes at this level, which might generate adverse effects. The mice treated with phosphatidylserines 5 μg/g for 4 days and the control mice (three control mice and three treated mice) were sacrificed, and their hearts and kidneys were collected. The lipids extracted from the organs were separated by HPLC.

The experiments conducted so far have not revealed any major differences in the types of lipids separated from the hearts of mice treated with phosphatidylserines compared to untreated mice, or the lipids separated from the kidneys of mice treated with phosphatidylserines, compared to the control mice.

The lipid fractions obtained by HPLC were investigated by gas cromatography, to identify the types of fatty acids from the lipids extracted from the myocardium and kidneys.

The chromatograms obtained to determine the types of fatty acids from the separated phospholipids have not revealed any significant differences between the fatty acids from the hearts and kidneys of the mice treated with phosphatidylserines, compared to the control mice.

The experiments have not revealed any metabolic changes at the level of the lipids extracted from the organs of mice treated with phosphatidylserines, due to the absence of any significant changes, both concerning the types of phospholipids, and the fatty acids from the lipids extracted from the hearts and kidneys of treated mice.

CONCLUSIONS:

1. Cellular activity is significantly influenced by membrane behaviour. Membrane function may be modulated by the characteristics of the lipid bilayer, also depending on the types of fatty acids from the composition of lipids. Disorders in the ratio between the types of fatty acids (saturated/unsaturated) have been described in numerous pathologies. This has led to the current interest in investigating the fatty acids composition of membrane lipids, as a possible therapeutic target. The studies presented in this paper refer to the analysis of fatty acids from the cellular lipids, allowing for the characterization of certain cellular types in terms of the fatty acids content and the saturated/unsaturated fatty acids ratio. To improve the knowledge in this area, we conducted experiments, both on various types of cells (normal and tumoral cells), and on mouse organs.

2. The present thesis developed an efficient and consistent method for the identification of fatty acids from the cell culture lipids, by gas cromatography; this method was chosen due to its rapidity, high resolution and sensitivity. The fatty acids identified, and the percentage of each fatty acid vary depending on the type of cells analyzed, the fatty acid content of cell lipids undergoing changes during the evolution of the cell culture.

3. The treatment of tumoral cells with DHA has determined changes at the level of the fatty acids from cellular lipids. In HeLa and MCF-7 cells, the treatment induces an increase in the rate of saturated fatty acids, while in DU145 cells, it induces a decrease in such rates. In all three cell types investigated, the rate of monounsaturated fatty acids decreases. The rate of polyunsaturated fatty acids increases in HeLa and

DU145 rates, following the treatment with DHA, while no changes are found in MCF-7 cells.

4. The treatment of MCF7 cells with DHA 100 μM has determined changes at a behavioural, morphological and biochemical level. The MCF-7 cell line used has a phenotype which does not synthesize caspase 3, hence the apoptosis may not be induced in this case. Therefore, the MCF-7 line was chosen to study the consequences of the treatment with DHA at the concentration of 100 μM.

5. Changes have been revealed at the level of the saturated/unsaturated fatty acids ratio, induced by the treatment of MCF7 cells with DHA. In MCF7 cells, DHA determines a decrease in the oleic acid content (monounsaturated fatty acid grown in cancerous cells), as well as an increase in palmitic acid, which is a saturated fatty acid.

6. The decrease in the level of oleic acid following the treatment of MCF7 cells with DHA is not due to any possible modifications of expression of SCD1.

7. The treatment of MCF-7 cells with DHA impacts the fatty acids content of cellular lipids on total lysate, and differentially, within the investigated cellular fractions. Stearic acid increases only within the plasmalemmal fraction. Palmitoleic acid is detected only within the plasmalemmal fraction, where it drops following the DHA treatment.

8. MCF-7 cells begin to accumulate lipid deposits soon after the beginning of the DHA treatment. The number and size of the lipid deposits in the cytoplasm increases significantly with the duration of the treatment administered to the cells.

9. DHA induced a significant decrease in the proliferative capacity of MCF7 cells.

10. Real time video-microscopy monitoring has revealed that the DHA treatment determines a more rapid adherence and spreading on collagen of the MCF7 cells. Lower effects were recorded in terms of cellular motility examined, both in terms of the migration speed, and of the directionality of migration. DHA treatment has proven that it may also influence the invasive capacity of MCF7 cells.

11. DHA treatment, respectively UVA exposure in a separate experimental model has reversible effects on the fatty acids content of cell lipids in dysplastic keratinocytes (DOK), and normal keratinocytes, respectively. (HaCaT). Concerted DHA+UVA treatment maintains the effects induced by DHA on the fatty acid composition of cell lipids.

12. The experiments aimed at investigating the concerted effects of DHA and UVA maintain the changes induced by DHA on the fatty acid composition of cell lipids.

13. The effects induced by UVA on cell proliferation and motility are reversed by DHA, in our experimental conditions, as shown by the concerted treatment and exposure.

14. The results obtained through our experimental model suggest a protection effect induced by DHA, against UVA irradiation on keratinocytes.

15. The receptor deficiency for SDF-1α induces an increased accumulation of unsaturated fatty acids in the lipid inclusions from cardiomyocytes, compared to the normal myocardium. Such increased accumulation of unsaturated fatty acids explains the more accentuated osmiophilia of lipid drops in heterozygote mice, compared to those from the normal myocardium.

16. Starting from the idea that the modulation of the fatty acid composition of cell lipids impacts cellular metabolism, the present doctoral thesis has shown that the supplementation of the cell growth environment with polyunsaturated fatty acids has determined changes at a behavioural, morphological and biochemical level. Also, DHA treatment has had a protective affect against UVA irradiation on keratinocytes. An interesting path to investigate further would be whether the morphological changes noticed are due to a real differentiation of the tumoral cells towards the normal cells, or the DHA treatment merely confers certain characteristics similar to the normal cells, without adjusting tumoral behaviour.

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