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THESIS

SUMMARY

LIPID RAFT PROTEOMICS AND SIGNALING MOLECULAR MECHANISMS IN HYPERLIPIDEMIC EXPERIMENTAL MODELS

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INTRODUCTION

Atherosclerosis is a disease mainly of the large and medium caliber arteries with severe clinical manifestations (atherosclerotic plaque) and often with tragic end (cardiac ischemia, lower limb gangrene, ischemic encephalopathy, myocardial infarction, cerebral infarction). Hyperlipidemia is a major risk factor in atherosclerosis, statin treatment being the most common measure to counteract the increased circulating lipid levels. The endothelial cells are the first to come into contact and are activated by pro-atherogenic factors, such as elevated levels of cholesterol and triglycerides in the circulating lipoproteins, which results in endothelial dysfunction. Pulmonary endothelium is directly involved in a number of vital functions of the body (solute exchange, regulation of vascular tone, vasculogenesis, angiogenesis) and although it does not normally develop atherosclerotic plaques, it can be activated by pro-atherogenicstress factors, (hyperlipidemicdiet, hypertension, deregulated production of nitric oxide, etc.) with implications in altering signaling pathways in the atherosclerotic plaques prone areas.

Studies have shown the importance of signaling mechanisms in the biological membranes, these structural and functional barriers actively involved in cell homeostasis. Membrane microdomains represent dynamic membrane nano-assemblies with a special protein and lipid content involved in transport, cholesterol homeostasis, etc. This particular structure determines their characteristic of being insoluble in non-ionic detergents and flotation in a density gradient following ultracentrifugation at 200000xg. Their protein profile is enriched in signaling proteins, which suggests their active involvement in not only physiological but also pathological molecular processes.

This paper aim is to investigate the alteration of the molecular mechanisms in pulmonary endothelium detergent resistant membrane microdomains following a hyperlipidemic stress on two laboratory experiment models: the ApoE deficient mouse and Golden Syrian Hamster fed with a hyperlipidemic diet.

FIRST PART – CURRENT STATE OF KNOWLADGE REGARDING LIPID RFATS AND SIGNALING MOLECULAR MECHANISMS

The "fluid mosaic" was hypothesized in 1972 (Singer and Nicolson, 1972) to explain the structural and functional organization of the plasma membrane and answered questions regarding its functions: transport processes, protection, cell-cell contact, signaling mechanisms.

Subsequent studies (Pike, 2003; Engelman, 2005) have shown that the plasma membrane is more mosaic than fluid and proposed a new model for membrane organization, in which it appears "patched" with portions segregated in different thickness and composition.

However, the concept of membrane microdomains appeared much earlier (Klausner et al., 1980; Simionescu et al., 1981, van Meer and Simons, 1983, etc.) and the “lipid raft” term was proposed in 1997 (Simons and Ikonen, 1997), describing floating islands on the membrane surface. Lipid rafts sites are enriched in cholesterol, glycosphingolipids, saturated phospholipids and specialized proteins, whose rigid packaging allow phase separation. Today, they are seen as dynamic nano-ordered protein assemblies, rich in sphingolipids and cholesterol, in which the metastable state can be activated under the influence of stimuli to attract and achieve specific lipid-lipid, protein-lipid and protein-protein interactions resulting in the formation of higher-order signaling platforms (Hancock, 2006).

Lipid raft functions in different cell types have been the study of many groups of researchers over the years. These include polarization, cell signaling, endocytosis, cholesterol homeostasis, cell-cell and cell-pathogen adhesion processes (Jacobson et al., 2007), but their most valued role is the ability to concentrate molecules involved in cell signaling. It was revealed that the coalescence of membrane microdomains results in a more efficient signaling process. Also a major feature of these microdomains, especially of caveolae (membrane subdomains) is the transport function through endocytosis and transcytosis of specific molecules in particular in endothelial cells (Palade et al., 1981, Vasile et al., 1983; Nistor and Simionescu, 1986). For example,

caveolaeare the main route of the albumin transport in endothelial cells, using a transcytosis process (Predescu et al. 1988 Antohe et al. 1991). Also, using caveolae as a transport vehicle, aminopeptidase P antibody passes the endothelium from the blood flow to the lung tissue (Oh et al. 2007).

Observations that many membrane proteins involved in cell signaling, adhesion and migration processes are located in detergent resistant membrane microdomains, together with data from studies on sterols, sphingolipids and proteins nano-assemblies in living cells, increased the interest in the biological functions of tlipid rafts and their role in inflammation and immune response. It seems that the rafts enriched in cholesterol and sphingolipidsare major regulators of atheroma pathophysiology by the activation of signaling pathways involved in the generation of atherosclerotic lesions. The type and plasma membrane lipid composition *per se* is a major determinant of protein localization in lipid rafts, and subtle changes in plasma membrane lipid composition and especially cholesterol content can result in the overall alteration of signaling pathway cascades.

Atherosclerosis is a chronic, multi-factorial disease and is one of the leading causes of death especially in developed countries (Naghavi et al., 2003). Although for a long time this pathology was seen as slow and irreversible, new knowledge in the field reveals that it is a multi-regional, dynamic and when early treated reversible process. Endothelial cells are specifically involved in the development and progression (or regression) of atherosclerotic lesions. Vascular endothelium is the first layer that interacts with different cellular stress pro-atherogenic or biochemical factors in the blood plasma, resulting in the endothelial and vascular dysfunction (Blankenberg et al., 2003; Simionescu and Antohe, 2006, Pavlides et al., 2014).

The pulmonary endothelium is directly involved in vital functions of the body, such as the exchange solution, fibrinolysis, coagulation, regulation of vasculogenesis and angiogenesis, interaction with platelets and leukocytes (Lucas, 2008). Although atherosclerotic plaques are not characteristic to this region, various studies have suggested that lung endothelium can be modulated for health improvement using angiotensin converting enzyme inhibitors and statins (Lucas, 2008 Osto et al., 2007). Published data have shown that atherosclerotic risk factors such as lipid rich diet (Uyy et al., 2013; Haraba et al., 2011), hypertension (Sellers et al., 2008), reactive oxygen

species (Lang et al. 2002), a disturbed production of nitric oxide (South et al., 2007), over-production of cytokines, chemokines (Hamacher et al., 2002, Bechara et al., 2007) and impaired coagulation and fibrinolysis processes (Lucas et al., 1997, Russell et al., 2003), can activate the pulmonary endothelium, affecting multiple signaling pathways, with significant impact on the stability of atherosclerotic plaques in the vascular areas prone to atheroma formation.

Statins are blood lipid lowering drugs, which were developed and clinically tested for regulation of cholesterol biosynthesis. Several studies measuring hemostatic parameters have also demonstrated the beneficial effects of statins on endothelial cells, including promoting the pro-fibrinolytic state (Seljeflot et al., 2002).

Given the many still unresolved issues in this field, we focused on the molecular signaling mechanisms localized at the plasma membrane level using hyperlipidemicexperimental animal models (Golden Syrian Hamsters and ApoE deficient mice) and biochemical, immunological, mass spectrometry analysis and bioinformatics tools. Using the existing methods for isolatingdetergent resistant membrane microdomains, weobservedthe DRM protein profile alteration induced by hyperlipidemic diet (associated or not with ApoE protein deficiency).

SECOND PART – ORIGINAL CONTRIUTIONS

To highlight the molecular mechanisms involved in membrane microdomains signaling affected by hyperlipidemia, we used two experimental hyperlipidemic animal models: the ApoE deficient mouse and the Syrian Golden hamster, subjected to hyperlipidemic diet. Controls were represented by a group of C57 Black mice and a group of hamsters with standard diet. Also in this study we focused on statin treatment effects following a high fat intake. Thus, after 6 weeks and 6 months of high lipid diet, the ApoE deficient mice and Syrian Golden hamsters exhibited significantly higher levels of cholesterol and triglycerides compared to control groups. Statin treatment resulted in significant decreases in serum levels of cholesterol and triglycerides. Microscopy experiments revealed lipid deposits in the heart valves successive cryosections in both hyperlipidemia groups of animals.

Membrane microdomains were isolated and purified on the basis of their insolubility by non-ionic detergents, and selective flotation in a density gradient (Sargiacomo et al. 1993). Of the 12 collected fractions following an ultracentrifugation process (200,000xg), the 4^{th} and 5^{th} fractions presented a higher concentration of protein, cholesterol and glycerolipids. These two fractions were combined and called non-ionic detergent resistant membrane microdomains (DRM), and subsequently used in qualitative and quantitative experiments. It was also demonstrated that the isolated DRMs were of endothelial origin, through detection of angiotensin converting enzyme peak activity in the two fractions. In addition, we observed an increased activity in the ApoE deficient mice group that received a hyperlipidemicdiet and the statin treatment one, thus suggesting endothelial activation due to hyperlipidemicstress. DRM isolation validation was performed using immunological experiments and mass spectrometry data.

DRMs isolated from the hamsters groups were solubilized and purified for protein extraction. The proteinswereprelabeled with fluorescent cyanine dyes and subjected to isoelectric focusing separation (according to the isoelectric point) and single dimension electrophoresis (for separation according to molecular weight) using the 2D-DIGE

approach. Using an imaging system based on laser scanning together with high-performance bio-informatics analysis, we detected ~ 1500 different protein spots in the range of 4-9pH. Also, the 2D-DIGE technique allowed us to obtain a good reproducibility between the analyzed samples, which resulted in a high degree of confidence in subsequent quantitative analysis which revealed 48 differently expressed protein spots between the control group and those receiving statin treatment, 85 spots with altered abundance determined by the hyperlipidemia diet and statin treatment. In total, over 200 protein spots were observed with significantly altered intensity. These spots were automatically excised from gels and processed for mass spectrometry analysis using a MALDI-TOF system and the Peptide Mass Fingerprinting technique. This led to the identification of structural proteins (actin variants, vimentin, tubulin, myosin), cytokines (interferon, interleukins), various signaling molecules (GTPases, receptors, HSPs, etc.) of trafficking and cellular transport proteins (annexin ATP synthase, membrane channels, etc.), and numerous enzymes (synthases, reductases, dehydrogenases, nucleotidase, caspases, etc.). Among these molecules, we observed that the hyperlipidemic diet resulted in the over-expression (beta actin, vimentin, interferon, interleukin-1 precursor, inositol phosphatase F, caspase-12, annexin A3 and fatty acid synthase), or under-expression (tubulin, Ras, Grp94) of some molecules. Statin therapy resulted in abundance decrease for some protein spots to a level comparable to the control (HDL binding protein, annexin A3, Grp94), and an inverse effect for others(MHC class II antigen, CRA kinase, protein trafficking of nitric oxide synthase).

High performance techniques based on mass spectrometry coupled with nano-liquid chromatography were applied for qualitative and relative quantitative analysis of mice isolated DRMs. Using the MudPIT approach we identified 1279 proteins in the control group, 1233 in the hyperlipidemic one and 1239 in the group of mice that received statin therapy. Gene Ontology based analysis revealed that, in terms of *Cellular Components*, most of the identified proteins were indeed of membrane origin. However, we also identified with high confidence proteins of nuclear, cytoskeletal, cytosolic, mitochondrial and extracellular origin. The distribution of proteins based on *Biological Process* demonstrated the proteins' primary role in metabolic processes, regulation of biological processes, response to stimuli, cell organization and biogenesis,

cell communication, development and cell differentiation. Classification based on *Molecular Functions* demonstrated a main representation of proteins in molecular interaction events, but also in catalytic activity, interaction with nucleotide, metal ions binding activity and molecular structure. The relative quantification analysis revealed 654 significantly differentially expressed proteins in the hyperlipidemicand statin treatment groups relative to the control. Their spatial distribution by Principal Component Analysis revealed a very good differentiation between the three DRM protein groups. 29 of these proteins were closely related to cytoskeleton-DRM interaction sites. Their analysis revealed that hyperlipidemia stress statin therapy affectedthree over-represented KEGG signaling pathways: *Regulationof Actin Cytoskeleton, Focal Adhesion and Adherence Junction*.

Moreover, immunological, biochemical and mass spectrometry studies were employed to highlight a number of DRM resident or associated protein molecules with significantly altered abundance caused by the hyperlipidemic stress and statin therapy. Thus, it was demonstrated that the cardiopulmonary dysfunction affected the expression of caveolin-1 (significantly over-expression), and PTRF (significantly under-expression). Also we demonstrated co-fractionation and altered expression of dynamin (under-expression caused by the hyperlipidemic condition),filamine (over-expressiondue to the hyperlipidemicstress), Hsp70 (significantly increased expression in the hyperlipidemic mice group) and Hsp90 (significantly decreased expression in the hyperlipidemic group) in the isolated DRMs. These results led to the correlation of secreted HSPs serumand tissue levels, emphasizing the possible role of DRM in their transport and regulation.

High-performance nano-chromatography mass spectrometry experiments were performed for the DRM protein profiles hyperlipidemic mice and hamsters comparison. The high percentage (~ 65%) of identified hamster proteins attributed to the MusMusculusorganism together with their similar characteristic chromatographic profiles, demonstrateda high degree of sequence homology between the two organisms. Also, the Gene Ontology data analysis performed with Protein Center, revealed similar protein profiles, the majority of them being of membrane, extracellular, cytoskeletal, nuclear and mitochondrial origin, with roles in metabolic processes, response to stimuli, cellular organization and biogenesis, involved inprotein and

nucleotide binding and catalytic activity. The relative quantitative analysis revealed 830 proteins whose abundanceswere altered by hyperlipidemia and statin therapy. The 422 different proteins uniquely assigned to the mice group were found in the following overrepresented KEGG signaling pathways: Antigen Processing and Presentation, TCA and Drug Metabolism. In the hamster groups alone, 288 proteins were found with significantly altered expressions that were involved in Adhesion Junctions and Oxidative Phosphorylation. Interestingly, hyperlipidemia acted on a common pool of 120 proteins in both animal models. These were involved in Leukocyte Transendothelial Migration, Tight Junctions,Oxidative Phosphorylation and Phagosome pathways.

References:

1. Antohe F, Heltianu C, Simionescu M. 1991. Albumin binding proteins of endothelial cells: immunocytochemical detection of the 18kDa peptide. Eur. J. Cell Biol., 56, 34-42.
2. Bechara C, Chai H, Lin PH, Yao Q, Chen C., 2007. Growth related oncogene-alpha (GRO-alpha): roles in atherosclerosis, angiogenesis and other inflammatory conditions. Med SciMonit 13:RA87-90.
3. Blankenberg S, Barbaux S, Tiret L., 2003. Adhesion molecules and atherosclerosis. Atherosclerosis 170:191-203.
4. Engelman DM. 2005. Membranes are more mosaic than fluid. Nature 438: 578–580.
5. Hamacher J, Lucas R, Lijnen HR, Buschke S, Dunant Y, Wendel A, et al., 2002. Tumor necrosis factor-alpha and angiostatin are mediators of endothelial cytotoxicity in bronchoalveolar lavages of patients with acute respiratory distress syndrome. Am J RespirCrit Care Med 166:651-6.
6. Hancock JF. 2006. Lipid rafts: Contentious only from simplistic standpoints. Nat Rev Mol Cell Biol 7: 456–462.
7. Haraba R, Suica VI, Uyy E, Ivan L, Antohe F., 2011. Hyperlipidemia stimulates the extracellular release of the nuclear high mobility group box 1 protein. Cell Tissue Res 346:361-8.
8. Jacobson K, Mouritsen OG, Anderson RGW. 2007. Lipid rafts: At a crossroad between cell biology and physics. Nat Cell Biol 9: 7–14.
9. Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. 1980. Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. J. Biol. Chem. 255, 1286–1295
10. Lang JD, McArdle PJ, O'Reilly PJ, Matalon S., 2002. Oxidant-antioxidant balance in acute lung injury. Chest 122:314S-20S.
11. Lucas R, Lou J, Morel DR, Ricou B, Suter PM, Grau GE., 1997. TNF receptors in the microvascular pathology of acute respiratory distress syndrome and cerebral malaria. J LeukocBiol 61:551-8.
12. Lucas R., 2008. Recent advances on the role of the endothelium in pulmonary function and disease. VasculPharmacol 49:111-2.
13. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J et al., 2003. From vulnerable plaque to vulnerable patient: a call for a new definitions and risk assessement strategies: part I. Circulation 108:1664–1672
14. Nistor A, Simionescu M., 1986. Uptake of low density lipoproteins by the hamster lung. Interactions with capillary endothelium. Am Rev Respir Dis.; 134(6):1266-72.
15. Oh, P., Borgstrom, P., Witkiewicz, H., Li, Y., Borgstrom, B.J., Chrastina, A., et al., 2007. Live dynamic imaging of caveolae pumping targeted antibody rapidly and specifically across endothelium in the lung. Nat. Biotechnol. 25, 327–337.
16. Osto E, Coppolino G, Volpe M, Cosentino F., 2007. Restoring the dysfunctional endothelium. Curr Pharm Des 13:1053-68.
17. Palade GE, Simionescu M, Simionescu N., 1981. Differentiated microdomains on the luminal surface of the capillary endothelium. Biorrheology, 17, 563-568, 1981.
18. Pavlides S, Gutierrez-Pajares JL, Iturrieta J, Lisanti MP, Frank PG., 2014. Endothelial caveolin-1 plays a major role in the development of atherosclerosis. Cell Tissue Res 356:147-57
19. Pike, L.J. 2003. Lipid rafts: bringing order to chaos. J. Lipid Res. 44, 655–667
20. Predescu D, Simionescu N, Simionescu M, Palade GE. 1988. Binding and transcytosis of glycoalbumin by the microvascular endothelium of the murine myocardium. Evidence that the glycoalbumin behaves as a bifunctional ligand. J. Cell Biol., 107, 1729-1738.
21. Russell JA., 2003. Genetics of coagulation factors in acute lung injury. Crit Care Med 31:S243-7.
22. Sargiacomo M, Sudol M, Tang ZL, LisantiMP., 1993. Signal transducing molecules and GPI-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol 122:789–807
23. Seljeflot I, Tonstad S, Hjermann I, Arnesen H., 2002. Improved fibrinolysis after 1-year treatment with HMG CoA reductase inhibitors in patients with coronary heart disease. Thromb Res 105:285-90.
24. Sellers MM, Stallone JN., 2008. Sympathy for the devil: the role of thromboxane in the regulation of vascular tone and blood pressure. Am J Physiol Heart CircPhysiol 294:H1978-86.

25. Simionescu M, Antohe F., 2006. Functional ultrastructure of the vascular endothelium—changes in various pathologies. In: Moncada S, Higgs A (eds) Handbook of physiology and bichemistry, vol 176/I. Springer, Berlin, pp 41–69
26. Simionescu N, Simionescu M, Palade GE., 1981. Differentiated microdomains on the luminal surface of the capillary endothelium. I. Preferential distribution of anionic sites. J. Cell Biol., 90, 605-613.
27. Simons K, Ikonen E. 1997. Functional rafts in cell membranes. Nature 387: 569–572.
28. Singer, S.J. and Nicolson, G.L. 1972. The fluid mosaic model of the structure of cell membranes. Science 175, 720–731
29. Sud N, Sharma S, Wiseman DA, Harmon C, Kumar S, Venema RC, et al., 2007. Nitric oxide and superoxide generation from endothelial NOS: modulation by HSP90. Am J Physiol Lung Cell MolPhysiol 293:L1444-53.
30. Uyy E, Ivan L, Boteanu RM, Suica VI, Antohe F., 2013. High-fat diet alters protein composition of detergent-resistant membrane microdomains. Cell Tissue Res 354:771-81.
31. van Meer, G. and Simons, K. 1983. An efficient method for introducing defined lipids into the plasma membrane of mammalian cells. J. Cell Biol. 97, 1365–1374
32. Vasile E, Simionescu M, Simionescu N., 1983. Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ. J Cell Biol. Jun;96(6):1677-89.