

**ROMANIAN ACADEMY**  
**Institute of Cellular Biology and Pathology**  
**„NICOLAE SIMIONESCU”**

**THESIS**  
**-Book summary-**

**FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF**  
**MEMBRANE MICRODOMAINS IN NORMAL STATE AND**  
**PATHOLOGY**  
**(diabetes, hypercholesterolemia, atherosclerosis)**

**Coordinator**  
**Ph. Antohe Felicia**

**PhD Student**  
**Elena Uyy**

**BUCHAREST**  
**2012**

## CONTENTS

### PART I - STATE OF KNOWLEDGE ON MEMBRANE MICRODOMAINS

<b>I.1. Plasma membrane and lipid microdomains</b>	
<b>I.2. The caveolae as highly specialized membrane microdomains.....</b>	<b>7</b>
I.2.1. Introduction .....	10
I.2.2. Caveolae and their involvement in cardiovascular physiology .....	16
<b>I.3. Caveolae in endothelial cell .....</b>	<b>17</b>
I.3.1. Introduction.....	17
I.3.2. Caveolae involvement in the regulation of vascular permeability.....	19
I.3.3. Role of caveolae / caveolins in plasma macromolecules transport and homeostasis.....	26
I.3.4. Caveolin-1 and angiogenesis .....	29
<b>I.4. Caveolae and endothelial cell dysfunction.....</b>	<b>30</b>
I.4.1. Introduction.....	30
I.4.2. Endothelial cell dysfunction in cardiovascular diseases and role of caveolae in:.....	31
I.4.2.1. diabetes.....	36
I.4.2.2. hypercholesterolemia / atherosclerosis .....	42
I.4.2.3. arterial hypertension.....	49
I.4.2.4. cancer.....	58
<b>I.5. Receptors and signaling pathways involved in the relationship between caveolae / caveolins and endothelial cell dysfunction .....</b>	<b>61</b>
I.5.1. Caveolae associated receptors .....	61
I.5.2. Signaling pathways .....	63
I.5.3. Role of caveolae in Ca <sup>2+</sup> mobilization mechanisms.....	65
I.5.4. Caveolae, folate receptor, receptor clustering and potocytosis .....	66
I.5.5. Therapeutic potential of folate conjugates .....	67
<b>I.6. Conclusions.....</b>	<b>69</b>

### PART II - ORIGINAL CONTRIBUTIONS

<b>II.1. Introduction and objectives .....</b>	<b>71</b>
<b>II.2. Experimental methodology .....</b>	<b>73</b>
II.2.1. Experimental design.....	73
II.2.2. Microscopy techniques .....	75
II.2.2.1. Electron microscopy and morphometric analysis .....	75
II.2.2.2. Optical microscopy .....	75
II.2.3. Preparation of cytoplasmic, nuclear and membrane protein extracts .....	76
II.2.4. Preparation of membrane domains enriched in caveolin-1 by sucrose gradient ultracentrifugation .....	77
II.2.5. Western Blotting .....	77

II.2.6. ELISA.....	78
II.2.7. ARN isolation and RT-qPCR.....	78
II.2.8. LC-MS/MS.....	79
II.2.9. Statistical analysis .....	79
<b>II.3. Experimental studies .....</b>	<b>80</b>
<b>II.3.1 Caveolin-1 is associated with structural changes of pulmonary endothelial cells in diabetes .....</b>	<b>80</b>
II.3.1.1. Introduction .....	80
II.3.1.2. Experimental design .....	81
II.3.1.3. Results and discussion .....	82
II.3.1.3.1. Characterization of experimental models .....	82
II.3.1.3.2. Structural changes of pulmonary endothelial cells in diabetes .....	82
II.3.1.3.3. Biochemical characterization of Caveolin-1 enriched membrane microdomains (ACE activity, cholesterol level, total protein).....	85
II.3.1.3.4. Caveolin-1 and -2 protein over-expression in diabetes .....	88
II.3.1.3.5. Caveolin-1 gene up-regulation in diabetes .....	91
II.3.1.4. Conclusions.....	93
<b>II.3.2. Protein expression of heat shock proteins (HSP) and other " lipid raft" proteins in experimental hyperlipidemia .....</b>	<b>95</b>
II.3.2.1. Introduction.....	95
II.3.2.2. Experimental design .....	97
II.3.2.3. Results and discussion .....	97
II.3.2.3.1. Hyperlipidemic diet induced atherosclerotic plaque progression .....	97
II.3.2.3.2. Structural changes of lung endothelial cells in hyperlipidemia...	101
II.3.2.3.3. Characterization of caveolin-1 enriched membrane microdomains .....	102
II.3.2.3.4. Characterization of cytosolic / nuclear / membrane protein fractions .....	106
II.3.2.3.5. Hyperlipidemia induces the up-regulation of caveolin-1 protein in "lipid rafts".....	108
II.3.2.3.6. Caveolin-1 gene expression in atherosclerosis .....	109
II.3.2.3.7. Atherosclerosis induces the down-regulation of PTRF protein expression in "lipid rafts" enriched in caveolin-1 .....	110
II.3.2.3.8. Atherosclerosis induces the protein up-regulation of RAGE co-fractionated with caveolin-1 and the activation of AKT signal pathway ...	111
II.3.2.3.9. Folate receptor protein up-regulation in lung endothelium in atherosclerosis .....	114
II.3.2.3.10. Up-regulation of the proteins involved in dynamin mediated endocytosis that co-fractionate with caveolin-1 (dynamin, filamin A, clathrin).....	115
II.3.2.3.11. Correlation of serum heat shock proteins released and their	

location in membrane caveolin-1 enriched fractions in hyperlipidemia.....	118
II.3.2.4. Conclusions.....	121
<b>II.3.3. Increased uptake of folate conjugates by activated macrophages in experimental hyperlipemia .....</b>	<b>125</b>
II.3.3.1. Introduction.....	125
II.3.3.2. Experimental design .....	127
II.3.3.3. Experimental procedures.....	127
II.3.3.3.1. The preparation of tissue cryosections with atherosclerotic lesions .....	128
II.3.3.3.2. The uptake of folate-Texas Red in cultured peritoneal macrophages .....	129
II.3.3.3. The uptake of folate- FITC/Texas Red by the U937 monocytes.....	129
II.3.3.4. Results and discussion .....	130
II.3.3.4.1. The specific uptake of folate-Texas Red in cultured peritoneal macrophages .....	130
II.3.3.4.2. The uptake of folic acid by atheroma plaque associated cells.....	133
II.3.3.4.3. The specific uptake of folic acid-FITC/Texas Red by the U937 cell line .....	135
II.3.3.5. Conclusion.....	137
<b>II.4. General conclusions.....</b>	<b>139</b>
<b>II.5. Bibliography.....</b>	<b>143</b>
<b>II.6. List of published and communicated scientific papers .....</b>	<b>161</b>

## **KEYWORDS**

**Endothelial cells,**

**"Lipid Raft"**

**Caveolin-1,**

**PTRF,**

**Folate receptor**

**Diabetes,**

**Hyperlipidemia,**

**Atherosclerosis,**

## **PART I**

### **STATE OF KNOWLEDGE ON MEMBRANE MICRODOMAINS**

The "fluid mosaic" model of cell membranes structure (as well as endomembranes) is based on a heterogeneous, asymmetric, two-dimensionally organized lipid fluid bilayer. The bilayer contains proteins which give it a mosaic feature (proteins are floating, or are immersed in a lipid sea), membrane associated cytoskeleton (a structure located on the internal face of the membrane) and the glycocalyx (a structure exposed on the outer surface of membranes, made of oligo-(poly)-saccharide chains of glycolipids, glycoproteins and proteoglycans).

In addition to the "fluid mosaic" model, in terms of structure and function there are defined notions of membrane domains and / or microdomains. Microdomains composition and existence (caveolae, "lipid rafts", clathrin coated structures) have a dynamic nature that responds to the ever changing need of the cells, imposed by internal biochemical activities, or by external stimuli. Membrane components such as lipids, proteins or carbohydrates present both structural and metabolic roles. This duality of roles is valid for both classes of biochemical membrane components and molecular entities (same molecule can perform both structural and metabolic functions) (Pike et al, 2009).

Significant progress has been achieved in molecular and cellular biology in 2009 when Pike and his collaborators have formulated several questions whose answer is sought even in the present. In short the questions address the following mechanisms: 1) How the membrane protein level is modifying in response to stimuli coming from the environment that affect the composition and behavior of the "lipid rafts"?, 2) What are the physiological functions of the "lipid rafts"?, 3) How the continuous flow of membrane lipids, coming from/to the plasma membrane to/from the internal compartments, is performed , 4) How does this flow affect the domain formation and how can diet or drug therapy modify the lipid composition by altering lipid domains function?

## PART II

### ORIGINAL CONTRIBUTIONS

Experiments performed *in vitro* on membrane systems such as the *black hole* model do not explain all the complex functions of intact cells. In this context, *in vivo* experiments were carried out for a better understanding of membrane microdomains pathophysiology. Our data showed that:

In insulin-dependent diabetes, endothelial cells express a high number of caveolae, exposing a greater luminal surface and a well developed inner biosynthetic membrane complex. These changes correlate with increased ACE activity, cholesterol level and caveolin-1 expression which indicates an EC response to the induced stress by elevated serum glucose and serum lipid levels (Uyy et al., 2010)

Association of hyperlipidemia diet with type I diabetes emphasizes stress effects in lung tissue.

Increased cholesterol in plasma membrane induced by type I diabetes and / or hyperlipidemia diet demonstrated an enrichment of the cell membrane with "lipid rafts" with a modified biochemical composition which significantly affects fluidity and permeability of the cell membrane (Uyy et al., 2010, Uyy et al., in press 2012).

In the pathogenesis of insulin-dependent diabetes mellitus, increased expression of caveolin-1, cholesterol concentration and increased ACE enzymatic activity may cause inhibition of eNOS located on the internal face of the membrane, followed by reduced production of NO, which ultimately lead to the emergence dysfunction of endothelial cells (Uyy et al., 2010)

The results obtained have a high potential to be used in practical applications through simultaneously targeting molecules as ACE and caveolin-1, on endothelial cells surface affected by hyperglycemia and hyperlipidemia, a valuable mechanism which could be a further therapeutic strategy in diabetes mellitus type I (Uyy et al., 2010).

Experimental models designed using a hyperlipidemic diet on Golden Syrian hamsters and APO E *knockout* mice have demonstrated the development of atherosclerotic lesions and installation of the pathology accompanied by significant changes in the endothelial cell ultrastructure and function (Haraba, Uyy et al., 2011, 2011, Uyy et al., in press 2012)

Hyperlipidemic diet induced changes in the expression of membrane proteins involved in transport vesicle fission, that co-fractionate with caveolin-1. These results support the

concept of membrane associated caveolae rapid disassembly as endothelial cell response to stress hyperlipidemia and also redistribution of caveolin-1 in non-caveolae "lipid rafts" at the plasmalemma level or intracellular membranes (PTRF and dynamin protein down-regulation and caveolin -1 and filamin A protein up-regulation) (Uyy et al., in press 2012).

The changes induced by hyperlipidemic diet in the expression of proteins that co-fractionated with caveolin-1 involved in transport vesicle fission (PTRF, dynamin, clathrin and filamin A) may be an indirect response of increased internalization of macromolecules by dynamin dependent clathrin mediated endocytosis at the expense of the caveolar one in pathological conditions induced by atherosclerosis in pulmonary microvasculature (Uyy et al., in press 2012).

The onset of atherosclerosis induced an increase of RAGE and folate receptors (FR) expression that co-fractionate with caveolin-1 in pulmonary endothelial membrane by activating AKT signaling pathway. These mechanisms maintain and amplify the inflammatory processes in the lung tissue (Haraba, Uyy et al., 2011, 2011).

Statin therapy significantly decreases the tendency of folate receptor and RAGE to accumulate in the membrane fractions isolated from endothelium lung and attenuates the AKT signaling pathway activation and inflammation associated with atherosclerosis.

Caveolae disassembly induced by hyperlipidemia and by redistribution of caveolin-1 in non-caveolae "lipid rafts" doesn't appear to influence the expression of the two proteins (RAGE and FR) in caveolin-1 positive membrane fractions enriched in "lipid rafts" but can be correlated with increased expression of caveolin-1 protein.

Atherosclerosis induces an over-expression of HSP 70 and a down-regulation of HSP 90 that co-fractionate with caveolin-1 in membrane fractions isolated from lung tissue (Uyy et al., in press 2012).

The statin therapy reversed significantly the hyperlipidemia induced changes by reducing the serum secretion of HSP 60 and 70 and by increasing the secretion and co-fractionation of HSP 90 with caveolin-1 (Uyy et al., in press 2012).

The hyperlipidemic condition induced a change in the protein expression of HSP 70 and HSP 90 that co-fractionate with caveolin-1 in membrane fractions which correlated with the protein expression of HSP 70 and HSP 90, secreted in serum isolated from the same experimental group, which leads us to hypothesize the involvement of caveolin-1-positive "lipid rafts" in their secretion (Uyy et al., in press 2012).



Because the experimental models used presented similarities with the human pathology, the data obtained may be relevant to the modulation of molecular processes in patients with type I diabetes and / or atherosclerosis.

The quantitative (spectrofluorimetric) and qualitative (fluorescence microscopy) analysis revealed a significant increase in the retention of folate - conjugates by peritoneal macrophages harvested from the hyperlipidemic hamsters as opposed to the animals which were maintained on either normal (high folate) or folic acid deficient diet (Antohe, Puchianu et al. 2005).

Histological analysis confirmed the prevalent uptake of folate conjugate by atherosclerotic plaques populated by macrophages, compared with the distal regions which presented a low fluorescence level (Antohe, Puchianu et al. 2005)

The experiments performed on U937 macrophages in culture using hyperlipidemic medium confirmed the results obtained *in vivo* (Antohe, Puchianu et al. 2005)

Increased folate uptake by U937 macrophages can be explained by the increased expression of the folate receptors (FR) on activated macrophages. This increase of FR expression can be exploited in folic acid compounds targeted therapies by using various drugs directed to activate macrophage rich atherosclerotic lesions (Antohe, Puchianu et al. 2005)

The concept of membrane microdomains used to explain the endothelial cell dysfunction in various diseases is a very modern and common subject. In this regard, their protein content characterization and the therapeutic opportunities offered by the lipid membrane microdomains could expand the arsenal of treatment options in cardiopulmonary diseases.

## **Bibliography**

- Elena Uyy, Felicia Antohe, Luminita Ivan, Raluca Haraba, Dorel Lucian Radu, Maya Simionescu, Upregulation of caveolina-1 expression is associated with structural modifications of endothelial cells în diabetic lung, *Microvascular Research*, 79, 154-159, 2010.
- Elena Uyy, Luminita Ivan, Raluca Boteanu, Viorel Suica and Felicia Antohe, Heat Shock Proteins and Membrane Caveolins Overexpression în Experimental Hyperlipidemia trimisa spre publicare la *Biology of the cell* (FI 4,898)
- Felicia Antohe, Luminita Radulescu, Elena Puchianu, Michael D. Kennedy, Philip Low, Maya Simionescu - Increased uptake of folate conjugates by activated macrophages în experimental hyperlipemia, *Cell Tissue Res.*, 320(2):277-285, 2005. (FI 2,991)
- Pike L. J., The challenge of lipid „raft”s, *Journal of Lipid Research*, 2009, 50:S323-328

Raluca Haraba, Elena Uyy, Viorel I. Suica, Luminita Ivan, Felicia Antohe - Fluvastatin reduces the high mobility group box 1 protein expression în hyperlipidemia, International Journal of Cardiology, Jul 1;150(1):105-7, 2011, (FI 7,078)

Raluca Haraba, Viorel I. Suica, Elena Uyy, Luminita Ivan, Felicia Antohe, Hyperlipidemia stimulates the extracellular release of nuclear high mobility group box 1 protein, Cell Tissue Res. Dec; 346 (3) : 361-8. 2011 (FI 3,114)