Universidade de Aveiro Departamento de Química
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Phosphatidylethanolamines Glycoxidation: Products and Significance

Glicoxidação de Fosfatidiletanolaminas: Produtos e Importância

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves Reis Marques Domingues e do Doutor Pedro Miguel Dimas Neves Domingues, Professores auxiliares do Departamento de Química da Universidade de Aveiro

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“Whatever you do may seem insignificant, but it is most important that you do it.” Mahatma Gandhi

Dedico este trabalho à Mi Su

o júri

presidente

Prof. Doutora Nilza Maria Vilhena Nunes da Costa
Professora Catedrática do Departamento de Educação da Universidade de Aveiro

vogais

Prof. Doutor Francisco Manuel Pereira Peixoto
Professor Auxiliar com Agregação do Departamento de Química da Universidade de Trás os Montes e Alto Douro (Arguente)

Prof. Doutor Artur Augusto Paiva
Equiparado a Professor Adjunto da Escola Superior de Tecnologia da Sáude de Coimbra, Assessor do Centro do Sangue e da Transplantação de Coimbra | Instituto Português do Sangue e da Transplantação (Arguente)

Prof. Doutora Maria Manuel Oliveira
Professora Auxiliar do Departamento de Química da Universidade de Trás os Montes e Alto Douro

Prof. Doutor Francisco Manuel Lemos Amado
Professor Associado do Departamento de Química da Universidade de Aveiro

Prof. Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues
Professora Auxiliar do Departamento de Química da Universidade de Aveiro (orientadora)

Prof. Doutor Pedro Miguel Dimas Neves Domingues
Professor Auxiliar do Departamento de Química da Universidade de Aveiro (coorientador)

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"De Coimbra fica o tempo que não passa/Neste passar do tempo que não volta" Manuel Alegre

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palavras-chave

Glicação, oxidação, stress oxidativo, diabetes mellitus, fosfolipidos, espetrometria de massa, citocinas e sistema imune.

resumo

As fosfatidiletanolaminas constituem a segunda classe de fosfolípidos mais abundantes nos organismos. Elas estão presentes nas membranas biológicas e nas lipoproteínas. As alterações estruturais dos fosfolípidos, ocorrem devido ao stress oxidativo e podem manisfestar-se em alterações das suas propriedades e funções. Já são conhecidas algumas condições fisiopatológicas, nas quais os fosfolípidos oxidados estão envolvidos, por exemplo sinalização celular, resposta imunitária, apoptose e doenças relacionadas com o envelhecimento. Por esse motivo, o interesse no estudo dos fosfolípidos oxidados e suas funções tem crescido nos últimos anos. Contudo, a maioria dos estudos realizados, focam a oxidação das fosfatidilcolinas, tendo sido dedicada pouca atenção a outras classes de fosfolipídos, como as fosfatidiletanolaminas. As fosfatidiletanolaminas, podem ainda sofrer outras modificações, devido ao grupo amina livre presente na cabeça polar, como por exemplo a glicação. As fosfatidiletanolaminas glicadas já foram detectadas em condições de hiperglicemia, em pacientes diabéticos, e tem correlação com a hemoglobina glicada. Sabe-se que a glicação de biomoléculas, pode aumentar as modificações oxidativas, que por sua vez, podem ser responsáveis pelo estado inflamatório, existente na diabetes mellitus. Tanto o stress oxidativo, como a inflamação estão relacionados com a diabetes e as suas complicações. A espectrometria de massa tem sido utilizada como uma importante tecnologia na detecção e caracterização de modificações oxidativas de fosfolípidos. Assim, neste trabalho pretendeu-se estudar as modificações oxidativas induzidas em fosfatidiletanolaminas glicadas, e os seus efeitos biológicos nos monócitos e células dendríticas do sangue periférico. Pretendeu-se ainda, estudar as alterações que ocorreram nas espécies de fosfatidiletanolaminas do fígado de ratos diabéticos. Os resultados obtidos permitiram identificar vários produtos de oxidação de fosfatidiletanolaminas glicadas, nomeadamente novos produtos formados pela oxidação da cabeça polar glicada. A oxidação na cabeça polar glicada foi, ainda, confirmada pela realização de experiências com *spin traps* combinadas com espetrometria de massa. Posteriormente, as fosfatidiletanolaminas oxidadas, glicadas e glicoxidadas demonstraram ter efeitos pró-inflamatórios, confirmados pelo aumento da estimulação monócitos e de células dendríticas, expresso no aumento do número de células produtoras de citocinas em comparação com o estado basal. As diferentes modificações de fosfatidiletanolaminas induziram estímulos distintos nos dois tipos de células. Sendo as fosfatidiletanolaminas glicadas e as glicoxidadas, os compostos que induziram um maior estímulo. Estes resultados sugeriram que as fosfatidiletanolaminas glicadas e as glicoxidadas podem estar associadas com o estado inflamatório que decorre da hiperglicemia crónica. Ainda, a avaliação do perfil lipídico de extratos de fígado de ratos diabéticos demonstrou que a hiperglicemia induz inúmeras alterações das espécies de fosfatidiletanolaminas e das espécies de outras classes de fosfolípidos, em

simultâneo com sinais de lesão hepática. Em conclusão, este trabalho demonstra a relação existente entre, a hiperglicémia, o stress oxidativo, a glicação e oxidação de fosfatidiletanolaminas e ainda a inflamação e compliações diabéticas. Portanto a contribuição da lipidómica é importante para compreender os efeitos prejudiciais da hiperglicemia não controlada, e por isso, merece ser mais explorado.

keywords

Glycation, oxidation, oxidative stress, diabetes mellitus, phospholipids, mass spectrometry, cytokines and immune system.

abstract

Phosphatidylethanolamines (PE) are the second most abundant phospholipids found in biological membranes and in lipoproteins. In cases of oxidative stress, phospholipids may undergo structural changes that lead to alterations of phospholipids properties and functions. Oxidized phospholipids are known to be involved in various physiopathological conditions, such as signaling of inflammation, immune response, apoptotic events and in age related diseases. Because of such implications, the interest in the study of this subject is increasing. However, most of the studies performed only focused the role phosphatidylcholines and little attention has been paid to other phospholipid families, like phosphatidylethanolamines. Also, phosphatidylethanolamines can undergo other modifications, due to the free amino group present at polar head, such as glycation. Glycation of phosphatidylethanolamines was already reported to occur under hyperglycemia, in diabetic patients and to have correlation with glycated hemoglobin. The glycation of biomolecules increase oxidative modifications, which can be responsible for the low inflammatory state, typical of diabetes mellitus. Both oxidative stress and inflammation are related to diabetes and can be associated with diabetic complications. Mass spectrometry has been used as an important tool in the detection and characterization of these phospholipids' oxidative modifications. So work aimed to study the oxidative modifications induced in glycated phosphatidylethanolamines, and their biological effects in blood monocytes and dendritic cells. It also aimed to evaluate the alterations of liver PE species under hyperglycemia in diabetic rats. The results allowed identifying various oxidation products of glycated phosphatidylethanolamines, including some new products with oxidation in polar head and in glucose. Oxidation in glycated polar head was confirmed by spin trapping experiments combined with mass spectrometry. Furthermore the oxidized, glycated and glycoxidized phosphatidylethanolamines showed pro-inflammatory effects, confirmed by the increase monocytes and dendritic cell stimulation with increased frequency of cytokine-producing cells, relative to the basal level. The different PE modifications induced different stimulus in the different cells, being the glycated and glycoxidized compounds the ones that induced higher stimulus. The results suggested that glycated and glycoxidized phosphatidylethanolamines could be associated with inflammatory status present in chronic hyperglycemia. In addition, the evaluation of lipid profile of liver extracts from diabetic rats showed that under hyperglycemia occurred changes in PE and other phospholipid species accompanied by signs of hepatic damage. In conclusion, this work re-enforces the network between hyperglycemia, oxidative stress, PE glycation and oxidation, inflammation and diabetic complications. Thus the contribution of lipidomic to understand deleterious effects of uncontrolled hyperglycemia is a promising field of research that deserves to be further explored.

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Comunicações Orais/ Oral Comunications

Identification of changes in phospholipid profile of liver from streptozotocin-induced diabetic rats.
1st Workshop Lipidomics, Aveiro, Maio 2012.

Phosphatidylethanolamines glycation, oxidation and glycoxidation: effects on monocyte and dendritic cell stimulation.
1º Workshop de Imunologia e Citometria de Fluxo, Aveiro, Dezembro 2012

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CONTENTS

LIST OF ABBREVIATIONS

AGEs – Advanced glycated end-products

AGE-LDL – Low density lipoprotein with advanced glycated end-products

AMD – Age-related macular degeneration

CDP-Etn – Cytidine diphosphoethanolamine

CID – Collision-induced dissociation

CL – Cardiolipin

CTP – Cytidine triphosphate

DAG – 1,2-diacylglycerol

DCs – Dendritic cells

DM – Diabetes mellitus

DMPO – 5,5-dimethyl-1-pyrrolidine-N-oxide

DPPE or dPPE – 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine

EDTA – Ethylenediaminetetraacetic acid

EET – Epoxyeicosatrienoic acid

EI – Electron impact

EK – Ethanolamine kinase

EPT – CDP-Etn: 1,2-diacylglycerol ethanolaminephosphotransferase

ER – Endoplasmic reticulum

ESI – Electrospray ionization

ET – CTP: phosphoethanolamine cytidylyltransferase

Etn – Ethanolamine

FAB – Fast atom bombardment

FSC – Forward light scatter

GC – Gas chromatography

G-CSF – Granulocyte colony-stimulating factor

GluPE – Glycated PE

GluOxPE – Glycoxidized PE

gPLPE – Glycated PLPE

HDDE – 4-hydroxy-(2*E*,6*Z*)- dodecadienal

HDL – High density lipoprotein

HEEA – *N*-hexanoyl-ethanolamine

HEPE – *N*-hexanoyl-PE

HETE – Hydroxyeicosatetraenoic acid

HHE – 4-hydroxy-2*E*-hexenal

HILIC – Hydrophilic interaction liquid chromatography

HNE – 4-hydroxy-2(E)-nonenal

HPLC – High performance liquid chromatography

HUVECs – Human umbilical vein endothelial cells

ICAM-1 – Intercellular adhesion molecule-1

IFN-γ – Interferon gamma

IL-8 – Interleukin-8

$L^{\cdot}$ – Lipid carbon centered radical

LC-MS – Liquid chromatography-mass spectrometry

LDL – Low density lipoprotein

$LO^{\cdot}$ – Lipid alkoxyl radicals

LOH – Lipid hydroxides

$LOO^{\cdot}$ – Lipid peroxyl radicals

LOOH – Lipid hydroperoxides

LOX – Lipoxygenase

LPEAT – Lyso-PE acyltransferase

LPS – Lipopolysaccharide

mAb – Monoclonal antibody

MALDI – Matrix assisted laser desorption ionization

MCP – Multichannel plate

MCP-1 – Monocyte chemotactic protein-1

M-CSF – Macrophage colony-stimulating factor

mDC – Myeloid dendritic cell

MFI – Mean fluorescent intensity

MIP-1β – Macrophage inflammatory protein-1beta

MMP-2 – Metalloproteinase-2

MRM – Multiple reaction monitoring

MS – Mass spectrometry

MS/MS – Tandem mass spectrometry

m/z – Mass-to-charge ratios

OxPAPC – Oxidized 1-palmitoyl-2arachidonoyl-*sn*-glycero-3-phosphatidylcholine

OxPC – Oxidized phosphatidylcholines

OxPE – Oxidized phosphatidylethanolamines

PAF – Platelet activating factor

PAPC – 1-palmitoyl-2arachidonoyl-*sn*-glycero-3-phosphatidylcholine

PAPE – 1-palmitoyl-2arachidonoyl-*sn*-glycero-3-phosphatidylethanolamine

PB – Peripheral blood

PBS – Phosphate buffer saline

PC – Phosphatidylcholines

PCI – Protein C inhibitor

PE – Phosphatidylethanolamine

PEMT – Phosphatidylethanolamine N-methyltransferase

P-Etn – Phosphoethanolamine

PI – Phosphatidylinositol

PL – Glycerophospholipid

PL A – Phospholipase A

PLPE – 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylethanolamine

PMCA – Plasma-membrane Ca^{2+}-ATPase

POPE – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine

PS – Phosphatidylserine

PSD – Phosphatidylserine decarboxylase

PSS2 – Phosphatidylserine synthase 2

Q – Quadrupole

QIT – Quadrupole ion trap

QqQ – Triple quadrupole

*R*f – Retention/retardation factor

rf – Radio-frequency

RNS – Reactive nitrogen species

RPE – Retinal pigment epithelium

ROS – Reactive oxygen species

Ser – Serine

SM – Sphingomyelin

SSC – Side light scatter

STZ – Streptozotocin

THP-1 – Human acute monocytic leukemia cell line

TLC – Thin layer chromatography

TNF-α – Tumor necrosis factor-alfa

TOF – Time-of-flight

TG – Triglycerides

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

VCAM-1 – Vascular cell adhesion molecule-1

VEGF – Vascular endothelial growth factor

CHAPTER I

GENERAL INTRODUCTION

Lipidomics is a field of research, comprised in the vast metabolomics field. Lipidomics is specifically dedicated to the study of lipids and has been defined as the complete profile of lipids extracted from a cell, tissue or biological fluid (1, 2). However it is more than that, it is the comprehensive understanding of the biological roles of all lipids and lipid metabolism and function (1, 2). Thus, lipidomics intersects proteomics and genomics, in the way that includes proteins and genes that regulate lipid processes. The major challenges that lipidomics faces are the diversity and complexity of compounds that lipids embrace. Lipids "comprise an extremely heterogeneous collection of molecules from a structural and functional standpoint" (2). As result of this structural diversity, the last classification system developed by LIPID MAPS Consortium divided lipids into eight categories, namely fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids (3). Each category is further divided into classes, subclasses and subgroups. Furthermore, such structural diversity reflects distinct functions on biological systems. Those biological functions range from roles in cell structure and organization, signaling events, energy storage, trafficking and sorting of macromolecules. Besides, in the last years, lipidomics field had a rapid development facilitated by advances in analytical techniques, in particular mass spectrometry (MS). MS became the core technology used in most lipidomic studies due to its high sensitivity and ability of providing structural information through tandem mass spectrometry experiments (MS/MS). The knowledge of how lipids contribute to cell and body function could be important to understand the lipid involvement in disease pathogenesis, which ultimately would lead to improvements in diagnostic and therapeutics (1, 2, 4).

1. GLYCEROPHOSPHOLIPIDS: STRUCTURE, PROPERTIES AND FUNCTION

Glycerophospholipids (PL) are present in all organisms, representing approximately 60 mol% of total lipids of a cell. As all lipid molecules, PL are soluble in organic solvents and contain long hydrocarbon chains (5, 6). In turn, glycerophospholipids are distinguished from the other lipids by the presence of a phosphate (or phosphonate) group esterified to the third hydroxyl group of a glycerol (2, 3).

In general, the glycerophospholipids are derivatives of *sn*-glycero-3-phosphoric acid that contains at least one fatty acid residue attached to glycerol moiety and a polar head group attached to phosphoric acid (1). The diversity of glycerophospholipids is enormous, because of all the possible fatty acid and polar head group combinations in the glycerol backbone structure. The nature of molecules linked to phosphate forming different polar head groups is the basis for classification of PL into different classes (1, 5, 7). The major classes found are phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidic acids, phosphatidylglycerols, cardiolipins and phosphatidylinositols. Each class have

different biological characteristics, which is important for membrane dynamics (8). Additionally, the nature of the covalent linkage (ester, vinyl ether and alkyl ether linkage), between the aliphatic chain and *sn-1* position of glycerol backbone, allow the subdivision into subclasses. Thus *O*-acyl, *O*-alkyl or *O*-alkyl-1'-enyl residues attached to glycerol characterize three PL subclasses. The most predominant subclass is the diacyl-, that corresponds to ester linkage at *sn-1* position of glycerol (*O*-acyl residue), however in some tissues plasmenyl- and plasmanyl- subclasses (vinyl ether and alkyl ether linkage, respectively) can be found in high abundance (1, 5). Furthermore, at *sn-2* position of glycerol backbone is always an ester residue and the species that lack of the aliphatic chain in this position are denominated as lyso-PL (1). Besides the phospholipid characteristics are mainly dependent on the nature of the polar head group, they also depend on the fatty acyl chains, which differ on chain length and number and location of unsaturations (7). Figure I: 1 shows a representation of the glycerophospholipids core structure and the different classes and subclasses.

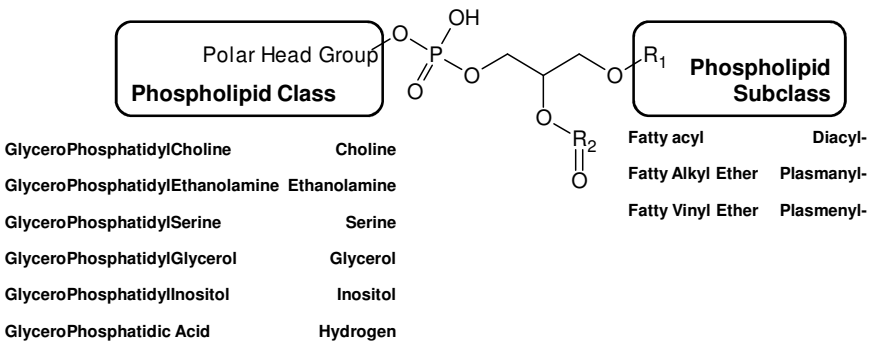

FIGURE I: 1 – Glycerophospholipids general structure. The different polar head groups esterified at the *sn*-3 position are indicated, as well as the correspondent phospholipids classes. R1 and R2 represent the esterified fatty acids at the *sn*-1 and *sn*-2 position, respectively. Subclasses of phospholipids, formed by different linkage at the *sn*-1, are also represented. Adapted from Pufler and Murphy (7).

The presence of phosphate group in glycerophospholipid structure provides important amphipathic properties. These amphipatic properties of phospholipids allow the spontaneous arrangement into micelles or bilayer vesicles in an aqueous environment. In the micelles or bilayers, the hydrocarbon tails form a hydrophobic core surrounded by polar heads that remain associated with water, creating an appropriate environment to membrane protein function and interactions (6). The PL characteristic bilayer arrangement, make them key components of biological membranes. The hydrogen bonds and electrostatic interactions between polar head groups and the hydrophobic interaction between the acyl chains are critical for proper membrane organization and function. Furthermore PL distribution among lipid bilayer is not homogeneous and several lipid microdomains can be formed, like highly ordered "lipid rafts", rich in phosphatidylinositols, which are very important for cell signaling. Also the PL distribution between

leaflets is determined by polar head group. While phosphatidylcholines (with a larger head group) fit better in the outer leaflet of membrane, phosphatidylethanolamines (with a smaller head group) fits better in the inner leaflet, and lyso-phospholipids tend to favor the outer leaflet. Besides being the building blocks for biological membranes, they have other important biological functions, which are compartmentalization of cytoplasm, intracellular and extracellular adhesion of proteins, membrane trafficking events, cytoskeletal support, function as second messengers and as precursors of lipid mediators (5-7).

1.1. PHOSPHATIDYLETHANOLAMINES

Phosphatidylethanolamines have an ethanolamine molecule in the head group and constitute the second major glycerophospholipid class, after phosphatidylcholines. However in most prokaryotic cells, PE is a major PL class, together with cardiolipins and phosphatidylglycerols. PE is frequently the main lipid component of microbial membranes, whereas PC is not usually present (5, 9). In mammalian cells, PE account for 20 to 50% of the total phospholipids, being the total amount dependent on the tissue and/or organelles in study (10). PE is found in particularly high amounts in retinas (11), in brain and mitochondria (10). PE is also found in LDL (low density lipoprotein) and in human plasma, where they represent 5 % - 6% of total phospholipids (12). Additionally PE class can be discriminated in three PE subclasses, namely diacyl-PE, plasmenyl-PE and plasmanyl-PE, which differ in the nature of the covalent linkage at the *sn*-1 position of glycerol, as represented in Figure I: 2.

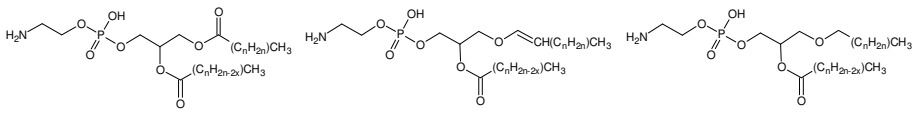

FIGURE I: 2 – Schematic representation of the different PE subclasses that can be found in animal tissues. The diacyl-PE subclass contains two *O*-acyl residues attached to glycerol. Plasmanyl and plasmenyl- contain one *O*-acyl residue at *sn*-2 and, respectively, one *O*-alkyl and *O*-alkyl-1'-enyl residue at *sn*-1 position of glycerol. Where n represents the number of carbons and x represents the number of double bonds.

1.1.1. PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS; HYDROLYSIS AND REMODELING

In cells, there are two major pathways for *de novo* biosynthesis of phosphatidylethanolamine, namely cytidine diphosphoethanolamine (CDP-Etn) pathway and phosphatidylserine decarboxylation pathway (described below). Besides these two pathways, PE

can also be synthesized by enzymatic addition of an acyl chain to lyso-PE, mediated by lyso-PE acyltransferase (LPEAT) and by enzymatic base exchange with phosphatidylserines, mediated by PS synthase-2 (PSS2) (9, 10). In Figure I: 3 the referred pathways for PE synthesis are represented.

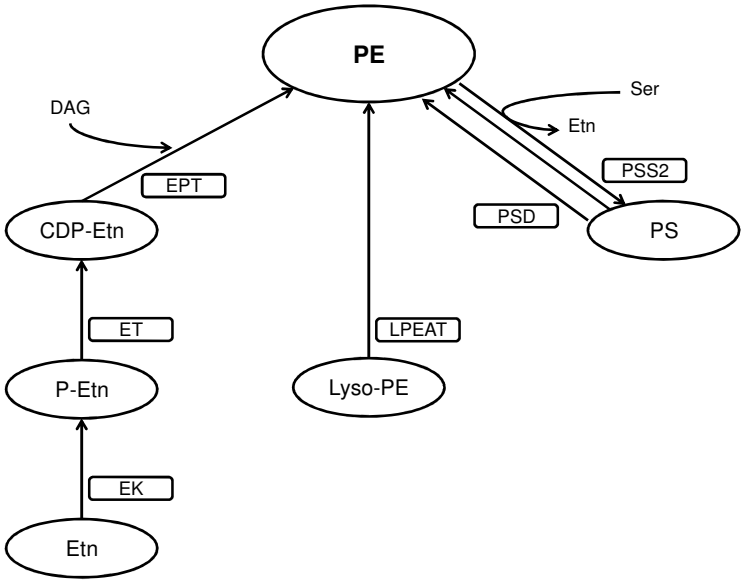

FIGURE I: 3 – The metabolism of phosphatidylethanolamine (PE) in mammalian cells. PE synthesis via CDP-ethanolamine pathway initiates with ethanolamine (Etn) phosphorylation by ethanolamine kinase (EK), producing phosphoethanolamine (P-Etn), which is subsequently converted to CDP-ethanolamine (CDP-Etn) by the action of CTP:phosphoethanolamine cytidylyltransferase (ET). CDP-Etn is then combined with 1,2-diacylglycerol (DAG) in a reaction catalyzed by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT), an enzyme of the endoplasmic reticulum and nuclear envelope. PE synthesis via phosphatidylserine decarboxylation pathway occurs only in mitochondria, by the action of phosphatidylserine decarboxylase (PSD), which decarboxylates PS to PE. PE can also be produced from lyso-PE, in a reaction most likely catalyzed by an acyl-CoA-dependent acyltransferase (LPEAT). In endoplasmic reticulum exchange reactions between ethanolamine (Etn) for serine (Ser) head groups catalyzed by base-exchange enzyme, PS synthase-2 (PSS2), converts PE into PS and vice-versa. Adapted from Vance (10).

For CDP-ethanolamine biosynthetic pathway, two precursors are needed, ethanolamine, that comes from dietary sources and diacylglycerol formed from phosphatidic acid through the action of phosphatidic acid phosphohydrolase. The first step in phosphatidylethanolamine biosynthesis is the phosphorylation of ethanolamine by the cytosolic enzyme ethanolamine kinase (EK). The phosphorylated ethanolamine further reacts with cytidine triphosphate (CTP) leading to formation of cytidine diphosphoethanolamine (CDP-Etn), in a reaction catalyzed by CTP: phosphoethanolamine cytidylyltransferase (ET). Finally, in the endoplasmic reticulum membranes, the CDP-ethanolamine: diacylglycerol ethanolaminephosphotransferase (EPT), catalyses the reaction of CDP-ethanolamine with diacylglycerol to form phosphatidylethanolamine (9, 10, 13).

The PS decarboxylation biosynthetic pathway is restricted to mitochondria, where the mitochondrial enzyme, PS decarboxylase is located, at external leaflet of inner membrane. Thus the inner membrane of mitochondria has higher PE content than the other organelles, being most of all PE made *in situ* through this pathway. A reaction catalyzed by PS decarboxylase (PSD) utilizes newly-synthesized PS to form PE. The PS is produced in endoplasmic reticulum (ER), so needs to be transferred from ER to the mitochondrial outer membrane, cross the outer membrane and then transferred to the outer leaflet of inner mitochondrial membrane, before the decarboxylation. This pathway reveals how PE and PS are inter-related and how their concentrations are tightly regulated (9, 10, 13).

These two major biosynthetic PE pathways are essential in living systems and the elimination of either pathway is embryonically lethal (10). Still, the relative contribution of each pathway seems to be dependent on the cell type. Furthermore, each pathway occurs in different organelles, allowing PE compartmentalization. The PE formed by either pathway are substantially different, while the ethanolamine pathway, preferentially synthesizes PE containing mono- and di-saturated acyl chains at *sn*-2 position, the PS decarboxylation pathway preferentially produces PE containing polyunsaturated acyl chains at *sn*-2 position (9, 10).

In addition to PE biosynthesis, the PE species, as well as the other phospholipids, are also subject to processes of enzymatic hydrolysis and remodeling, which control the final fatty acyl composition. Deacylation reactions are catalyzed by phospholipases A1 and A2, which hydrolyzes PL at *sn*-1 and *sn*-2 positions, respectively. After deacylation, reacylation reaction can occur mediated by various acyl-CoA:lysophospholipid acyltransferases. Phospholipases C or phosphodiesterases cleave the glycerophosphate bond. Finally, phospholipase D enzymes hydrolyze glycerophospholipids to generate phosphatidic acid and the respective head group (1, 9, 14).

1.1.2. Phosphatidylethanolamine Functions

Studies with *E. coli*, revealed the importance of phosphatidylethanolamine, whose presence is not required for life maintenance, but whose absence inhibit life processes. Studying *E. coli* membranes, in which PE is a major component, allowed the discovery of most of PE characteristics and functions (9, 13). PE alone tends to form inverted hexagonal phase by itself due to reduced head group size and hydratation, which confers to PE a cone shape. Polyunsaturated fatty acids, like arachidonic and docosahexaenoic acids, found in higher proportions in PE than the other phospholipids, favor this shape, increasing membrane fluidity. This is a critical factor that confers to PE ability to induce membrane fusion and cytokinesis (1, 10, 13). In the bilayers, PE is found preferentially at inner leaflet because of the negative curvature that PE promotes. The

membranes lacking PE show changes in their physical properties (1, 7, 8, 15). PE exerts a lateral pressure that stabilizes membrane proteins in their optimum conformations and, its ionizable amine group, can establish connections with proteins by hydrogen bonds (9). Due to the neutral or zwitterionic nature, in the pH range 2 to 7, it is believed that in bacterial membranes, phosphatidylethanolamine have a role in simply dilute the high negative charge density of the anionic phospholipids. This is important for lipid-protein charge interactions, either in transmembrane domains or cytoplasmic loops, determinants of membrane protein topology, which is the orientation of protein transmembrane domains with respect to the plane of the membrane bilayer. The cytoplasmic loops of integral proteins contain preferentially positive charged residues, and PE is believed to have a key function on that. PE inhibits the location of negative amino acids on the cytoplasmic side and it has an appropriate charge density to balance the charges of the membrane surface and the protein. However in some circumstances for support of protein function, PE allows the presence of negatively charged residues on the cytoplasmic side (9, 15).

It seems that PE is involved in supporting or even stimulating membrane transport systems. PE acts as a 'chaperone', assisting the transition from the cytoplasm to the membrane, guiding proteins to adopt the correct folding, which is important for protein proper function (9, 13). Still, after correct position in membrane, PE continues to be needed to orient enzymes correctly in the inner membrane in a dynamic process that is sensitive to changes in membrane properties (9, 15). Further cellular internalization of the serpin, protein C inhibitor (PCI) is essentially supported by plasma membrane PE, whose conical shape represents an important factor for the interaction with PCI. The protein translocation across the plasma membrane, mediated by cell surface-exposed PE, is rapidly directed to the nucleus, both *in vitro* and *in vivo* (16). In the eye, phosphatidylethanolamine reacts with free all-*trans*-retinal in the photoreceptor outer segment membrane to form retinylidene-phosphatidylethanolamine, a high affinity substrate of ABCA4 protein (a member of ABC transporters), that transports the retinoid compounds across the disk membranes (9, 17).

Additionally, phosphatidylethanolamine is important in the sarcolemmal membranes of the heart because the sarcolemmal disruption is a consequence of altered transbilayer distribution of PE. Also, the ATP deficiency, observed during ischemia, leads to PE externalization (10, 18). As well, the PE externalization is found in irradiated cells (19). The PE high content of the inner mitochondrial membrane has been proposed to be important in optimization of oxidative phosphorylation (1). Nascent intracellular very-low-density lipoproteins are enriched in PE when compared to secreted very-low-density lipoproteins, revealing the involvement of PE in hepatic secretion of the density lipoprotein (9, 10). In very low density lipoproteins, but not in high-density lipoproteins, PE are the main active compounds leading to stimulation of the contact system coagulation and dependent thrombin formation, and thus, blood coagulation (20).

The PE subclass, PlasmenylEthanolamine (with a vinyl ether substituent at *sn*-1) is found at high concentrations, up to 70%, in some tissues, like the human brain (10, 21). These phospholipid species have important functions protecting cells from the effects of oxidation, as facilitators of membrane fusion and as reservoirs for arachidonic acid release (5). Arachidonic acid is an important precursor of biologically active lipid mediators like prostaglandins and leukotrienes (1). Plasmenylethanolamines seems to have some role in the prevention of atherosclerosis since plasmenylethanolamines serum levels are directly correlated with the levels of high density lipoprotein (HDL), directly correlated with low density lipoprotein (LDL) particle size and inversely correlated with LDL (22, 23). Finally, the plasmenylethanolamines were reported to be decreased in Alzheimer's disease, changing membrane integrity, permeability, and loss of synapses and that decrease have correlation with the severity of dementia (24, 25).

"The understanding of the pathologic processes to which lipids contribute has solified the importance of lipids, lipid metabolism and lipid oxidation" (6). So far, the lipids are known to be involved in many metabolic diseases such as obesity, atherosclerosis, stroke, hypertension and diabetes (2). In a way that can be due to altered biosynthesis, metabolism or structure. Besides, PL can undergo considerable structure transformations, such as oxidation, modification with sugar residues and other functional groups that could be relevant to disease development and progression.

2. OXIDATIVE STRESS: OVERVIEW

Aerobic organisms cannot live without oxygen, however living with oxygen is dangerous, because oxygen could be as indispensable as toxic for aerobic organisms (26). In consequence of normal aerobic metabolism, some oxygen reactive intermediates are formed with potential to damage cellular constituents. Oxidative stress is defined as an imbalance between oxidant and antioxidants, in favour of the oxidants, that may lead to damage (27-29).

Major oxidants in living systems are free radicals and, because they contain unpaired electrons, are very reactive species (28). Hydroxyl radical ($HO^{\bullet}$) and superoxide anion ($O_2^{\bullet -}$) are some examples of these free radicals, also named reactive oxygen species (ROS). Reactive nitrogen species (RNS), such as nitric oxide ($NO^{\bullet}$) are other examples of free radicals. Peroxynitrite ($ONOO^-$) it is not a free radical, but it is also an important oxidant in biological systems. Oxidants can be formed by different mechanisms, such as cell metabolism (aerobic respiration, immune response, and others), different types of radiation (X-ray, ultraviolet light, ultrasound and microwave irradiation) and by Fenton and Fenton-like reactions (reactions

catalyzed by transition ion metals) (28-30). Furthermore, oxidants are likely to come from endogenous (for example: immune cell activation, inflammation and excessive exercise) or exogenous (for example: from air and water pollution, cigarette smoke, alcohol and food) sources (31). All biomolecules are potentially targets of oxidants, at the site of their generation or at distant places depending on the half-life of the oxidant species (29).

Because of the damage produced by oxidants, the organisms had to adapt themselves, creating numerous antioxidant defenses. Antioxidants are defined as any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays, prevent or removes an oxidative damage to a target molecule (28, 32). There are three lines of defense against oxidative damage, being the first the prevention, by protecting against the formation of oxidant molecules. The second line of defense is interception by non-enzymatic and/or enzymatic antioxidants. The non-enzymatic antioxidants comprise not only the vitamins, like vitamin C and E, but also small molecules like glutathione, uric acid, ceruloplasmin or ubiquinone. The enzymatic antioxidants included superoxide dismutase, catalase and glutathione peroxidase families, among others. Once oxidative stress occurs, there are also mechanisms of protection, which repair directly or indirectly (replacement) the damage, and are considered the third line of defense (26, 29).

Healthy organisms produce and need free radicals, but the production of oxidants is usually equilibrated by antioxidant defenses. In some cases, moderate levels oxidants could function as signal transduction and be benefic for the organism. But in other cases, this balance is disrupted, as result of increased oxidant production, or decreased antioxidant defenses, or even both. In those cases, cellular response can be varied, ranging from increased proliferation, prevention of cell division, senescence and cellular death (by apoptosis or necrosis) (32). Thus, oxidative stress has been associated with a wide variety of degenerative processes, diseases and syndromes. It is accepted that oxidative DNA damage is responsible for mutagenesis, cell transformation and cancer (31, 33). There are also evidences that oxidative stress have a role in cardiovascular diseases, including atherosclerosis, arteriosclerosis, heart attacks, strokes and ischemia/reperfusion injury (31). Central-nervous-system disorders were also investigated to be related to oxidative stress, such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's and Alzheimer's diseases (31, 34). In addition, chronic (rheumatoid arthritis, lupus erythematosus and psoriatic arthritis) and acute inflammatory diseases, ocular diseases (such as cataract) (35), diabetes (36), age-related disorders and the aging process itself are correlated to oxidative stress (31, 37).

The first studies of biomolecules oxidative damage were focused on lipid peroxidation, but over time, the subject of oxidative damage have been changed from lipids to proteins and DNA. However, lately there has been an increasing interest in the study of phospholipid oxidation, not only because their association in the membranes, but also because their involvement in other

biological processes. So far, oxidized phospholipids are known to be involved in age-related and chronic diseases, atherosclerosis, inflammation and immune responses (38).

2.1. Oxidized Phospholipids: Structure and Function

Phospholipids oxidation produces a wide variety of oxidation products which may have different biological consequences (11, 38). Phospholipid oxidation are known to occur in the unsaturated fatty acids through different mechanisms (explained in the next chapter), leading to formation of long chain oxidation products, short chain/truncated oxidation products and adducts formed by cross-linking reactions. Phosphatidylcholine is, by far, the most studied phospholipid class, with different oxidation products identified either *in vitro* or *in vivo*, using several methodological approaches, as reviewed elsewhere (38). The biological role of these oxidized species depends on the nature of the changes and on their location (11).

The main consequences of phospholipid oxidation are comprised to alterations of membranes properties. Membranes containing oxidized phospholipids become rigid, lose selective permeability and membrane potential, alter lipid-protein interactions and under extreme conditions, can lose their integrity (11). Oxidized phospholipids are a strong active component of oxidized LDL particles, which are involved in the promotion of atherosclerotic lesions by increasing the monocyte binding to the endothelium and foam cell formation (11, 39-48). Furthermore, oxidized phospholipids induce apoptosis and platelet aggregation due stimulation of platelet activating factor (PAF) receptor with PAF-like fragments formed by PC oxidation (11, 44-47, 49). Dual effects can be observed in blood coagulation, where oxidized phospholipids can either stimulate or inhibit protein C activity (46). The role of oxidized phospholipids in inflammation or immune system is wide, since oxidized phospholipids can generate either acute and chronic inflammatory responses, but can also have anti-inflammatory properties (11, 45-47).

Among the pro-inflammatory effects of oxidized phospholipids is the stimulation of endothelium to express adhesion molecules and chemokines, increasing the adhesion of monocytes. Besides the action on endothelial cells, oxidized phospholipids can induce pro- and anti-inflammatory effects on leukocytes depending on the type of inflammation and differentiation/activation state of leukocytes. The best known anti-inflammatory property of oxidized phospholipids is the inhibition of acute inflammatory responses produced by LPS (bacterial lipopolysaccharide), but they also inhibit oxidative burst produced by inflammatory cells and induce anti-oxidant and anti-inflammatory genes. Other actions of oxidized phospholipids include modulation of vascular smooth muscle cell phenotype, promotion or inhibition of angiogenesis, calcification of atherosclerotic lesions, and regulation of endothelium permeability and adaptive immune system (function of dendritic cells and T lymphocytes) (45, 46).

Most of these studies, regarding phospholipids oxidation, are focused in phosphatidylcholines (11, 44-46, 50, 51). Indeed, the biological activity of oxidized phospholipids was proposed to be dependent on the structural feature of the *sn*-2 fatty acyl chain and not on the phospholipid class (52, 53). However, the unique properties/functions of phosphatidylethanolamines referred before, suggest that biological consequences of phosphatidylethanolamines could be specific. In fact, there are few studies regarding specifically phosphatidylethanolamines oxidation and the deleterious consequences of oxidized PE.

2.1.1. Phosphatidylethanolamine Oxidation Products: Mass Spectrometry Identification and Characterization

PE could suffer modifications under oxidative conditions, PE (and other phospholipids) hydroperoxides and hydroxides were detected and quantified using fluorescence high performance liquid chromatography (HPLC), in liver extracts treated with a radical generator (54) and in small intestine after mice total body irradiation (55). However, little structural information, about oxidation products can be obtained in such studies. Mass spectrometry is thus a valuable tool that has been employed for identification and characterization of phosphatidylethanolamines oxidation products either *in vivo* or *in vitro* samples.

Oxidized phosphatidylethanolamines, containing hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs), were detected in human red blood cells (56) and in rat liver (57). Such oxidative derivatives of arachidonic acid were also found in other PL classes. In rat liver, the EET-containing phospholipids were detected by HPLC (57). In red blood cells, the oxidized phospholipids were analyzed by HPLC-mass spectrometry after PL hydrolysis. The HETE or EET carboxylate anions $[M-H]^-$ were detected at *m/z* 319. The fragmentation of $[M-H]^-$ ions allowed the distinction between HETE and EET, the identification of six positional isomers of HETE (hydroxyl group present at carbons 5, 8, 9, 11, 12 or 15) and the identification of four positional isomers of EET (epoxy group located at carbons 5-6, 8-9 11-12 or 14-15). The HETE and EET were found in PE, PC and PS, with the highest concentrations of HETEs being found in PE. Additionally the treatment of intact red blood cells with a lipid peroxidation initiator substantially increased the amount of phospholipids containing HETE and EET. Still, the levels of phospholipids containing EET have a greater increase than the ones of HETE, which led to the conclusion that epoxy formation resulted from a non-enzymatic mechanism (56).

Similar studies were preformed with monocytes, macrophages and platelets, but in these studies arachidonic acid containing phospholipids were oxidized by lipoxygenase (LOX) enzymatic mechanism (58-60). Such oxidation of arachidonic acid into HETE were absent in monocytes, macrophages from $LOX^{-/-}$ knockout mice, confirming the occurrence oxidation through the

enzymatic mechanism (58, 59). First, Maskrey *et al*. (58) studied the oxidation products of phosphatidylethanolamines which had arachidonic acid at the *sn*-2 position and some function in inflammation was found (see below). In activated human monocytes, a hydroxyl oxidation product (hydroxyeicosatetraenoic acid (HETE)) was formed by the action LOX. This specie was esterified in oxidized phospholipids, and was detected using precursor ion scan for ion at *m/z* 319 (carboxylic anion of HETE), in the negative mode. In this study, only the PE class was found to have species containing HETE acyl chain, a result that was confirmed by liquid chromatography (LC)-MS. Four PE species, with esterified HETE at *sn*-2 position, were identified using this approach. Further LC-MS/MS of oxidized PE fraction indicated that the four identified products had the same modification on arachidonic acid, namely 15-HETE product. The authors concluded that the difference between the oxidation products was in *sn*-1 acyl chain. The oxidation products were identified as 18:0/15-HETE-PE, 18:0plasmenyl/15-HETE-PE, 18:1plasmenyl/15-HETE-PE, and 16:0plasmenyl/15-HETE-PE. In human activated platelets (58, 60) and in murine peritoneal macrophages (59) these four PE species were also found to be formed but with a different HETE isomer, specifically 12-HETE, because of an existence of different LOX isoform. Although the products found in platelets and macrophages were the same, they were formed by different mechanisms. While oxidation products containing HETE were formed in platelets by esterification of freely oxidized HETE, in macrophages these products were formed by direct oxidation of phospholipids (59, 60). Activated platelets also generated non-esterified thromboxane B2 (58), and two PC containing HETE (16:0/12*S*-HETE-PC and 18:0/12*S*-HETE-PC) (60), in addition to the three plasmenylethanolamines and the diacyl PE containing HETE. Temporal studies of HETE formation in platelets revealed that after one hour, the enzymatic precursor of HETE, the hydroperoxy (12-HpETE), was not efficiently reduced to 12-HETE, thus the formation of decomposition products from 12-HpETE, including esterified carbonyls, isoprostanes, epoxides, and short chain aldehydes can be observed (60). Moreover, it was evaluated the externalization of oxidized PE species to the outer membrane leaflet upon stimulation of murine peritoneal macrophages (59) and platelets (60) derivatized with a cell-impermeable reagent. It was found that either macrophages and platelets, not only increased the amount of PE containing HETE, but also externalize these oxidized PE (59, 60).

Still on the oxidation achieved by the action of LOX, Morgan *et al*. (61) studied the phospholipids containing docosahexaenoic acid (DHA). The oxidation of docosahexaenoic acid (DHA) in phospholipids led to formation of hydroxyldocosahexaenoic acid (HDOHE), that under MS/MS originated the product ion at *m/z* 343.2 (negative ion mode). LC-MS/MS precursor ion scan of *m/z* 343.2, allowed the detection of four oxidized phospholipids containing HDOHE in activated platelets, all of them belonging to PE class. The four oxidized PE corresponded to two diacyl PE and two plasmenylethanolamines bearing either palmitic or stearic acid at *sn*-1 position and HDOHE at *sn*-2. Furthermore, isomers of HDOHE were also identified after saponification of phospholipids, where 14-HDOHE was the most abundant isomer, but 17- and 20-HDOHE isomers

were also found. The presence of 14-HDOHE isomer suggested that the oxidation was generated by platelet 12-LOX (61).

Two studies were published reporting the oxidation of PE under Fenton conditions. Khaselev and Murphy (62) first characterized the composition of commercial bovine brain glycerophosphatidylethanolamines and their subspecies. Further, they oxidized the phospholipid mixture with Cu (II) and H_2O_2. The oxidized phospholipids were analyzed by MS and MS/MS in the negative ion mode. They found oxidation of plasmenylethanolamines that resulted in loss of the *sn*-1 alkenyl chain with formation of the corresponding 1-lyso-2-acyl-phosphatidylethanolamine. In addition, they also detected diacyl phosphatidylethanolamines oxidation products formed by insertion of one and two oxygen molecules. They concluded that plasmenylethanolamines oxidation was somehow dependent of *sn*-2 acyl composition and that plasmenylethanolamines were more susceptible to oxidation than diacyl phospholipids (62). Domingues *et al*. (63) studied *in vitro* oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PE (PLPE), which is a phospholipid from phosphatidylethanolamine family. From the analysis of PLPE oxidation products obtained by hydroxyl radical generated under Fenton reaction, using liquid chromatography coupled mass spectrometry; they were able to indentify several oxidation products, including short chain products and long chain products. They identified long chain products with insertion of one to four oxygen molecules and long chain product with insertion of one to three oxygen molecules, but with a decrease of 2 Da units. These later products, with a decrease of 2 Da units, contain keto groups, while the first only contain peroxy and/or hydroxy groups. Several short chain products, of truncated *sn*-2 acyl chain, with different lengths (7-12 carbons), and aldehyde or carboxylic function were also identified. The PE oxidation products and their isomers were analyzed by LC-MS/MS. Structural characterization allowed to propose several structures for the oxidation products found. Like Gugiu *et al*. (64), it was reported that the major oxidation products, occur by oxidation in carbon 9 of *sn*-2 linoleoyl acyl chain. However they also found extensive oxidation in carbons 11, 12 and 13, especially when carbon 9 was already oxidized (63).

Gugiu *et al*. (64) quantified the phosphatidylethanolamines and oxidized phosphatidylethanolamines present in rat retina. They also studied the oxidation of phosphatidylethanolamines *in vitro* with different oxidation systems (Cu (II), UV radiation and myeloperoxidase). Using mass spectrometry, LC-MS/MS and MRM (multiple reaction monitoring) they identified short chain products with terminal aldehyde (after derivatization with methoxylamine hydrochloride) and short chain products with carboxylic acid function (after derivatization with pentafluorobenzyl bromide). They found, in rat retinas, predominantly six oxidation products resulting from oxidation of 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-PE, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PE and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PE. Each PE originated two short-chain oxidation products with the same carbon length but with different terminal functions. Additionally, different PE originated short-chain oxidation products with different carbon length of *sn*-2 acyl chain, as summarized in the Table I: 1. The same oxidation products were obtained by *in*

vitro oxidation of the same phosphatidylethanolamines with the three different systems. Although the extent of oxidation obtained with each system was different, therefore the UV radiation system induced more oxidation, followed Cu (II) and finally the myeloperoxidase system (64).

TABLE I: 1 – Short-chain oxidation product of PE found in rat retinas and *in vitro* oxidation (64). It is represented the PE oxidation products, the PE that originates them, and the modifications of *sn*-2 acyl chain. The *sn*-2 acyl chain is characterized by its carbon length and terminal function.

Non-modified PE	Oxidation products	Carbon length	Terminal function
1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-PE	1-palmitoyl-2-(4-oxobutyroyl)-*sn*-glycero-3-PE 1-palmitoyl-2-(succinoyl)-*sn*-glycero-3-PE	4 carbons	Aldehyde Carboxylic acid
1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PE	1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-PE 1-palmitoyl-2-(glutaryl)-*sn*-glycero-3-PE	5 carbons	Aldehyde Carboxylic acid
1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PE	1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-PE 1-palmitoyl-2-(azeleyl)-*sn*-glycero-3-PE	9 carbons	Aldehyde Carboxylic acid

More recently oxidation products of PE were detected in RAW 264.7 cell extracts, after incubation with a radical generator (65-67). With the amine group derivatized for increased sensitivity, the compounds were analyzed by MS after HPLC separation. The results revealed a decrease in the endogenous PE due to the formation of oxidation products. The oxidation products formed include lysoPE, plasmenyl- and phosphatidylethanolamines that contained a *sn*-2 chain shortened aldehyde and plasmenyl- and phosphatidylethanolamines that contained E2-isoprostanes, F2-isoprostanes, hydroxyoctadecadenoic acid or hydroxyeicosatetraenoic acid esterified at *sn*-2 position. Oxidation products containing *sn*-2 chain shortened aldehyde had different lengths depending on the fatty acid that was oxidized, thus carbon-5, carbon-7 and carbon-9 were resultant from oxidation of arachidonic acid, adrenic acid and linoleic acid, respectively (65).

Plasmenylethanolamines oxidation with UV irradiation and Fe^{2+}/ascorbate led to the degradation of such compounds with release of various classes of aldehydes (68). The release of aldehydes from plasmenylethanolamines, as result of ozone oxidation, with a concomitant

formation of lysophosphatidylethanolamines was also observed (69). Those free aldehydes immediately disappeared from solution due to possible reaction with other molecules (68).

2.1.2. Secondary Oxidation Products: Aldehyde Modification of PE Polar Head

During lipid peroxidation (and non-lipid oxidation) aldehydic short-chain products are generated. Those small molecules are very reactive and can easily react with other molecules, forming adducts as secondary oxidation products. Most studies demonstrated the formation of adducts between lipid peroxidation products and proteins and DNA (26, 38). However, it was also demonstrated that PEs are the primary component becoming modified by these aldehydes (70-72).

2.1.2.1. 4-hydroxy-alkenals

Several lipid oxidation products studies are known to react with PE amino group forming either Schiff bases or Michael adducts (70). The 4-hydroxy-2(E)-nonenal (HNE) is the lipid oxidation product most recognized to generate adducts with PE. Bacot *et al*. (73) related the formation of 4-hydroxy-2(E)-nonenal (4-HNE) from 15-HETE, of 4-hydroxydodeca-(2*E*,6*Z*)-dienal (4-HDDE) from 12-HETE and 4-hydroxy-2*E*-hexenal (4-HHE) from docosahexaenoic acid. All three short aldehydes were found *in vivo*, in human plasma, and the degradation of 12-HETE into 4-HDDE was observed in human platelets. In human plasma, the 4-HNE was the most abundant product followed by the other two, which have proximal abundances (73).

The reaction between HNE and PE was evaluated and analyzed by LC-MS in negative ion mode. Three adducts, pyrrole, Schiff and Michael adducts, were found to be produced in this reaction, being the Michael adduct the main derivative (74). The different reactivity of the three aldehydes, 4-HNE, 4-HDDE and 4-HHE, with the amino group of PE were evaluated with brain extracts (73). It was found the formation of aldehyde-PE adducts and that 4-HDDE was more reactive than 4-HNE, which, in turn, was more reactive than 4-HHE. Furthermore the reactivity of these compounds was more intense in the plasmenylethanolamines than in the diacyl-PE (73). More recently, the same group (75) investigated the presence of the same Michael adducts in oxidative stress conditions. Human blood platelets, after *in vitro* induced oxidation, and retinas of streptozotocin-induced diabetic rats were tested for the presence of 4-HNE, 4-HHE and 4-HDDE free aldehydes and aldehydes-PLPE adducts applying GC-MS. In platelets, under moderate oxidative stress induced by diamide, there was a significant increase of 4-HNE and 4-HHE free aldehydes but only PE-4-HNE adduct was detected. However, under more aggressive oxidative

stress, induced by aspirin combined with mercaptosuccinic acid, the 12-LOX pathway was specifically targeted because there was a significant increase of 4-HDDE and only slight increase of 4-HNE and 4-HHE free aldehydes. Besides the higher increase of 4-HDDE, the PE-4-HNE adduct was formed in higher abundance than PE-4-HDDE adduct and only trace amounts of PE-4-HHE adducts were detected. In the rat retinas, all adducts, PE-4-HNE, PE-4-HDDE and PE-4-HHE, were increased, and the higher increase was obtained for PE-4-HHE (75).

2.1.2.2. ISOLEVUGLANDINS

The isolevuglandins are dialdehydes formed from arachidonic acid oxidation that are extraordinarily reactive toward primary amino groups of biomolecules, including PE (70). Detailed characterization of two isolevuglandins adducts with PE, by mass spectrometry (LC-MS and LC-MS/MS, in negative ion mode) were performed by Bernoud-Hubac *et al*. (76). The two adducts found had different *m/z* values and the product ions were identified as isolevuglandin-PE pyrrole and dehydrated reduced isolevuglandin-PE Schiff base adducts (76). Isolevuglandin-PE pyrrole adducts were found to be further oxidized by air generating hydroxylactam and lactam end products (77). MS characterization of isolevuglandin-PE hydroxylactam-PE and lactam-PE adducts were performed by direct infusion MS in the negative ion mode. LC-MS/MS allowed the detection of lysoPE-lactams and lysoPE-hydroxylactams formed *in vitro* through myeloperoxidase-catalyzed oxidation of PAPC in the presence of lysoPE. However, these adducts were not detected in human plasma during MRM experiments. The detection and quantification of isolevuglandin-PE and hydroxylactam-PE *in vivo* were only achieved using MS after phospholipase A2-catalyzed hydrolysis. Elevated levels of isolevuglandin-PE and hydroxylactam-PE, but not lactam-PE adducts were found in plasma from patients with age-related macular degeneration and in mouse liver with ethanol-induced injury (77).

2.1.2.3. OTHER LIPID AND NON-LIPID OXIDATION PRODUCTS

There are also other lipid oxidized molecules that were documented to form adducts with PE. Zamora and Hidalgo (78) studied, using ESI-MS, the ability of the 4,5-epoxy-2-alkenals, secondary lipid peroxidation products of docosahexaenoic acid, to form adducts with PE. Stadelmann-Ingrand *et al*. (79) studied the Schiff base adducts formation between PE and the fatty aldehydes produced by plasmenyl-phospholipids degradation under oxidative conditions (UV radiation and Fe(II)/ascorbate), in brain homogenates. The adducts were further characterized by ESI-MS and GC-MS (79). Zemski Berry and Murphy (80), determined the chromatographic and mass

spectrometric properties of acrolein-PE adducts. The acrolein reacted with PE to form acrolein-PE Michael adduct, which could further react with a second acrolein to form a piperidine adduct by aldol condensation. The formation of aldol condensation product, over the Michael adduct, occurred even when the ratio of acrolein to PE was less than one. It was established a sensitive assay, LC-MS after sample reduction, for the screening of such adducts piperidine that allowed the detection acrolein-PE adducts in HL-60 cells exposed to acrolein (80). Tsuji *et al*. (81) reported the modification of PE with an hydroperoxy derivative of linoleic acid, which was generated by LOX enzymatic reaction. Interestingly, this is an oxidation product that is not shortened, which reacts with PE and leads to the formation of *N*-hexanoyl-PE (HEPE). The HEPE was hydrolyzed with phospholipase D, originating *N*-hexanoyl-ethanolamine (HEEA) that was analyzed by LC-MS/MS in positive ion mode. This approach was successfully used to detect HEPE products in oxidized erythrocytes ghosts and in oxidized LDL (81).

Previous studies reported herein, utilized a directed approach to identify or detect specific aldehyde-PE adducts. However Guo *et al*. (71) recently established an untargeted approach to discover new aldehyde-PE species. Aldehydes formed from arachidonic acid and PC-containing arachidonic acid oxidation in the presence PE standard were identified and characterized by MS. High resolution mass spectrometry and LC-MS/MS allowed the detection of more than 10 species, formed with different relative abundances. These compounds were further detected and measured (by MRM, see below) in oxidized HDL after deacylation of the acyl chains (71), a similar approach to that utilized before in HUVECs aldehyde-PE measurements (82).

The modification PE amino group is not exclusive of aldehydes formed for lipid oxidation products, but can also occur from protein or glucose oxidation products. For instance, PE-glucose and PE-acetone adducts had also been detected in human tissues, especially those of diabetic patients (9, 83).

Concerning protein oxidation products, Heller *et al*. (84) demonstrated the Schiff base formation between amino group of phosphatidylethanolamine and *p*-hydroxyphenylacetaldehyde. The *p*-hydroxyphenylacetaldehyde is a tyrosine oxidation product generated by the myeloperoxidase system of phagocytes, in LDL. In an approach that involved phospholipid hydrolysis and polar head analyses by GC-MS, this adduct were measured in LDL isolated from human atherosclerotic tissue (84).

Since PE oxidation was detected *in vivo,* it is important to understand what are the consequences of the presence of those oxidized PE under biological conditions.

2.1.3. Biological Consequences of Oxidized Phosphatidylethanolamines

So far PE was demonstrated to play important biological functions; thereby the alterations in PE structure, due to oxidation and/or glycation are likely to be related to cellular dysfunction and disease development.

HETE containing phospholipids (especially PE), are known to be formed from oxidation of arachidonic acid, in activation of monocytes, macrophages and platelets, and to have immune functions (58-60). Some of these studies also described that the formation of HETE-PE and externalization in monocytes and macrophages were dependent on the calcium ionophore stimulation (58, 59). In $LOX^{-/-}$ macrophages, ionophore also caused a large increase in native PE externalization, thus externalization of PE in wild-type macrophages, was targeted toward oxidized species (59). HETE-PE, in culture of human monocytes, inhibited LPS stimulation of tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF) generation (59). Thus esterified HETE products could play a role in signaling immune regulation and inflammation (58). Also LOX-dependent phospholipid peroxidation was associated with the Th2 and not Th1 lymphocytes inflammatory profile *in vivo* (59). Externalization of PE and HETE-PE could stimulate coagulation by enhancing activity of prothrombinase complex, in a process controlled by LOX (60). In fact, it was demonstrated that platelets from a patient with the bleeding disorder Scott syndrome, externalized less PE and HETE-PE was not externalized, even though these lipids were generated in platelets. This suggested that the lack of HETE-PE may contribute to the bleeding fenotype, leading to the assumption that HETE-PE could also be responsible for thrombotic disorders. HETE-PE were therefore considered to be physiological products, formed by acute receptor/agonist-dependent activation of human platelets. So HETE-PE was found to be important in thrombin generation/homeostasis and platelet function during health and disease (60).

Still on the procoagulant effect of oxidized PE, Zieseniss *et al*. (85) analyzed the influence of the atherogenic oxidized low density lipoproteins (LDL) on the activity of the platelet prothrombinase complex, a major contributor to overall thrombin formation *in vivo.* First they reported that oxidized LDL when incubated with platelets, enhanced the synthesis of the protease, and the native LDL did not. They also demonstrate that oxidized LDL stimulated the prothrombinase activity by promoting the formation of a procoagulant surface. In order to understand what were the oxidized LDL compounds involved in thrombin generation, they analyzed every compound activity after several separation steps. This allowed the identification of oxidized PE as the lipoprotein component which mediated the strongest stimulation of the platelet thrombin generation. Among oxidized diacyl-PE and plasmenylethanolamine, the stimulation of thrombin generation was more accelerated with the first one, probably due to, the already reported, plasmenylethanolamine resistance to oxidation (85).

More recently, oxidized PAPE (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-PE) was shown to bind, with high affinity, protein C inhibitor (PCI), in a process that competes with heparin for the same PCI binding site (86). The binding of oxidized PAPE to PCI, stimulate PCI to inhibit the activity of activated Protein C and thrombin. The results suggested that oxidized PAPE may act as inducible "heparin analogs". Thus oxidized PAPE (through PCI) could have both a role in arterial thrombosis, promoting blood coagulation and thrombus formation at sites of tissue injury, and have additional role in regulation of proteolysis at sites of injury and inflammation (86).

After identification of PE short-chain oxidation products in the retina, Gugiu *et al*. (64) proposed some potential biological role for these products in the retina. Since phosphatidylcholines oxidation is thought to trigger endocytosis by CD36 interaction, they hypothesized that phosphatidylethanolamines oxidation could also do the same. CD36 is involved in the regeneration of retinal pigment epithelium (RPE) which was damaged by photogenerated radicals. They proposed that PE oxidation products, could act as signaling molecules for the cell regeneration, contributing to retinal homeostasis. In age-related macular degeneration (AMD) exists an excessive production of PE oxidation products, so authors suggested that the excess of PE oxidation products could be responsible for the pathological degradation of photoreceptor cells that is observed in AMD (64).

2.1.3.1. PLASMENYLETHANOLAMINES INFLUENCE THE OXIDATION RATES

Plasmenylethanolamines (non-modified) was found to have biological roles, influencing the oxidation of other molecules. Maeba and Ueta developed a high sensitivity method to detect plasmenylethanolamines in human plasma (87). The effects of plasmenylethanolamines in oxidation was studied and it was found that the cholesterol oxidation is prevented in the presence of plasmenylethanolamine and plasmenylcholine (88). However the plasmenylethanolamine had greater ability to reduce cholesterol oxidation in bilayers. So they suggested that plasmenylethanolamine could play a role in preventing cholesterol oxidation in biomembranes by reducing the oxidizability of cholesterol in bilayers, acting as a physiological antioxidant for cholesterol (88). It was also reported the role of plasmenylethanolamines in lowering the oxidizability of membranes, because of the high oxidizability of plasmenylethanolamines, either in the presence or absence of cholesterol (89, 90). Other studies also reported the anti-oxidant properties of plasmenylethanolamines, that acted as scavengers of reactive oxygen species and chelators of transition metal ions, thus inhibiting lipid peroxidation (68, 91-95).

2.1.3.2. ALDEHYDE-PE ADDUCTS: BIOLOGICAL ROLE

The aldehyde-PE adducts share most of the effects produced by oxidized PE. The pro-atherogenic role of Schiff adduct, PE-HNE, was also evaluated by Zieseniss *et al*. (85). It was suggested that the imino group between the ethanolamine head group and the aldehyde was the major determinant for the stimulation of the prothrombinase activity. The Schiff base PE-aldehyde adducts, were the most active components in modified lipoproteins, and were found to increase thrombin formation strongly, suggesting a relevant role for oxidized LDL in the development of thrombosis *in vivo*. Also 4-HNE, PE-4-HDDE and PE-4-HNE adducts could alter the platelet aggregation and functioning (75). That involvement in platelet function may be important to understand the mechanisms leading to the formation of thrombi at the site of the unstable atherosclerotic plaques (85).

PE-adducts with unfragmented oxidized linoleic and arachidonic acids were capable of increasing mouse peritoneal macrophage viability and could have potential beneficial or adverse effects on plaque size and stability in atherosclerosis (96). Moreover, incubation of human umbilical vein endothelial cells (HUVECs) with PE-isolevuglandin adducts led to a dose-dependent increase in the expression levels of ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin and in the mRNA levels of both MCP-1 (monocyte chemotactic protein-1) and IL-8 (interleukin-8) (72). This led to an increase binding of THP-1 (human acute monocytic leukemia cell line) monocytes through pro-inflammatory mechanisms. Also, all the aldehyde-PE species identified by Guo *et al*. (71) were examined for the ability to stimulate THP-1 monocyte adhesion to endothelial cells. Several species were able to increase adhesion to endothelial cells, suggesting the PE potential to mediate inflammation associated with oxidative injury. Although other biological contributions cannot be excluded. Thus, establishing a pathway linking oxidative stress to inflammation may be useful for reducing inflammation (71). In conclusion several PE-aldehyde adducts were potent activators of endothelial cells, and therefore have the potential to mediate inflammation associated with oxidative injury, contributing to atherogenesis (71, 72)

Bacot *et al*. (73, 75) detected PE-4-HNE, PE-4HDDE and PE-4-HHE adducts in plasma, blood platelets, brain and retina. They supposed that the adduct-containing membranes have altered membrane fluidity and activity of anchored proteins, as well as the accessibility of ligands and other functional components that might affect cell function (75). Similarly, Bernoud-Hubac *et al*. (76) proposed that PE alterations by isolevuglandins binding could induce different phospholipid membrane distribution, affect membrane fluidity, and result in changes in membrane structure and function. Alterations in membrane function, like ability to stabilize membrane proteins due to PE-isolevuglandin adducts was also proposed by Li *et al*. (77). Membrane structure and the interactions of membrane PEs with membrane-bound or cytosolic proteins could be profoundly

altered by the conversion of the positively charged amino group of the PEs into a negatively charged group upon aldehyde adduction (77). The presence of PE-isolevuglandin adducts in membranes changed their properties towards increasing negative (72) or positive (71) membrane curvature. Besides PE-isolevuglandin adducts were quickly trafficked to endoplasmic reticulum, where potentially alter the membrane curvature. Thus ER stress response signals might be triggered by those adducts invoking IL-8 and MCP-1 expression, HUVEC activation and THP-1 binding. This is a process that was blocked by inhibitors of the ER stress response and revealed important links between oxidized lipids, ER stress, inflammation, and atherosclerosis (71, 72).

PE-isolevuglandin adducts, their oxidized derivatives and acrolein-PE adducts were proposed as biomarkers to access the risk of development of membrane disorders or other diseases stimulated by oxidative stress such as AMD (75, 77, 80). PE-isolevuglandins, *p*-hydroxyphenylacetaldehyde-PE and HEPE-PE formation could be an important pathway for converting LDL to a form of oxidized LDL, that can modulate signaling events, foam cell formation *in vivo*, atherosclerosis and other oxidative stress-related disease (76, 81, 84). Furthermore glucose-PE adduct formation in atheroma plaques and glycated PE involvement in LDL oxidation suggested that glycated PE could also be implicated in the onset of atherosclerosis in diabetic patients (12). Altogether these studies revealed the intricate connection that involves oxidative stress, membrane function, cell activation and inflammation that could lead to development of oxidative stress-related diseases.

3. DIABETES MELLITUS

Diabetes mellitus (DM) is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (97). Diabetes became a serious public health problem, with the increasing numbers of prevalence, affecting millions of people worldwide (98, 99). In Portugal (2009) the prevalence of diabetes was 11.7% in population between 20-79 years old, a number that increases to one third of population (34.9%) if a pre-diabetes stage is also considered (100). Several pathogenetic processes are involved in the development of diabetes. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action (97, 101). These two groups of pathogenetic processes lead to the distinction of two types of diabetes, namely type 1 and type 2 diabetes mellitus. Type 1 DM is caused by an absolute deficiency of insulin secretion. Type 2 DM, much more prevalent, is caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. Since the destruction of beta cells of the pancreas with consequent insulin deficiency may also be seen in type 2 DM, the distinction between type 1 and

type 2 DM could be difficult, thus it is very important to understand the pathogenesis of the hyperglycemia (101).

Deficient insulin action, from inadequate insulin secretion and/or diminished tissue responses at one or more points in the complex pathways of hormone action, leads to abnormalities of carbohydrate, lipid and protein metabolism. The chronic hyperglycemia (both levels and length of exposure) observed in diabetes generate pathological and functional changes even before the diagnosis is made. Long-term hyperglycemia leads to damage, dysfunction, and failure of various organs, especially eyes, kidney, nerves, heart, blood vessels. These long-term complications of DM include progressive development of the specific complications of retinopathy with potential blindness, nephropathy leading to renal failure, peripheral neuropathy and/or autonomic neuropathy. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease (97, 101). These long-term vasculopathy associated with DM are traditionally classified as macro- or microvascular depending on the underlying pathophysiology (102-105). Microvascular disease tends to occur predominantly in tissues where glucose uptake is independent of insulin activity, like kidney, retina and vascular endothelium. The development of microvascular complications arise from a combination of different factors that include hyperglycemia, dyslipidemia, oxidative stress and/or formation of advanced glycation end-products. Microvascular complication origin altered blood flow and changes in endothelial permeability, extravascular protein deposition and coagulation resulting in organ dysfunction. Macrovascular diseases include coronary heart disease, cardiomyopathy, stroke and atherosclerosis being the last one also related to oxidative stress and oxidized phospholipids. Furthermore, interrelation between microvascular and macrovascular pathologies may create a vicious cycle of tissue damage, that drives the leading cause of morbidity and mortality in diabetic patients. Consequently controlling hyperglycemia is important to protecting the body from diabetic complications, to improving longevity and quality of life (102-105).

3.1. Oxidative Stress in Diabetes

Current evidence argues against a generalized increase of oxidative stress in human diabetes, associated to hyperglycemia. Moreover, it is not well known whether oxidative stress is a primary event that occurs early in the course of the disease or whether it is a secondary phenomenon that merely reflects end-stage tissue damage (36). During diabetes, persistent hyperglycemia causes increased production of free radicals, especially ROS, from glucose auto-oxidation and increased formation of lipid peroxides. In addition, persistent hyperglycemia is also responsible for non-enzymatic protein glycosylation and advanced glycated end-products formation (AGEs). Among those, glucose oxidation is believed to be the main source of free radicals, although the interrelationship between glycation and oxidative stress is also noted, leading to

emergence of the term glycoxidation. On the other hand, antioxidant defense system is also altered in DM, with impaired glutathione metabolism, alteration in antioxidant enzymes, catalase and superoxide dismutase and decreased ascorbic acid levels (105-108). Furthermore, proteins like superoxide dismutase can be inactivated by glycation, turning them unable to detoxify free radicals, exacerbating oxidative stress in diabetes (28). Thus hyperglycemia is a major contributor to oxidative stress and increased oxidative stress is in turn suggested as mechanism underlying development and progression of diabetes and as one major contributor to long-term diabetic complications (105-108).

Oxidative stress conditions can result in the activation of various stress pathways such as NF-κB, JNK/SAPK, p38 MAPK, among others, also activated in normal insulin signaling. This cross talk between oxidative stress-induced pathways and normal insulin signaling creates the possibility for multiple disruptions in the ability of insulin to maintain its normal functions (105). Hyperglycemia, in DM, increase the ROS generation and oxidative stress markers (including lipid peroxidation), with an accompanying decrease in antioxidant levels. Increased lipid hydroperoxides were detected in several tissues, as kidney, liver, heart, brain, plasma, red blood cells, among others, of rats with induced diabetes, an indirect evidence of intensified free-radical production (107). Also in human plasma, lipid hydroperoxides, malondialdehyde and isoprostanes levels were found to be increased in diabetic patients (28, 109). The increase in the lipid peroxidation can be correlated with glucose levels (not without some controversy) and be prevented by controlling glycemia with insulin (105). The role oxidized LDL and oxidatively modified lipids in atherosclerosis, and their increased levels in diabetes are key factors for development of atherosclerosis as a diabetic complication (110). Lipid peroxides induce formation of pro-inflammatory cytokines, contributing for the high inflammatory state observed in DM (111).

3.2. PHOSPHATIDYLETHANOLAMINES IN DIABETES

Phosphatidylethanolamines, together with phosphatidylserines, are aminophospholipids, which have a free amino group present in the polar head, making them susceptible to non-enzimatic glycation, similar to that occurred in proteins. Thus the concept of the Maillard reaction was expanded to include glycation of aminophospholipids (9, 83).

The Maillard reaction is a process that initiates with the formation of an unstable Schiff base, which reaches a steady state within hours and is reversible. Then the rearrangement of the Schiff base into an Amadori product occurs, reaching a steady state in approximately 28 days and is also a reversible reaction (9, 107). When molecules have slow turnover rates, these Amadori products undergo multiple dehydration reactions and rearrangements to irreversibly form AGEs, like carboxymethyl- and carboxyethyl-adducts. AGEs also have potential to trigger pathological

processes and can be involved in the genesis of many of the irreversible complications of diabetes, including expanded extracellular matrix, cellular hypertrophy, hyperplasia, and vascular complications (9, 107). AGE proteins, unlike AGE lipids, have been intensively studied; however AGE lipids have also been found in diabetic plasma lipoproteins. Some AGEs involvement in oxidative stress is already known. AGEs can bind metal ions, making them available for glucose oxidation, damaging tissues. Schiff base adducts of methylglyoxal generate radical cations and superoxide without metal ion involvement (105).

First studies evidencing the glycation of phosphatidylethanolamines emerged in the early 90's, with the studies of Pamplona *et al*. (112). Later Ravandi *et al*. detected glycated PE both *in vitro* (12, 113) and *in vivo*, in atheroma (12), LDL (12), red blood cells (114) and plasma (114) from diabetic patients. Glycated PE was also characterized by LC-MS and MS/MS in the positive and the negative ion mode (12, 113-115). Amadori PE adduct formation in LDL, *in vitro*, originated two major species, namely 18:0/18:2-glycated PE and 18:0/20:4-glycated PE. These glycated PE were also the major species found *in vivo* glycated LDL, among other glycated PE species formed, including diacyl- and plasmenyl-PE. Furthermore, in glycated LDL, they verified that glycation of PE was more extensive than the glycation of apoprotein (12). Moreover, other researchers also reported the presence of glycated PE in red blood cells and plasma incubated with glucose, and that glycated PE can be degraded by phospholipases (116). In human red blood cells from diabetic patients, glycated PE was detected and quantified by LC-MS and found that glycated PE levels have a positive correlation with the glycated hemoglobin levels (117). The occurrence of glycated PE in plasma of diabetic patients (118) and in plasma, red blood cells, liver, kidney, pancreas, cerebrum and cerebellum of diabetic rats (119) was detected and quantified *in vivo* using LC-MS/MS neutral loss scan and MRM experiments (118, 119). The PE glycation in rats were apparently dependent on the PE fatty acid content. It was found that 18:0/20:4-PE was the first PE to be glycated followed by 18:0/18:2-PE. Additionally, glycation was suggested to be used as sensitive marker of the early stages of diabetes, since accumulation of carboxymethyl-lysine (protein advanced glycation end-product) and glycated hemoglobin was somewhat slower than glycated PE (119). Furthermore, carboxymethyl-PE were already detected and quantified *in vivo*, in human red blood cells from diabetic patients (120). *In vitro* synthesized carboxymethyl-PE and carboxyethyl-PE were studied by LC-MS and LC-MS/MS in order to develop a method for further detection *in vivo*. LC-MS/MS with MRM experiments allowed the detection of various species of carboxymethyl-PE and carboxyethyl-PE in plasma and red blood cells of healthy and diabetic subjects, without significant differences (121).

Glycation is a reaction that may play an important and primary role in initiating lipid peroxidation *in vivo* (83). Bucala *et al*. (122) first reported the involvement of lipid content of glycated LDL, in LDL susceptibility to oxidation. However, it was not identified any lipid glycated product (122). Since then, some studies have reported the involvement of glycated PE in lipid peroxidation, however the reason why is not completely understood. Ravandi *et al*. (12)

demonstrated that PE glycation is more easily oxidized probably because an increased exposure to the oxidizing agent, produced by the increase of polar head group bulk. Also, glycated PE promoted the oxidation of other molecules oxidation (cholesteryl esters in LDL, PC and non-glycated-PE) with formation of hydroperoxides, aldehydes, isoprostanes and lyso-PL compounds (12). The generation of lipid peroxidation in red blood cell membranes by glycated PE occurred even in the absence of metal ions (117). On the other hand, Oak *et al*. (123) synthesized Amadori-PE and evaluated their ability to induce lipid peroxidation in the presence of transition metal ion. With this purpose, they incubated linoleic acid alone, with non-glycated-PE and with Amadori-PE, under oxidative conditions. By measuring the amount of oxidation they recognized an increase of the linoleic acid oxidation in the presence of Amadori-PE, but not in the presence of non-glycated-PE. So they concluded that Amadori-PE is capable of generating reactive oxygen species, which trigger lipid peroxidation, in a processes that is inhibited by superoxide dismutase, mannitol, catalase and EDTA (123). Furthermore, glycated PE levels had a positive correlation with PC and PE hydroperoxides in human plasma (118) and in rat plasma and liver (119). Thus glycated PE was supposed to be correlated with atherosclerotic complications of diabetic patients with associated hyperglycemia, as result of oxidative stress modifications (12, 117-119, 123).

Little attention has been paid to other biological consequences of glycated PEs than the promotion of lipid peroxidation. A study indicated that glycated PE and LDL containing glycated PE affect LDL metabolism, by increasing cell-associated lipoprotein when compared with LDL containing non-glycated PE (115). The increased LDL uptake by macrophages was accompanied by cholesteryl esters and triacylglycerols accumulation in THP-1. Additionally the cholesteryl esters and triacylglycerols accumulation had correlation with the increase in PC oxidation. The effect of presence of glycated PE in LDL was comparable to that obtained when macrophages were incubated with glycated LDL, and were not inhibited by acetylated LDL. So it was proposed that the mechanisms involved in that interaction of LDL containing glycated PE and macrophages were not dependent of LDL or scavenger receptor, but would be probably dependent on the receptor of anionic phospholipids, namely CD36 or AGE receptor (115). In addition, incubation of HUVEC with glycated PE enhanced, in a dose depended manner, the proliferation, migration and tube formation of these cells, when compared cells treated with non-glycated PE (124). Similarly to the vascular endothelial growth factor (VEGF), glycated PE was found to enhance width and length of endothelial tubes. Furthermore, HUVEC incubated with glycated PE was found to secrete more metalloproteinase (MMP-2), in a dose-dependent manner. This study indicated that glycated PE could be important inducers of angiogenesis (124).

Glycated PE was also thought to induce changes in membrane properties and functioning. *In vivo*, lipid glycation is likely to induce changes in the biosynthesis and turnover of membrane phospholipids, the physical properties of membranes and the activity of membrane-bound enzymes, besides the susceptibility to oxidative stress (118). The influence of PE glycation in membrane protein, PMCA (plasma-membrane Ca^{2+}-ATPase) was evaluated by Levi *et al*. (125).

Only the effects of glycation were studied, and further oxidation was avoided with utilization of a saturated PE, nitrogen atmosphere, and a metal ion chelator. They showed that PE glycation, but not PMCA glycation, significantly decreased the affinity of lipids for the protein, in two different micellar environments. Additionally, significant decrease on lipid affinity for the transmembrane surface of erythrocyte protein, band 3, and Na^+/K^+-ATPase in the presence of glycated PE was also found. Furthermore, PMCA was inactivated faster in the presence of glycated lipids than in the presence of control PE and PC phospholipids. Therefore PE glycation triggered a structural rearrangement of the protein that decreased their stability and increased the sensitivity to thermal unfolding. This indicated that under hyperglycemic conditions (uncontrolled diabetes) or long-term exposure to glucose (aging), glycation of lipids of cellular membranes may cause a significant change in the structure and stability of membrane proteins which may affect the normal functioning of the membranes and therefore of the cells (125).

AIMS

The association between free radicals, glycation, diabetes, and its complications is not completely understood. Until now, the role of oxidized PEs has been associated with some pathophysiological conditions, including atherosclerosis, inflammation, Alzheimer disease, among others. However the mechanisms and the specific products that produce the reported effects were not always clear. Also, the formation of adducts among glucose and PE, as consequence of hyperglycemia, the increased susceptibility to oxidation of glycated PE, and the oxidation of other glycated or non-glycated molecules, such as other phospholipids, peptides and proteins induced by glycated PE are important features reveling the interconnections of oxidative stress and diabetes. Thus, studies are need to clarify the mechanism underlying the promotion of oxidation by glycated PE, and ultimately, to explore the mechanisms by which increased oxidative stress accelerates the development of complications in diabetes.

The main goal of this study was to develop a model, based on mass spectrometry analysis, to better understand the mechanisms involved in the glycated PEs oxidation, to better understand the relation structure-activity. Evaluation of biological effects of the products resulting from PE glycation, oxidation and glycoxidation on the immune responses were also studied, using flow cytometry.

To achieve this goal, the work was divided into three main objectives:

- Develop a new sensitive and efficient way of identification of the oxidation products of different glycated PEs formed by oxidative stress models, based on LC-MS and MS/MS (Chapter III).
- Evaluate the biological effects of oxidized PEs and glycoxidized PEs on stimulation of monocytes and dendritic cells (Chapter IV).
- Indentify and characterize alterations of PE species found in diabetic liver applying LC-MS and MS/MS methodologies (Chapter V).

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CHAPTER II

PROCEDURES AND OVERVIEW OF ANALYTICAL STRATEGIES

1. OBJECTIVE

The global strategy used to achieve the objective of this work can be divided in two main tasks. The first task comprises the structural characterization of oxidation products of glycated PE and the evaluation of the effects of oxidation products of glycated PE on the stimulation of monocytes and dendritic cells. For that purpose phosphatidylethanolamine standards were initially glycated, purified by high performance liquid chromatography (HPLC) and then oxidized by the hydroxyl radical generated under the Fenton reaction. The oxidation products formed were analyzed by mass spectrometry (MS) with or without previous HPLC separation. To evaluate the effects of the different modified phosphatidylethanolamine (PE) in monocytes and myeloid dendritic cells (mDCs), the non-modified PE, oxidized PE, glycated PE and glycoxidized PE were then incubated with peripheral blood for the evaluation of cell stimulation and cytokine expression employing flow cytometry technique. The structure of this work is summarized in the Figure II: 1.

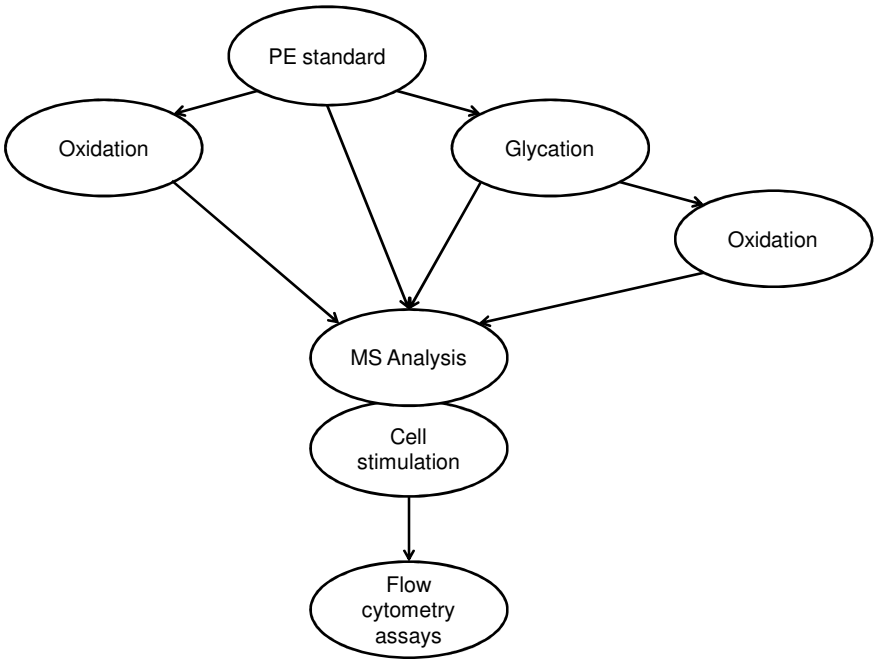


FIGURE II: 1 – Flow chart of the work done for the identification of oxidation products of glycated PE and the evaluation of glycated PE and glycoxidized PE on stimulation of monocytes and dendritic cells.

The second task comprises the evaluation of phospholipid profile of livers from rats with streptozotocin (STZ)-induced diabetes. This work was performed to evaluate the presence of glycated and glycoxidized PEs, capable of inducing oxidative stress and inflammation in diabetic subjects. The general approach for this task is represented in the Figure II: 2. Briefly, livers from

rats with streptozotocin (STZ)-induced diabetes and from non-diabetic rats, were homogenized and the phospholipids were extracted with the Folch method (1). In liver extracts, total phospholipids and amount of phospholipids *per* class were quantified. Analyses of phospholipid species were then performed by MS, using different approaches. Direct MS analyses of the total lipid extract were done in a Q-TOF2 mass spectrometer (Micromass, Manchester, UK.) instrument to obtain MS spectra and product ion spectra. Neutral loss and parent ion experiments were done in a triple quadrupole Waters Quattro Premier™ XE (Micromass, Manchester, UK. located at *Instituto de Biologia Molecular e Celular*). MS analysis in a linear ion trap LXQ (ThermoFinnigan, San Jose, CA, USA) was done after TLC and HPLC separation.

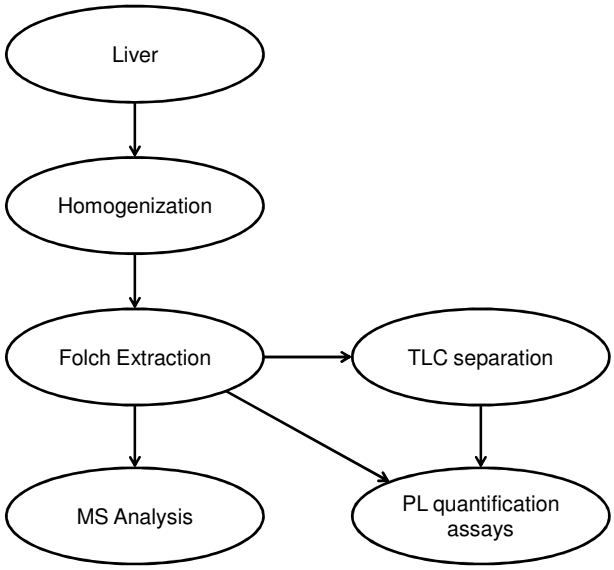


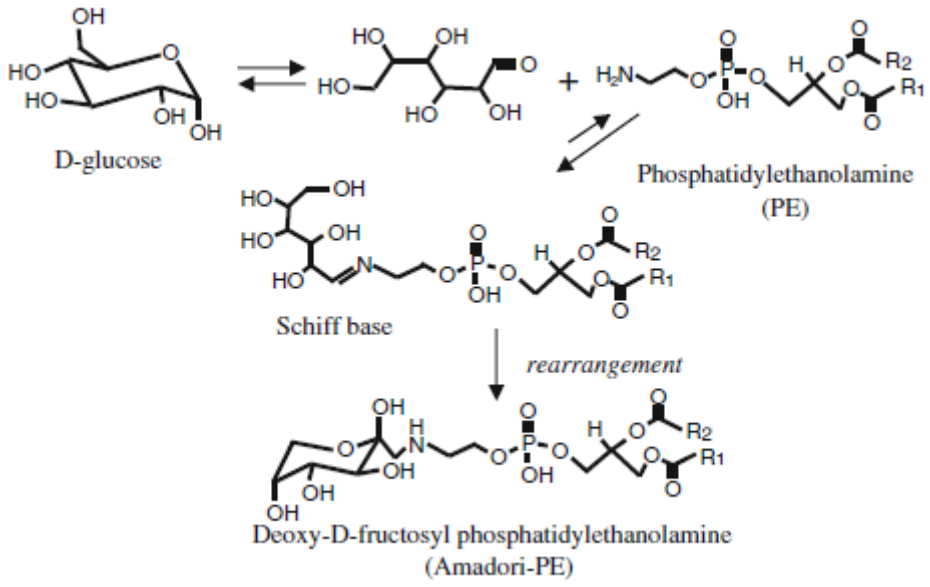
FIGURE II: 2 – Flow chart of the work done for the evaluation of phospholipid profile of livers from rats with streptozotocin-induced diabetes.

2. Overview of the Experimental Approaches

2.1. PE Glycation: Preparation of Glycated PE Standards

In vitro systems for PE glycation were reported in some previous studies (2-5). Also, *in vivo* glycation was reported to occur (6-10). The objective of glycation reactions performed in this work was to produce considerable amounts of glycated PE, which would allow to do oxidative experiments and the identification of oxidation products by mass spectrometry strategies. So we

opted to increase the yield of the glycation reaction in a shorter incubation period, instead of mimicking *in vivo* conditions. The glycation of PE standards was prepared in a methanol/water solution with a final concentration of 3 µM for PE and 0.5 mM for glucose, and reacting in boiling water during 30 minutes. To avoid oxidation of PE under the glycation process the reaction were conducted under nitrogen atmosphere. After glycation, the recovery of glycated PE and elimination of glucose from the reaction mixture was achieved by a modified Folch extraction (1). The organic phase containing glycated PE was collected for further purification by HPLC prior to oxidation. The schematics of the glycation reaction are presented at the Figure II: 3.



FIGUREII: 3 – Scheme for the glycation of phosphatidylethanolamine. The glucose reacts with the amino group of PE to form a Schiff base which undergoes an Amadori rearrangement to yield Amadori-PE (6).

2.2. Mechanisms of Phospholipid Oxidation: Procedure of Glycated PE Oxidation by the Hydroxyl Radical

In living systems, membrane phospholipids are continually exposed to oxidative situations (11). Phospholipid oxidation occurs mainly in the unsaturated fatty acyl chains (usually placed at the *sn*-2 position of glycerol), in a process that consists of three stages, initiation, propagation and termination. Lipid peroxidation is a chain reaction initiated by free radicals (like $HO^{\cdot}$ which is the most known potent oxidant) that have the capacity to abstract a hydrogen atom from bis-allylic carbon of unsaturated fatty acyl chains, forming lipid carbon centered radical ($L^{\cdot}$). This carbon centered radical undergoes further molecular rearrangement, forming a conjugated diene. When oxygen is present, the $L^{\cdot}$ reacts with oxygen, forming a peroxyl radical ($LOO^{\cdot}$). This $LOO^{\cdot}$ can react

with other molecules, propagating the reaction by abstracting a hydrogen radical with formation of a lipid hydroperoxide (LOOH). These lipid hydroperoxides (LOOH) can easily decompose into several reactive species including: lipid alkoxyl radicals (LO˙), ketenes, alkanes, lipid epoxides, and alcohols (LOH). Also lipid alkoxyl radicals can also decompose into alcohols (LOH), short alkanes, short alkenes, short aldehydes or short carboxylic acids. The short (or truncated) fatty acyl chains are formed by chain cleavage in the vicinity of the local of modification (11-18). For example PAF-like lipids are oxidation products of plasmenyl PC that contain a truncated fatty acid at *sn*-2 (17, 19). Furthermore in oxidation of fatty acids containing more than three double bounds like, arachidonic acid, the LOOH may undergo cyclization and rearrangement reactions that originate more complex oxidation products, namely prostaglandins (from unesterified arachidonic acid), isothromboxanes, isofurans, isolevuglandins and isoprotanes (18, 20). In general, the long-chain products are formed first and then could suffer further rearrangements that could lead to cyclization or cleavage of the modified fatty acyl chains as the reaction represented at Figure II: 4. The lipid peroxidation products have distinct structural features that may render distinct activities (17).

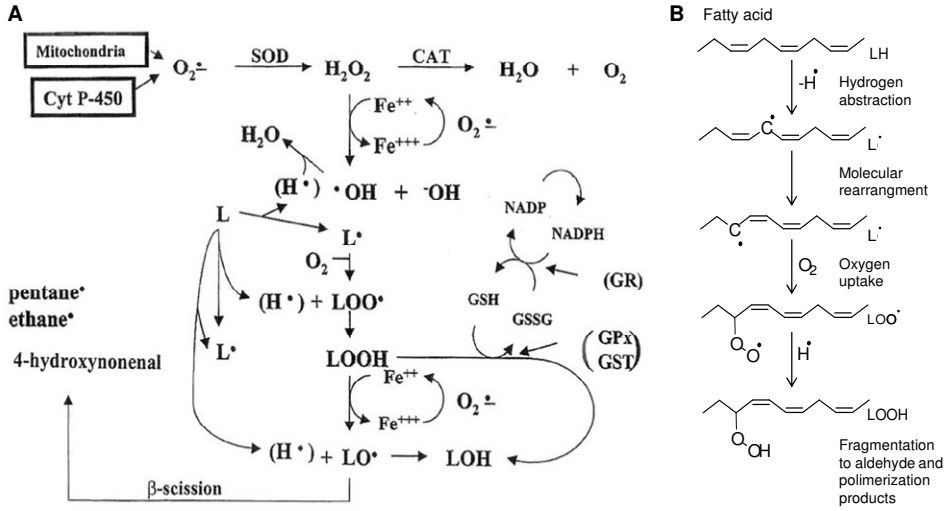


FIGURE II: 4 – Lipid peroxidation reaction (A) (21) and molecular alterations (B; adapted from Betteridge (12)). L˙ represents lipid alkyl radicals, LOO˙ represents lipid peroxyl radicals and LO˙ represents lipid alkoxyl radicals.

Lipid peroxidation occur *in vivo* systems, but these reactions can be mimicked by *in vitro* systems, in order to identify the oxidation products formed herein and understand their reactivity and functions. The phospholipid oxidation had been studied under diverse *in vitro* oxidation systems. Depending on the oxidation system employed, the lipid peroxidation products formed can be different. *In vitro* lipid peroxidation can be reproduced by enzymatic reactions (using lipoxygenase or mieloperoxidase) or non-enzymatic reactions, which can be radicalar or non-

radicalar (ozone and singlet molecular oxygen) (14, 15). Non-enzymatic radicalar oxidations are produced by radiation (22), Fenton and Fenton-like reactions (23) and from azo initiators (compounds that generate radicals by their decomposition) (22).

In this study, oxidation of glycated PE was achieved by the hydroxyl radical generated under Fenton reaction. Glycated PE vesicles (final concentration of 50 mM) were prepared in ammonium bicarbonate buffer (5 mM, pH 7.4). Then $FeCl_2$ and H_2O_2 were added to liposome mixture to a final concentration of 0.5mM and 50mM, respectively. The reaction mixture was kept at 37ºC, in the dark for at least 5 days, with daily MS monitoring either in a Q-TOF2 (Micromass, Manchester, UK.) instrument or in a linear ion trap LXQ (ThermoFinnigan, San Jose, CA, USA). The new oxidation products identified in MS spectra were further analyzed by tandem mass spectrometry.

2.3. Cell Stimulation with Glycated PE and Flow Cytometry Analysis

Glycated PE and glycoxidized PE obtained by the procedure explained before, were further evaluated for the ability to stimulate monocytes and dendritic cells. For that purpose fresh peripheral blood were collected from healthy donors. Blood was incubated in a liquid medium supplemented with the phospholipid vesicles, prepared in PBS, and Brefeldin-A, to prevent the release of cytokines from outside the cells, at 37ºC in a 5% CO_2 humid atmosphere, in a sterile environment for 6 hours. After that samples were labeled with monoclonal antibodies for detection of monocytes (CD 33 and HLA-DR), dendritic cells (CD33, HLA-DR, CD14, CD19, CD56 and CD3) and intracitoplasmastic cytokines (IL-1β, IL-6, IL-8, MIP-1β and TNF-α). This allows flow cytometry identification of monocytes and dendritic cells and analysis of the expression of each cytokine under study.

2.4. Preparation of Liver Extracts for Lipidomic Analysis

Proper preparation of samples is important for elucidation of the biochemical and biophysical properties of membrane lipids and their cellular functions. Attention should be paid to prevent biochemical changes, thus quick procedures, at low temperatures and in the presence of antioxidants are commonly used strategies. When the samples are organs and tissues, it is necessary to destroy their integrity, with homogenization steps, prior to lipid extraction, in a form that they can be subsequently fractionated. The homogenization is highly influenced by the operational criteria which differ among tissues. Various homogenization methods have been employed based on mechanical disruption (Potter-Elvehjem, rotating blade devices such as Waring blendor and Ultraturax, glass beads) or other shear forces (pressure/cavitation, freeze-thaw). Also

during homogenization step, the preservation of the membranes as they exist *in vivo* is accomplished with the usage of buffers that mimic the pH and solute composition of the living cells. The lipid extraction methods from samples exploit the high solubility of hydrocarbon chains in organic solvents, in a procedure that depends on the chemical nature of the lipid components and the kind of complexes or associations in which they occur in the cell. The separation is accomplished with addition of immiscible solvents to the aqueous sample, like chloroform or benzene containing solutions. Membrane associated lipids require polar solvents such as ethanol or methanol to disrupt the hydrogen bonding networks or electrostatic forces between lipids and proteins and to precipitate proteins. Special care should be taken to minimize risk of lipid oxidation and hydrolysis and avoid rapid protein denaturation to improve lipid recovery. There are various extraction protocols and the chosen protocol should take in consideration not only the nature of lipids and sample, but also the analytical method used (24-26).

In this study, the livers of STZ-induced diabetic rats were dissected out, after 4 months of the intraperitoneal injection of streptozotocin. Homogenization of livers was performed with PBS (phosphate buffered saline), mimicking the *in vivo* pH, and employing mechanical disruption (Potter-Elvehjem homogenizer and teflon pestle). Total lipid were extracted from homogenized livers by Folch method, reaching a final proportion of chloroform, methanol and water of 8:4:3, by volume (1). Total phospholipids were measured by determination of phosphorus amount, after digestion with perchloric acid, according to a well-established procedure (27). Total phospholipid levels were normalized to protein content determined by biuret method. Phospholipid amount within each PL class were determined by phosphorus assay, subsequent to TLC separation. Characterization of individual phospholipid within each class was achieved by mass spectrometry (MS) after separation by liquid chromatography (LC). HPLC separation were accomplished by the adaptation of an existing method (28) and MS experiments were done in a linear ion trap LXQ (ThermoFinnigan, San Jose, CA, USA).

3. Overview of Analytical Strategies

3.1. Mass Spectrometry Fundamentals

In the recent years, with the development of the “omics” fields, is required “high-throughput analytical tools” and mass spectrometry has essential role in this field. There are an increasing of “omics” fields, from genomics, proteomics, lipidomics and many others. Each specific “omic” field comprises a wide range of techniques applied to the study of a specific subject (29, 30). In most lipidomics studies, MS constitute the core technology employed either in a global or target manner. Global lipidomics consists in identification of all or the majority of lipids of a sample on a single

experiment format. Shotgun lipidomics or LC-MS analyses of crude extracts are approaches commonly used to identify and quantify as many lipid molecular species as possible. In contrast, targeted lipidomics concentrates on one class of lipid and protocols are designed and utilized in a class specific manner (31, 32).

Mass spectrometry is an analytical tool that measures the molecular weight of sample constituents, via measurement of mass-to-charge ratios (*m/z*), and gives structural information, by application of tandem mass spectrometry (MS/MS). Mass spectrometers are instruments that ionize the samples (ion or molecules) and apply to them electrostatic, magnetostatic or electromagnetic fields to separate the ions by their mass-to-charge ratios (*m/z*). The ionization and ion separation occur in the gas phase, so the analyzed compounds need to be vaporized or transferred into vacuum either before or during the ionization. Mass spectrometers are generally composed by three fundamental parts, which are the ionization source, the mass analyzer and the detector (29-31). The Figure II: 5 shows a schematic representation of a mass spectrometer.

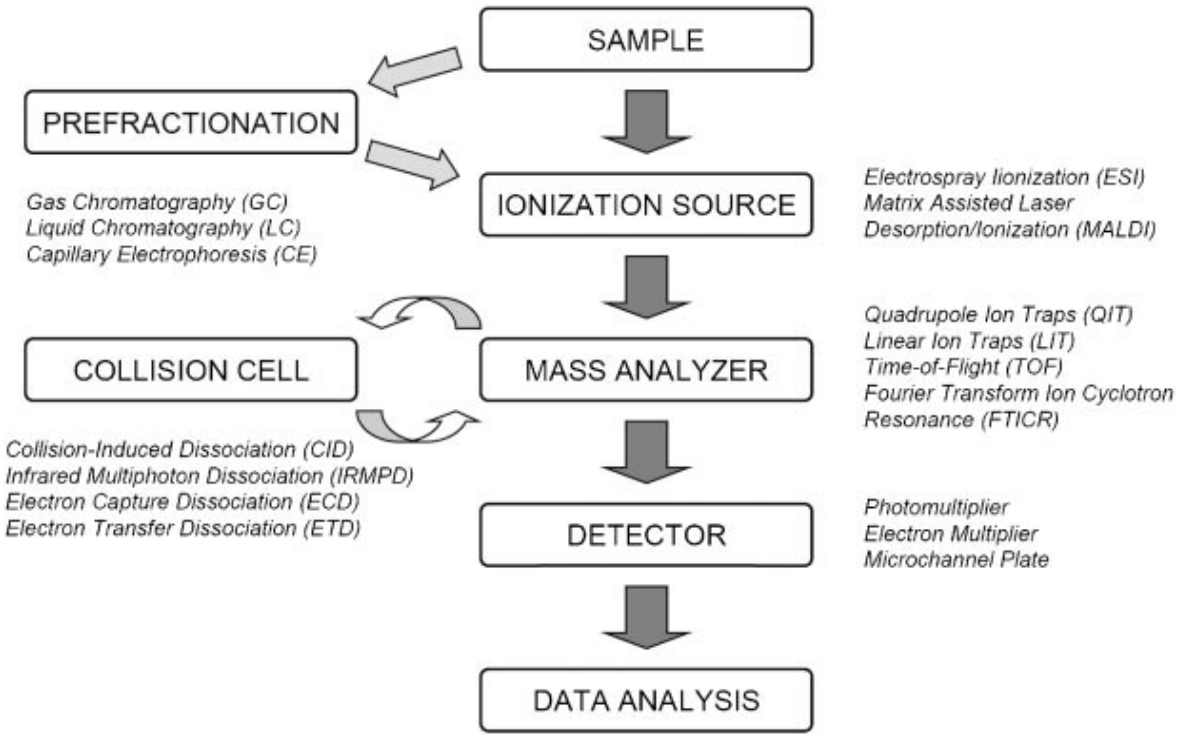


FIGURE II: 5 – Basic components of a mass spectrometer (29).

There are different ionization sources, mass analyzers and detectors and several combinations between the existent components are possible. Furthermore, the system for sample introduction on mass spectrometers can be conducted through direct injection or be easily coupled

with separation technologies, such as gas chromatography (GC) or high performance liquid chromatography (HPLC), which are especially important for the analysis of mixtures, as the cases of biological samples (29, 30). MS data is largely dependent on the sample introduction and the method of ionization, while the type of mass analyzer does not usually alter the observed chemical reactions (33).

3.1.1. IONIZATION SOURCES

Ionization source is the mass spectrometer component that promotes the ionization of the molecules so that they can be analyzed. There are different ionization sources that can be used, and the choice depends on the sample that needs to be ionized. Electron impact (EI) ionization, fast atom bombardment (FAB) ionization, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are some examples of the existent ionization sources. EI was the first ionization source, but this ionization method has some limitations, because promotes molecular fragmentation and only ionizes volatile compounds. The development of mass spectrometry technology, leaded to the emerging of the “soft” ionization methods, especially ESI and MALDI. These ionization sources produce minimal fragmentation, allowing the analysis of biomolecules and complex biological samples (29).

3.1.1.1. ELECTROSPRAY IONIZATION

Currently electrospray ionization is the most frequently used and well suited ionization technique for lipid analysis (24). ESI is the “softest” ionization technology, thus rarely disrupts the chemical nature of the analytes and even non-covalent bonds can be conserved. ESI is used for the analysis of large and non-volatile molecules/ions directly from liquid phase. In this ionization source, the analyte molecules or ions are present originally in solution and are transferred to the gas phase through either solvent or ion evaporation.

The ionization process is not fully understood, however it is known that the ionization is induced at atmospheric pressure by a strong electrostatic potential difference between the syringe needle and a counter (cone) electrode, which polarize the solution at the end of the needle and spray away the sample from the needle. Positively charged or negatively charged droplets can be formed depending on the applied potentials. The solvent evaporates, assisted by a flow of heated inert gas, and smaller droplets are formed until the ion desorption occurs. In this process multiple charged ions can be easily formed, which is a characteristic feature of ESI. These multiple charged ions allow the analysis of high molecular weight molecules, when it is used analysers that have a

lower mass limit. One advantage of ESI is that no matrix is needed, which is important for small molecules analysis, because the peaks associated with matrix ions (observed in MALDI) do not appear in the ESI-MS spectra (29, 30). The Figure II: 6 represents the schematic ESI source and the ionization process.

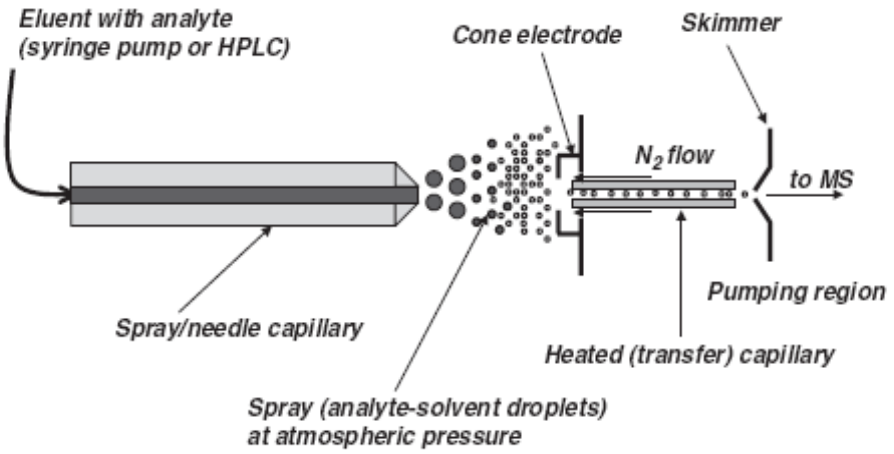


FIGURE II: 6 – Electrospray ionization (ESI) source (30).

3.1.1.2. MATRIX ASSISTED LASER DESORPTION IONIZATION

MALDI is another soft ionization technique. It is often used for analyses of proteins, but has already been used successfully in lipids (24). MALDI is most frequently used for phospholipid analysis after TLC separation, due to the easy MALDI-TLC coupling and for imaging MS (24, 25, 33-36). In turn, direct phospholipid analysis with MALDI is not so used, due to the complexity of the spectra formed (24, 25, 34). In MALDI the sample is mixed with a matrix, crystallized in a solid phase and the ionization is promoted by laser light. By irradiation of the matrix, which is in great excess to the sample molecules, with a laser beam, the molecules are excited and released to the gas phase. In the gas phase, the matrix and the sample interact with each other, and ionization occurs by proton transference. In this process, mostly singly charged ions are formed. The matrix is an essential part in this ionization method and there are different matrices. The selection of the matrix depends on the compounds to be analyzed. In lipid analysis, 2,5-dihydroxybenzoic acid (DHB) is the most common matrix used (37). In Figure II: 7 the principle of the matrix assisted laser desorption ionization is shown.

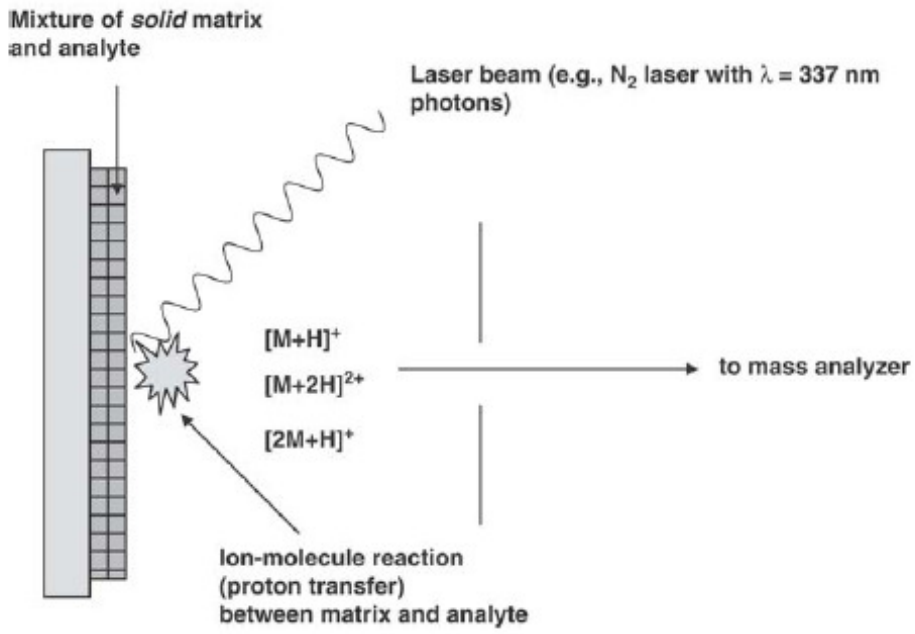


FIGURE II: 7 – Matrix assisted laser desorption ionization (MALDI) (30).

3.1.2. MASS ANALYZERS

The ions formed in the ionization source are further separated according to their *m/z* ratios in mass analyzers, normally by electric and/or magnetic fields. The properties that characterize mass analyzers include: mass accuracy (the error in the exact mass determination compared to the theoretical value), resolution (capacity to distinguish two ions with a small mass difference) and sensitivity (i.e., the number of ions detected), which depends on the transmittance (number of ions that reach the detector/number of ions produced in the source). It should be noted that mass analyzers must be compatible with the ionization source used. Quadrupole (Q), ion trap and time-of-flight (TOF) are the most common mass analyzers. Mass spectrometers can have only one type of mass analyzer or have combinations of analyzers. The later ones are called hybrid mass spectrometers. Hybrid mass spectrometers allow tandem mass spectrometry (MS/MS) experiments and are frequently used in lipid identification and quantification (24, 29, 30). The hybrid Q-TOF is an example of a hybrid mass spectrometer that was utilized during the work developed within this thesis.

3.1.2.1. QUADRUPOLE

The quadrupole (Q) mass analyzer is formed by four parallels metal rods organized in a quadrupolar structure. The opposite rods form pairs, which are connected electrically through the same voltages. One pair has a specific voltage and the perpendicular pair has the same voltage with an opposite signal. Direct current (dc) and radio-frequency (rf) potentials are applied between the two opposite pairs of these electrodes to produce a high frequency oscillating electric field. Mass separation is made possible by the oscillatory motions of ions in this electric field. Only the ions which have a stable trajectory can pass through the analyzer and reach the detector, while the others collide with the rods and are lost, never reaching the detector. A stable trajectory is achieved with specific values of the dc and rf potentials which selectively filter the ions of interest based on their *m/z* ratios. A mass spectrum is usually obtained by changing both the dc and rf potentials while keeping their ratio constant, and detecting how many ions pass through the filter at each *m/z* value (24, 29, 30, 37)

Quadrupole analyzers are normally used with ESI sources, because of the continuous flow of the ions generated in the source. These analyzers have a good reproducibility but generally, low mass resolution and accuracy (24, 29, 30, 37).

3.1.2.2. ION TRAP

Ion trap mass analyzers comprise different instruments, namely linear or two-dimensional ion trap (LIT), three-dimensional ion trap (QIT) and orbitrap. In the two- and three-dimensional ion traps, the ions are captured in a field and then sequentially ejected for separation (24, 29, 30, 37).

The two-dimensional or quadrupole ion traps have the same principle of quadrupoles, but with an additional axial dimension by means of an electric field produced by end-cap electrodes. The end-cap electrodes block passage of ions in the axial direction and the ions stay between the rods until they are ejected (radial ejection) to the detector. The two-dimensional ion trap works similar to the three-dimensional one, but the first one has higher resolution and expanded dynamic range, with a larger volume, can store more ions before space-charge affects the performance (24, 29, 30, 37).

The QIT (three-dimensional or cylindrical ion trap instrument) is composed of a doughnut-shaped central ring electrode and two end-cap electrodes, all with hyperbolic geometry. The trapping electric field is formed by applying dc and rf potentials to the ring electrode and ground potential to the end-cap electrodes. By manipulation of the ratios of the dc and rf field strengths, and the analyte ions can be captured and successively ejected, due to mass selective instability,

yielding a mass spectrum (24, 29, 30, 37). The ion traps have usually more sensitivity than quadrupoles and allow the fragmentation in consecutive steps (MS^n) (24, 29, 30, 37).

In Orbitrap, a different analyzer, ions are also trapped and stored in a potential well. However, instead of ejecting the ions for external detection the frequency of the trapped oscillating ions is measured. Orbitrap mass spectrometers provide high mass accuracy and resolving power (24, 37).

3.1.2.3. TIME-OF-FLIGHT

The time-of-flight (TOF) analyzers separate the ions by applying electric fields that accelerate the ions, with a specific kinetic energy. The time that ions take to reach the detector is measured and is correlated with the *m/z* ratio. Since the kinetic energy is the same, the velocity of the ions is inversely proportional to the square root of their mass. Thus lighter ions have higher velocity and shorter arrival time than the heavier ions, which have lower velocities. The resolution of the TOF analyzer is limited by the initial velocity (kinetic energy) and spatial spread of the ions. Spatial spread can be compensated by delayed extraction technique (time-lag focusing) and the velocity spread is reduced using the reflectron. The reflectron consists of an electrostatic field in which the ions must penetrate and switch their trajectory. In reflectron, the ions with same *m/z* value, but different velocities have to travel different distances for all those ions reach the detector at the same time. This improves significantly the resolution and mass accuracy of TOF analyzers. TOF analyzers are usually coupled to MALDI ionization, but can also be coupled with ESI sources. In this case an orthogonal acceleration is usually applied for ions to go to the TOF analyzer. TOF analyzers have a good resolution, an excellent sensitivity and a large mass range (24, 29, 30, 37).

3.1.3. DETECTORS

After the sample ionization and ion separation according with their *m/z* values, the signal of each *m/z*-resolved ion is measured in a detector. The ions hit the detector and the detector converts their energy into a current signal that is registered. The most frequently used detectors are the electron multipliers, which amplify a weak current of incoming particles by using a series of secondary emission electrodes or dynodes to produce a considerably higher current at the anode. The microchannel plate or multichannel plate (MCP) is an electron multiplier detector that is often utilized in TOF instruments. A MCP is formed by parallel array of channel electron multipliers, which amplifies the ions signal. The signal is then registered by the electronic devices and

transferred to the computer, which translates the signal in a mass spectrum. A mass spectrum is a plot that shows the *m/z* ratios of the ions versus their relative abundance (24, 30, 37).

3.1.4. TANDEM MASS SPECTROMETRY

Tandem mass spectrometry (MS/MS), is the study of specific ion fragmentations allowing the structural characterization of originally non-fragmented ions (precursor ions), which cannot be achieved only by direct mass spectrometry. Mass spectra only give the molecular mass information and this is insufficient, because of the diverse molecular structures and also existence of isomers. The fragmentation pattern obtained is characteristic of structural features of each molecule. However, the MS/MS spectra of the same precursor ion can be quite different if they are acquired in different instrument configurations. Besides the given useful information about molecular structure, MS/MS is a powerful analytical approach for molecular quantification, especially multiple reaction monitoring (MRM) experiments (24, 29, 30, 38).

Tandem mass spectrometry is a technique that uses multiple stages of mass selection and mass separation, to elucidate the structural information of specific ions. Typically two or more mass analyzers coupled in series are need for MS/MS experiments. In this configuration, an individual ion can be selected and isolated, in the first analyzer; then fragmented, in the collision cell; and the product ions formed are subsequently measured, in the second analyzer. Ion trap analyzers are able to perform MS/MS without coupling with another analyzer, and are also able multiple stages mass analysis, the MS^n (24, 29, 30, 38).

A prerequisite for MS/MS technique is that the sample molecules can be ionized intact and that they can be induced to fragmentate in some predictable and controllable way after the first mass selection step. The ion activation step is crucial to the experiment and ultimately defines what types of product/fragment ions are formed. Collision-induced/activated dissociation (CID/CAD) is the classic method for ion fragmentation in lipid analysis. CID is produced by collision of a neutral gas (helium or argon) and the selected ions, leading to fragmentation. During the collision with the gas, part of the kinetic energy of mass-selected precursor ions, which was augmented due to ion acceleration through an electric potential drop, is converted into internal (vibrational/rotational) energy. When the internal energy of the precursor ion is high enough, ions will break in the weak bonds and fragment, allowing to obtain chemical information about most frequent and most favorable molecular fragmentation. Depending on the type of mass analyzer, either high-energy CID (KeV collision energy in TOF instruments) or low-energy CID (<100 eV in quadrupole and ion trap instruments) can be performed (24, 29, 30, 38).

The fragmentation can be performed either *in space* or consecutively *in time*. *In space* fragmentation, ions are analyzed sequentially as they travel through the instrument containing at

least two “nontrapping” analyzers. *In time* fragmentation is, only possible in ion trap analyzers, where ion selection, fragmentation and fragment separation occur in the same physical space. The selected ion is trapped in the analyzer (while all the other ions are ejected), the fragmentation is induced and the fragment ions are then ejected for further detection. *In time* fragmentation allows consecutively repeated steps of selection, fragmentation and analyzes, the MS^n experiments, which is a valuable tool (29, 30, 38).

Product ion scan gives information of all the fragment ions of a selected ion, and can be accomplished with ion trap (*in time* fragmentation) and hybrid (*in space* fragmentation) mass spectrometers. Other scan modes can be performed, but only with *in space* fragmentation (typically triple quadrupoles instruments). These include precursor ion scan, neutral loss scan and MRM, as summarized in the Table II: 1. Neutral loss and precursor ion scans are used for detection of certain class molecules with a certain common functional group. Precursor ion scan mode gives information of the precursor ions from which a given fragment is formed, by adjusting the third quadrupole with a selected *m/z* value, while the first quadrupole scans for the precursor ions. The neutral loss scan is used for searching compounds that lose a common neutral fragment. For that, both the first and the third quadrupoles are scanned in a synchronized way, with a selected fixed *m/z* difference between them. MRM is a useful tool to obtain structural information from different molecules in the same sample and to provide reliable quantification. In this case the first and the third quadrupoles are set up to monitor only a selected parent ion and a selected fragment, respectively. This two-step mass filtering process, analyzes a known fragment of a specific the compound, thus providing high specificity and impressive accuracy and precision (30, 38).

TABLE II: 1 – MS/MS scan modes applicable in a triple quadrupole instrument (QqQ). Adapted from Somogyi *et al.* (30).

Scan modes	Quadrupole 1 (Q1)	Quadrupole 2 (q2)	Quadrupole 3 (Q3)
Product ion	Select a desired *m/z*	Ion activation/dissociation	Scan for all fragment ions
Precursor ion	Scan for parent of a giving fragment (F)	Ion activation/dissociation	Select and monitor fragment F
Neutral loss	Scan for parents	Ion activation/dissociation	Scan with shift of mass of the neutral
Selected/Multiple Reaction Monitoring (SRM/MRM)	Select a desired *m/z*	Ion activation/dissociation	Select and monitor desired fragments of *m/z*

3.1.5. MASS SPECTROMETRY OF PHOSPHATIDYLETHANOLAMINES

Analysis of phospholipids can be performed with several analytical strategies, and MS is considered to be the most important. The combination of sensitivity, specificity, selectivity and speed makes MS an ideal technique for analysis of phospholipids (33). The ESI and MALDI are the ionization methods used in phospholipid MS analysis. The ionization and fragmentation of phospholipids is somewhat different in ESI (39-41) and MALDI (42-44), as reviewed before. Each PL polar head group have different structural features, leading to different ionization propensity under positive or negative modes (24, 26, 33, 40, 45).

The PE can be ionized in the positive and in the negative ion modes. In the positive mode, ionization is produced by gaining one proton, originating protonated molecular ions $[M+H]^+$, and/or by gaining one cation, of which sodium is the most frequent, and originates sodiated molecular ions $[M+Na]^+$. Ionization by addition of 2 sodium ions, forming the $[M-H+2Na]^+$ molecular ions can also occur, especially in MALDI. In the negative ion mode, the ionization occurs by the loss of one proton originating the deprotonated molecular ions $[M-H]^-$, and by the gain of anions (when salts, like chlorates, are added) forming the $[M+Cl]^-$. The addition of salts can also be done in the positive ion mode, lithium salts are normally added in such cases, originating lithiated molecular ions $[M+Li]^+$. This capability of the PE be ionized in the negative ion mode is very useful for the shotgun analysis of phospholipid mixtures. Such mixtures contain phosphatidylcholines, which have a permanent positive charge, and difficult the detection of other phospholipids due to ion suppression. Thus, the phospholipids that have a good ionization in the negative ion mode can be easily detected, in this mode, without the interference of phosphatidylcholines (24, 42, 46, 47).

The glycerophospholipid fragmentation patterns normally reveal information on the lipid polar head group and its acyl chain moieties. Generally PL polar head groups can be associated with a characteristic fragment, thus neutral loss and precursor ion scans would enable the identification of phospholipid classes in complex mixtures (24, 26, 33, 40, 45). The fragmentation of PL $[M+H]^+$ ions originates a predominant fragment ion, which corresponds to the loss of the phospholipids polar head (33), and allows the discrimination of phospholipid families. Besides the information of polar head group, the fragmentation of PL $[M+H]^+$ ions gives information of total number of carbons and double bound of the two acyl chains combined. Because of the variety of combinations of different fatty acyl moieties could add up to the same molecular mass, further structural information is needed. The individual fatty acyl substituents are simply accessed by fragmentation metal adducts or deprotonated ions, allowing the unambiguous determination of the lipid species. The identification of the length of each fatty acyl moiety, the total number of double bonds on each fatty acyl chain and the regiospecificity of the two fatty acyl chains are accessed. However, the fragment ions corresponding to carboxylate anions of fatty acids are specially obtained in the negative ionization mode (24, 26, 40).

In the particular case of PE, the characteristic fragment is originated from neutral loss of polar head, which corresponding to a neutral loss of 141 Da (24, 40, 42, 44, 47). The fragmentation of the PE $[M+H]^+$ ions yield basically that product ion, thus only limited information is obtained. For further information is necessary to do the fragmentation of the PE cation adducts ($[M+Na]^+$, $[M+Li]^+$) or $[M-H]^-$ ions. Besides the neutral loss of 141 Da, $[M+Na]^+$ and $[M+Li]^+$ precursor molecular ions originate more fragment ions, that result from loss of aziridine (- 43Da; part of the polar head) and losses of the *sn*-1 and *sn*-2 acyl chains. The loss of *sn*-1 acyl chain is more favorable than the loss of *sn*-2 acyl chains, reflected by higher relative abundance of the ions $[M+Na-R_1COOH]^+$ relative to $[M+Na-R_2COOH]^+$. These differences in the loss of the acyl chains are a valuable characteristic that allows the differentiation of the acyl chain position in the glycerol backbone (24, 40, 47). Fragmentation of the PE $[M-H]^-$ ions originates the carboxylate anions ($RCOO^-$) formed by the loss of *sn*-1 and *sn*-2 acyl chains, which also have differences in the relative abundance. However, the ratio between the two carboxylate anions is dependent on the collision energy applied. Thus the identification of the specific location of the acyl chains in the glycerol backbone, in the negative ion mode, must take in consideration this factor. At low collision energies, the *sn*-2 carboxylate anion was preferred (ratio greater than 2 to 1); however, at higher collision energies an inversion of that ratio was observed (24, 40).

Moreover, by CID fragmentation it is possible to distinguish the phospholipids subspecies; diacyl phospholipids forms two carboxylate anions and plasmenyls (vinyl ether) or plasmanyls (alkyl ether) phospholipids forms only one carboxylate anion. Further differentiation of plasmenyls and plamanyls phospholipids can be obtained by phospholipid hydrolyzation under mild acidic conditions, this attack vinyl ether bonds. By comparison of the spectra before and after hydrolysis, it is possible distinguish plasmenyls from plamanyls glycerophospholipids (48).

3.1.6. Mass Spectrometry of Oxidized Phospholipids

As reported before, PE oxidation can generate a huge variety of oxidation products. This variety of phospholipids oxidation products makes difficult the MS analysis of all species in samples, especially because of ionization and ion suppression effects. However, the high sensitivity of MS and the advantage of obtaining information at molecular level, explains the employment of MS to the analysis of oxidized phospholipids. Coupling mass spectrometry with liquid-chromatography is the best choice for the detection of a wide range of oxidized PL and isomers. LC-MS allows the qualitative and quantitative determination of long and short chain oxidation products and lyso-PL without the need for derivatization procedures, and shows good linearity and reproducibility across a wide range of concentrations.

Targeted approaches can be designed for analyzing specific oxidized products, like neutral loss scan of *m/z* 319 used to detect oxidation products containing HETEs (18, 19). Long chain oxidation products could be detected by the addition of oxygen molecules to phospholipids. Thus oxidation products containing hydroxyl and peroxyl groups can be identified by the addition of multiples of 16 Da to the mass of unoxidized phospholipids. In turn, keto and epoxy groups of oxidized phospholipids could be identified by the increment of multiples of 14 Da to the mass of unoxidized phospholipids. Truncated oxidized phospholipids are more heterogeneous molecules, thus some derivatization techniques can be applied for easier detection and improved sensitivity (19). These targeted approaches are necessary to detect oxidized PL in complex biological samples. However, it is necessary to know exactly what products are formed and what are their fragmentation patterns, thus, *in vitro* studies are very important for the development of new methodologies for detection of oxidized PL.

3.2. Chromatography Fundamentals and Application on Lipidomics

Lipid species are routinely analyzed by ESI mass spectrometry via direct infusion or integrated with front-end liquid chromatographic separations such as normal- and reverse-phase HPLC. Chromatographic separations such as TLC or HLPC can be combined with MS analysis using either an in-line or off-line approach. By coupling chromatographic separation to MS, the lipidomic analysis is considerably improved, by reducing the complexity of the lipid sample, reducing the signal suppression, improving the detection of minor species and allowing the detection of isomeric/isobaric species (24-26).

3.2.1. Thin Layer Chromatography

Thin layer chromatography (TLC) was the earliest chromatographic method used to assess phospholipids (since the early 1960s), and is routinely used today. TLC was developed from paper chromatography. The TLC separations are accomplished through a combination of a solid stationary phase and a liquid mobile phase. The stationary phase consists of a thin layer coated in a flat support. For the separation of phospholipids, the most common plates are glass plates coated with silica gel, normal-phase TLC. There are also other stationary phases that can be used in phospholipid analysis. The mobile phase consists of a solvent system that varies in polarity. A wide variety of organic mobile phases can be applied to separation of virtually all phospholipid classes. Chloroform, methanol and water are typical solvents used, but triethylamine, ethanol, hexane and isopropanol are also common solvents. Separation of phospholipid classes can be

accomplished in one dimensional or two dimensional TLC. After separation phospholipids must be visualized, so specific and non-specific stains could be employed for that purpose (24, 25, 49, 50).

The migration of analytes, like phospholipids, on the stationary phase depends on the partition coefficients, retention/retardation factor (*R*f), capacity factor of the individual constituents on the plate and selectivity of the mobile and stationary phases. During TLC separation, the molecules of the solvent compete with the molecules of the analyte for binding sites on the stationary phase while the mobile phase passes through the stationary phase. Different partitioning between the two phases results in differential rates of migration of the different mixture components. Thus, the identification of analytes is based on the retention factor (*R*f), which is the ratio of migration distance of the analyte from the origin to the migration distance of the flowing solvent from the origin. Each phospholipid class has its own *R*f value under specific experimental conditions. In such normal-phase TLC systems, used for separation of PL classes, the less polar compounds have more affinity with the mobile phase and so elute in front of the more polar compounds which have higher affinity with the stationary phase (49-51). The TLC system used in this work for phospholipid classes separation consisted of silica gel plates developed with chloroform/ethanol/water/triethylamine (30/35/7/35, by volume) (52).

TLC is not a very sensitive methodology, but is a rapid and inexpensive screening tool that could be used prior to more sensitive and sophisticated techniques. TLC is often used as a sample preparation step to separate phospholipid classes prior to HPLC or MS analysis. Additionally TLC can be easily combined with MALDI, in a TLC-MS methodology successfully employed in lipidomic studies. However, concerns about oxidative degradation during TLC separation, contamination, poor reproducibility and resolution should not be neglected (24, 25, 36, 49, 50, 53).

3.2.2. High Performance Liquid Chromatography

High performance liquid chromatography is the most popular technique currently used for the separation of lipid classes normally prior to MS analysis. In HPLC separation, the liquid sample is driven through a stationary phase, packed in a column, by liquid flow at high pressure (typically 50–400 bar) provided by mechanical pumps. The diverse components of the sample are differently retained in the column and reach the end at different times, in a process that depends on the molecular properties of the analytes, the composition of the mobile phase, and column packing (stationary phase). At the end of column various detection systems can be coupled, including MS, refractive index (RI), ultraviolet (UV), fluorescence and evaporative light scattering detection (ELSD), in a way that signal intensity vs. time chromatograms can be plotted (24, 25, 49, 54).

Different stationary phases with small particle size (3–5 µm are commonly used) and a well-defined pore diameter can be combined with different mobile phases in an isocratic or gradient

elution. Mobile phases can be selected from a wide range of solvents, including water, methanol, acetonitrile, chloroform and *n*-hexane. The selection of the mobile phase relies in solvent properties like polarity (which defines the eluent strength) and miscibility. Solvent strength is defined as the capability of the solvent to elute a given compound from the stationary phase. Thus, stronger solvents elute the analyte from the column faster. The solvent system used for phospholipid analysis also depends on the method of detection. Gradient elution programs are normally preferred due to the wide range of polarity of the phospholipid classes. Also, multiple chromatographic separation techniques may be required for comprehensive lipid identification and quantification, due to the great diversity of the physicochemical properties of lipid species (24, 25, 49, 54).

Normal-phase HPLC and revered-phase HPLC conditions have been developed for phospholipid analysis. In the normal-phase HPLC, the stationary phase is more polar than the mobile phase and the interaction between analyte and column has predominantly polar character (hydrogen bonding or dipole–dipole interactions, etc.). The most commonly used normal stationary phase is silica gel, where the phospholipids are separated by adsorption phenomena. In this conditions PL elutes depending on head group polarity, sequentially from non-polar to polar. Thus normal-phase HPLC is usually employed for separating glycerophospholipids based on their polar head groups (24, 25, 49, 54). The hydrophilic interaction liquid chromatography (HILIC) emerged as a variant of normal-phase HPLC (55). HILIC consists of combination between a polar stationary phase with an aqueous/polar organic solvent mobile phase, typically acetonitrile in which water is introduced to play the role of a stronger eluting solvent. Separations are achieved by different interactions, hydrophilic interactions are most predominant, but could be accompanied by others, such as hydrophobic or electrostatic interactions. Although HILIC are normally used for highly hydrophilic, ionic and polar molecules, such as peptides and monosaccharides, it can also be used for separations of amphipathic molecules like phospholipids (28, 55, 56).

Reverse-phase HPLC is the widely applied version of HPLC, and can be also employed in PL analysis. In the reverse-phase HPLC, the stationary phase is less polar than the mobile phase and the interaction between analyte and the stationary phase has a predominantly hydrophobic character. The most commonly used reverse stationary phase is silica gel covalently modified with octadecyl chains (C18), but other modifications can also be used, like butyl (C4) and pentyl (C5) chains. In this system individual molecular species, of a particular phospholipid class, are separated based on a partition mechanism and will elute based on lipophilicity. Thus glycerophospholipids are distinguished based on their fatty acids, chain length and number of double bonds. Short, polar, unsaturated fatty acids are eluted faster than longer and saturated fatty acids. Reverse-phage HPLC is specially used for separation of oxidized PL species from a specific class, which have an increased polarity caused by the position and number of oxygen atoms in the molecules (24, 25, 49, 54).

HPLC is a useful separation tool for lipid class separations (normal-phase) and for separating the molecular species (reverse-phase). HPLC offers speed, resolution, high sensitivity and specificity and quantification potential. However, is more expensive than TLC and "memory" effects may occur due to remaining contributions of a previous run (24, 25, 49, 50, 54).

In the work developed under this thesis, HLPC-MS experiments were done with the reverse-phase and normal-phase conditions. First, reverse-phase HPLC were employed to purify glycated PE from non-glycated PE and then LC-MS was employed for the separation of glycated PE oxidation products. The purification of glycated PE was achieved in a C4 column (25 cm × 4.6 mm, 7 µm) and a gradient elution with water and acetonitrile acidified (0.1% formic acid) solvents. The separation of oxidation products of glycated PE was achieved in a C5 column (15 cm × 0.5 mm, 5 µm), using the same water and acetonitrile acidified (0.1% formic acid) solvents but a applying a different gradient. Later, HILIC LC-MS was used in liver lipid samples, for separation of phospholipid classes. HILIC LC-MS was carried out in a silica column (15 cm × 1 mm, 3 µm) and a gradient elution formed by water, acetonitrile and methanol solvent system doped with 1mM of ammonium acetate.

3.3. Flow Cytometry Fundamentals

Flow cytometry is a very popular and widely used tool to determine the phenotype and characteristics of cells as they move in a liquid stream through a laser. It measures physical and chemical properties of the cells, given by the light-scattering properties (cell size and internal complexity/granularity) and color-discriminated fluorescence. The underlying principle of flow cytometry is that light is scattered and fluorescence are emitted when light from the excitation source strikes each individual moving particle. There are two distinct types of flow cytometers that can be used to acquire data from particles. One type can perform acquisition of light scattering and fluorescence only. The other type, the cell sorter, is capable of acquiring scattering and fluorescence data, but also has the powerful ability to sort and collect particles (57-61).

A typical flow cytometer consists of three functional units: (1) one or more laser light sources and a sensing system, (2) a hydraulic system, and (3) a computer system. Laser sources are used for fluorescence and light-scatter measurements by producing an intense beam of monochromatic light which in some systems may be tuned to several different wavelengths. The most common lasers used in flow cytometry are argon lasers, which produce light between wavelengths of 351 and 528 nm (particularly 488 nm). The sensing system comprises the sample/flow chamber and the optical assembly. The flow chamber is the instrumental for delivering the cells in suspension to the specific point that is intersected by the illuminating beam and the plane of focus of the optical assembly. The measurements are performed while the cells pass one by one through the detection

point (spot where the cell intercept the stationary laser light), due to a hydrodynamic focusing effect created by the sheath fluid. The light emitted by the cells or scattered from them, in the detection point, is filtered and directed to the detectors by a variety of mirrors and filters, where the electrical signals are measured and the data collected by the computer system (57-61).

Flow cytometers are designed to measure light scattered by a cell at two different and distinct angles, while at the same time measure one or more fluorescence emission signals. As the cell passes through the laser beam, light is scattered in all directions. The light scattered in the forward direction (FSC), at low angles (0.5–10°) from the axis, is proportional to the square of the radius of a sphere and so to the size and surface of the cell or particle. The light that enters the cell is reflected and refracted by the nucleus and other cell contents and is detected at 90° right angle, an orthogonal or side light scatter (SSC). The intensity of orthogonal light scatter is proportional to the granularity of the cell. Light scattering is related to structural and morphological cell features and may be a measure of a combination of parameters: (1) the size (projected surface area) of the particle, (2) the surface topography (rough or smooth), (3) the OD (OD will be influenced by the light absorbed and the refractive index will determine the light refracted through the particle), and (4) the internal structure of the particle (granular or uniform). Light scattering allows the determination of cell size and internal granularity and also the identification of cell viability (57-61).

Fluorescence is a key concept in flow cytometry and is proportional to the amount of fluorochrome present on particles. The fluorescence is conferred by dyes or fluorochrome conjugated monoclonal antibodies (mAbs) specific for molecules either on the surface or in the intracellular components of the cell. Each fluorochrome has a clear, distinct peak in its emission spectrum represented visually by a characteristic color. Particular care should be taken in choosing a fluorochrome capable of being excited by the wavelength of the laser in the instrument. When fluorochrome-labeled particles or cells pass through the laser beam, are excited and emit light with a specific spectral composition and fluorescence occurs. In most instruments, the collection of the fluorescence signal is shared with the orthogonal light collection system. Thus, the existence of optical mirrors and filters is essential to remove the unwanted wavelengths and allow only the desired wavelengths to pass through to the detector. The photons of required wavelengths reach the corresponding detector and are converted in an electrical impulse that is further converted in a numerical signal. Pulse peak, the highest sampled value; pulse area, the sum of all the sampled values; pulse width or other pulse characteristics are reconstructed and measured from individual samples of the impulse using computation system. Immunofluorescence allows the visualization of cellular features or structures by linking them to a fluorescent molecule before the analysis. Flow cytometry allows the use of multiple fluorochromes in a single experiment and then the simultaneous evaluation of different cell features (57-61).

The quantity and intensity of the fluorescence are recorded and combined by the computer system, in a way that individually analyzed particles are interpreted collectively. Data is exhibited

on a visual display unit as a frequency distribution that may be single-parameter, dual-parameter (usually referred to as dot plots), or multiparameter. Single-parameter histograms usually convey information regarding the intensity of fluorescence and number of cells of a given fluorescence, so that weakly fluorescent cells are distinguished from those that are strongly fluorescent. Dual-parameter histograms correlates distribution plots of two parameters, like forward scatter (FSC) versus side scatter (SSC). In these dual-parameter histograms, each cell is represented as a dot and is positioned on the x and y axes according to the intensities detected for that cell. FSC versus SSC plots allow identification of the different cell types within the preparation, based on size and granularity, and selection of populations of interest. Gating is a powerful analytic tool that is available on any type of dual-parameter histograms that allows the isolation of cell subsets. The process consists in the selection of regions of interest, “gates”, where a specified set of scatter properties is set to identify fluorescence signals from a desired group of particles only (57-61).

Flow cytometry rapidly provide quantitative and qualitative measurements from multiple fluorochrome stains (either for intracellular or surface components) along with several intrinsic measurements of particle characteristics. Physical and/or chemical characteristics can be determined, like differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH, enzyme activity, and DNA content. Thus, heterogeneous populations of cells can be visualized in a single dot plot, and morphologically or immunologically different subpopulations can be resolved (57-61).

Flow cytometry is advantageous because allows the analysis of an extremely large number of particles in a very short time (up to thousands of particles per second); makes individual measurements for each particle on the suspension, rather than bulk measurements of an average property for the entire population; and allows simultaneous and multiparametric measurements, capacity to collecting 5–20 parameters on each particle. Flow cytometry analysis is a powerful tool that has many applications in basic research and clinical practice/diagnostics, like blood cells analysis, identification and count of microorganisms (57-61).

In this work, flow cytometry was used for the evaluation of cytokine expression after cell stimulation with glycated and glycoxidized PE. Intracellular staining for specific cytokines requires blocking the protein secretion pathway, cell fixation and permeabilization before the mAbs labeling process.

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CHAPTER III

IDENTIFICATION OF THE OXIDATION PRODUCTS OF GLYCATED PES

INTRODUCTION

Phosphatidylethanolamines glycation is reaction between PE free amino group present at polar head and glucose. PE glycation was found to occur *in vivo* under hyperglycemia conditions and to be associated to oxidative stress. Thus the occurrence of glycated phosphatidylethanolamines could increase oxidative stress modifications and be related to diabetic complications. However, the fate of glycated phosphatidylethanolamines under oxidative stress is unknown and the role of glycated PE in increasing oxidation of other molecules, is not clearly understood.

The work presented in this chapter aimed to study the glycated phosphatidylethanolamines oxidation using a Fenton system and MS analytical technique. First were demonstrated the oxidation products formed from glycated PE analyzed and then the radical intermediaries formed under oxidation conditions were studied with spin trap experiments.

The results demonstrated, for the first time, the oxidation products of glycated phosphatidylethanolamines and allowed the identification of new oxidation sites located at glycated polar head. The structural characterization of glycated PE oxidation products and particularly the oxidation products of glycated polar head contributed to better understand of glycation and oxidation interrelation. Furthermore, glycated polar head oxidation products were found to be formed earlier than the ones that occur at unsaturated acyl chains, thus contributing to the exponential increase of oxidative stress and oxidative modifications that are known to occur under hyperglycemic conditions.

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ORIGINAL PAPER

Oxidation of glycated phosphatidylethanolamines: evidence of oxidation in glycated polar head identified by LC-MS/MS

Cláudia Simões · Vanda Simões · Ana Reis · Pedro Domingues · M. Rosário M. Domingues

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Abstract Phosphatidylethanolamine glycation occurs in diabetic patients and was found to be related with oxidative stress and with diabetic complications. Glycated phosphatidylethanolamines seem to increase oxidation of other molecules; however, the reason why is not understood. In this work, we have studied the oxidation of glycated phosphatidylethanolamines (1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (dPPE)) using a Fenton system. Liquid chromatography–electrospray ionization (ESI)–mass spectrometry and ESI–tandem mass spectrometry in both positive and negative modes were used for detecting and identifying the oxidation products. We were able to identify several oxidation products with oxidation in unsaturated *sn*-2 acyl chain of PLPE, as long- and short-chain products with main oxidation sites on C-7, C-8, C-9, and C-12 carbons. Other products were identified in both glycated PLPE and glycated dPPE, revealing that oxidation also occurs in the glycated polar head. This fact has not been reported before. These products may be generated from oxidation of glycated phosphatidylethanolamines (PE) as Schiff base, leading to short-chain product without the amine moiety, due to cleavage of glycated polar head and long-chain product with two keto groups linked to the glycated polar head or from glycated PE as Amadori product, short-chain products with –NHCHO and –NHCHOHCHO terminal in polar head. Oxidation of glycated phosphatidylethanolamines occurred more quickly than the oxidation of non-glycated phosphatidylethanolamines probably because of the existence of more oxidation sites derived from glycation of polar head group. Monitoring glycated polar head oxidation could be important to evaluate oxidative stress modifications that occur in diabetic patients.

Keywords Phospholipid · Phosphatidylethanolamines · Glycation · Oxidation · Mass spectrometry · LC-MS/MS

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C. Simões · V. Simões · A. Reis · P. Domingues · M. R. M. Domingues (✉)
Mass Spectrometry Centre, Department of Chemistry, University of Aveiro,
3810-193 Aveiro, Portugal
e-mail: mrd@ua.pt

Introduction

Glycation is associated with oxidative stress, mainly due to the formation of oxidation intermediaries like Schiff bases, Amadori rearrangement products, and advanced glycation end products (AGEs) [1, 2]. AGEs also play major roles in inflammation, and pro- and anti-apoptotic signaling in cells [3]. The glycation of amino acids and proteins and its biological consequence have been thoroughly investigated [4], but little attention has been dedicated to the study of phospholipid glycation. However, some studies found glycated phosphatidylethanolamines (PE) in plasma with high glucose concentrations [1, 5]. Glycated PEs were found to be the major LDL lipid glycation products and can be related with atherosclerosis and complications of diabetic patients with associated hyperglycemia [6, 7]. In fact, oxidative stress modifications were found to be increased in the presence of glycated phospholipids [6–8]. Altogether, these facts suggest that more efforts are needed in identifying the structural modifications and evaluating their possible implications on physiopathological processes.

Mass spectrometry (MS) is becoming increasingly important in phospholipid oxidation research. Detection,

identification, and characterization of oxidative modifications in phospholipids using MS and tandem mass spectrometry (MS/MS) has been used mainly for the study of phosphatidylcholine phospholipids [9]. Few papers reporting the oxidation in PE were published, but oxidation of glycated PE were not studied, as reviewed recently by Domingues et al. [9]. Oxidation of non-glycated PE has been reported in three studies: Domingues et al. [10] studied *in vitro* oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-PE (PLPE) by Fenton's reaction and identified several long- and short-chain oxidation products, Gugiu et al. [11] report the *in vitro* oxidation of PE using different oxidation systems (mieloperoxidase, Fenton's reaction, and UV) and report their detection in rat retinas, and Maskrey et al. [12] studied the oxidation of PE with arachidonic acyl chain at the *sn*-2 and found that oxidation products could contribute to signaling in inflammation. Published MS studies on glycated PE have mainly been focused on the study of fragmentation pathways with the purpose of identifying characteristic fragment ions in both positive [5, 6] and negative [6] ion modes. Ravandi et al. [6] studied in-source fragmentation of glycated PE, in both positive and negative ion modes. In positive ion mode, Nakagawa et al. [5] studied the Amadori-glycated PE in human plasma of healthy subjects and diabetic patients using MS and MS/MS. They identified loss of glycated polar head group (−303 Da) as the major fragment ion in positive mode [5, 6] and used neutral loss and multiple reaction monitoring of 303 Da to found glycated PE in diabetic and non-diabetic human plasma [5]. Although the role of glycated PE in oxidative stress seems to be important, the identification and characterization of glycated PE oxidation products have not been performed, and the mechanism through the oxidative modifications that occurs is still not known.

In this work, we characterize the oxidation products of glycated PLPE and glycated 1,2-dipalmitoyl-*sn*-glycero-3-PE (dPPE) by electrospray tandem mass spectrometry. For that, we have synthesized glycated dPPE and glycated PLPE and studied their fragmentation in both positive and negative ion modes, using electrospray ionization method. After that, we studied the oxidation products of glycated dPPE and glycated PLPE formed by the hydroxyl radical under Fenton reaction. Mass spectrometry, in both positive and negative ion modes, was used for identifying the glycated oxidation products, which were further characterized using LC-MS/MS.

Experimental

Chemicals The phosphatidylethanolamines dPPE and PLPE were purchased from Sigma-Aldrich (Madrid, Spain). $FeCl_2$ and H_2O_2 (30%, *v*/*v*) used for the peroxidation reaction were acquired from Merck (Darmstadt, Germany). All solvents used were of HPLC grade.

PE glycation PE were glycated in methanol/water solutions, and different solutions were tested in order to obtain high amounts of glycated PE. Best results were obtained with 1 mg of phospholipid diluted in methanol (500 μL) and 50 mg of glucose dissolved in water (250 μL). Solutions were mixed and left to react in boiling water during 30 min. After that, the phospholipids were extracted from reaction mixture using a modified Fölch method [13]. Briefly, 1 mL of chloroform and 100 μL of water were added to the reaction mixture, followed by centrifugation. The organic phase was collected, the extracts were purified by HPLC in order to isolate the glycated PE; the purity of glycated samples was confirmed by mass spectrometry and stored for further analysis. Briefly, 10 μL of diluted (1:8 (*v*/*v*), in 75% methanol) glycated PE was introduced into a Jones Chromatography C4 column (25 cm×4.6 mm, 7 μm). The mobile phase A consisted of 97.5% water and 2.5% acetonitrile with 0.1% (*v*/*v*) of formic acid. The mobile phase B consisted of acetonitrile with formic acid (0.1%, *v*/*v*). The solvent gradient was programmed as follows: gradient started with 55% of B and held isocratically for 5 min before being increased to 70% of B during 15 min, followed by other linear increase to 100% B over 10 min, and then returned to initial conditions (55% of B) during 10 min.

Preparation of oxidized glycated PE vesicles The phospholipid vesicles were prepared from stock solutions of 1 mg/mL dried under nitrogen stream. Phospholipid vesicles were prepared by adding ammonium bicarbonate buffer (5 mM, pH7.4) to the dried phospholipid [14]. Oxidative reactions were carried out by adding 0.04 mM $FeCl_2$ and 50 mM of hydrogen peroxide to the phospholipid vesicles in order to obtain a final concentration of phospholipids of 3 mM. This mixture was left to react at 37 °C in the dark for different periods of time (3 days for glycated PLPE reactions and 8 days for glycated dPPE reactions) with occasional vortexing. The phospholipid oxidation products were extracted using a modification of the Fölch method [13] with chloroform-methanol (2:1, *v*/*v*). Controls were performed using the same conditions of oxidative reactions, replacing hydrogen peroxide with water. The extent of oxidation was monitored by electrospray mass spectrometry (ESI-MS) and liquid chromatography electrospray mass spectrometry (LC-MS).

LC-MS conditions The phospholipid oxidation products were separated by LC performed on a HPLC system (Waters Alliance 2690) coupled to a Q-ToF 2 instrument (Micromass, Manchester, UK). Ten microliters of diluted

(1:8 (*v*/*v*), in 75% methanol) reaction mixture was introduced into a Supelco Bio Wide Pore C5 column (15 cm× 0.5 mm, 5 μm). The mobile phase A consisted of 97.5% water and 2.5% acetonitrile with 0.1% (*v*/*v*) of formic acid. The mobile phase B consisted of acetonitrile with formic acid (0.1%, *v*/*v*). The solvent gradient was programmed as follows: gradient started with 20% of B and increased to 70% of B during 15 min, followed by other linear increase to 100% B over 10 min, and held isocratically for 10 min. The flow rate through the column was 12 μL min^{-1}, obtained using a pre-column split (Acurate, LC Packings, USA). Mass spectrometry conditions were the same as the MS studies.

Q-TOF 2 conditions for MS studies The studies were carried out in the positive and negative modes on Q-TOF2 instrument (Micromass, Manchester, UK). Samples were introduced into the electrospray source at a flow rate of 10 μL min^{-1}. The cone voltage was set at 30 V and capillary voltage at 3 kV. Source temperature was at 80 °C and desolvation temperature at 150 °C. The resolution was set to about 9,000 (FWHM). MS/MS were acquired by collision-induced decomposition (CID), using argon as the collision gas (measured pressure in the penning gauge ~6× 10^{-5} mbar). The collision energy used was 25 to 30 eV. Data acquisition was carried out with a MassLynx 4.0 data system.

Results and discussion

Initially, we studied the electrospray tandem mass spectrometry fragmentation pathways of the non-oxidized glycated phosphatidylethanolamines in both positive and negative ion modes. Fragmentation of the glycated phosphatidylethanolamines using in-source fragmentation in the negative and positive ion modes was reported by Ravandi et al. [6]. Nakagawa et al. [5] also reported the fragmentation in positive ion mode using CID, using a QTrap instrument. The major fragment ion that was observed was formed by the neutral loss of 303 Da, corresponding to loss of the glycated polar head group. In the tandem mass spectra of glycated PLPE and glycated dPPE $[M+H]^+$ ions, we also observed a major ion resultant from the neutral loss of the glycated polar head. This can be seen in the MS/MS spectrum of glycated PLPE ($[M+H]^+$ ion), represented in Fig. 1. We were also able to identify other characteristic fragments, not mentioned before, like the ion at *m*/*z*304.1, which corresponds to glycated polar head and ion at *m*/*z* 716.5, formed by loss of the glucose moiety (−162 Da). Furthermore, we also observed the neutral loss of 84 Da (loss of three water molecules and H_2CO) and one, two, and three water molecules from the precursor ion and from the ion at *m*/*z*304. These fragmentation pathways were reported before in glycated amino acids [15] and are characteristic for the presence of a glucose moiety. In the negative ion mode, the fragmentation of the $[M-H]^-$ ion of PLPE (Fig. 2) shows three major fragment ions, two of them correspond to the fatty acid anions (R_1COO^- and R_2COO^-) and the other at *m*/*z*714.6, formed by loss of glucose moiety (−162 Da). These ions were also found in in-source fragmentation by Ravandi et al. [6]. We have also found other minor fragment ions: ions formed by glucose fragmentation including the loss of 90 Da ($C_3H_6O_3$), 120 Da ($C_4H_8O_4$), and 150 Da ($C_5H_{10}O_5$); ions formed by loss of the fatty acyl chain and the ion formed by loss of 205 Da, which corresponds to the loss of aziridine with the glucose moiety (43+162 Da).

Subsequently, the glycated PLPE was oxidized by Fenton reaction, as described in the "Experimental" section.

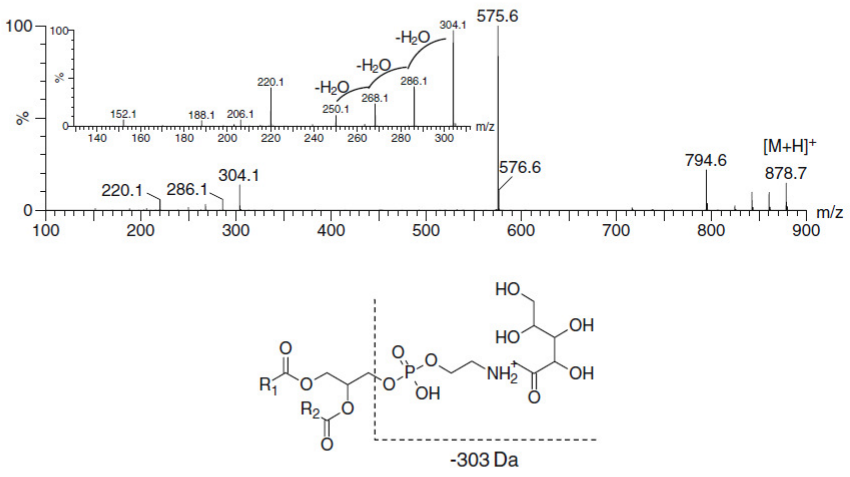


Fig. 1 ESI-MS/MS spectra of $[M+H]^+$ ions of glycated PLPE (*m*/*z*878.6) and the schematic representation of glycated PE

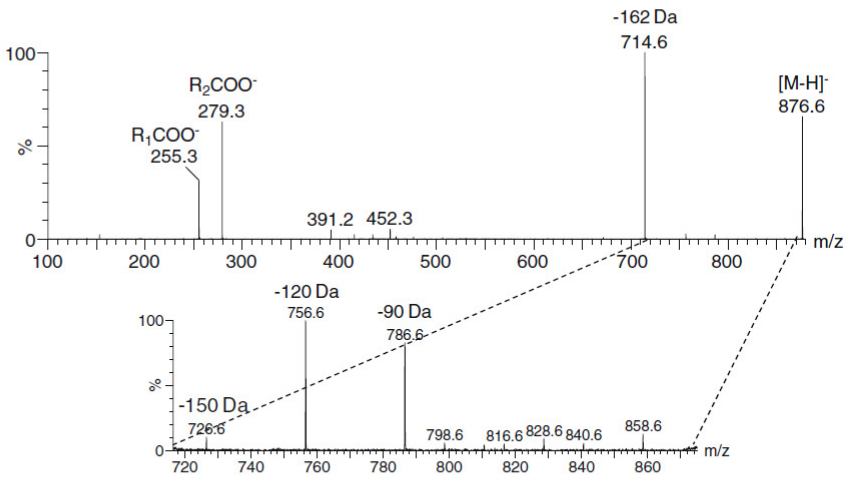


Fig. 2 ESI-MS/MS spectra of $[M–H]^-$ ions of glycated PLPE (m/z876.6)

The oxidized mixtures were first analyzed in the positive ion mode using direct injection and also by LC-MS and LC-MS/MS, and the results obtained were compared with the results previously reported during PLPE oxidation [10] (Fig. S1, see Electronic supplementary material). Different classes of oxidation products can be found in Table 1:

1. Short-chain oxidation products without the glucose moiety, formed by cleavage of unsaturated *sn*-2 acyl chain at m/z610.4 ($R_2'C_8OOH$), 624.4 ($R_2'C_9OOH$), and 596.4 ($R_2'C_7OOH$) (R_2' means oxidized *sn*-2 acyl chain, C_n identifies the chain length, and =O and OOH represent the aldehyde or the carboxylic functions).
2. Short-chain oxidation products with the glucose moiety, at m/z756.4 ($R_2'C_8$=O), 758.4 ($R_2'C_7OOH$), 770.4 ($R_2'C_9$=O), 772.4 ($R_2'C_8OOH$), and 786.4 ($R_2'C_9OOH$) (Figs. S2 and S3, see Electronic supplementary material). Among them, the most abundant oxidation product is at m/z786.4, which corresponds to the glycated derivative of the ion at m/z624.4 ($R_2'C_9OOH$). This specie ($R_2'C_9OOH$) is the most abundant oxidation product in non-glycated PLPE oxidation [10].
3. Long-chain product ions without the glucose moiety at m/z732.5 (716.5+16 Da), 746.5 (716.5+30 Da), and 764.5 (716.5+48 Da), previously observed in the oxidation of non-glycated PLPE, as was confirmed by LC-MS/MS [10].
4. Long-chain products with the glucose moiety at m/z892.5, 894.5, 908.5, 910.5, 926.5, and 928.5 corresponding to insertion of one to three oxygen atoms (Fig. S4, see Electronic supplementary material). The tandem mass

Table 1 Resume of main oxidation products of glycated PLPE observed in positive mode and their main fragment ions observed in MS/MS spectra

	Oxidation products without glucose			Oxidation products with glucose		
	$[M+H]^+$ (m/z)	$[M+H—141Da]^+$	$R_2C{=}O^+$	$[M+H]^+$	$[M+H—303Da]^+$	$R_2C{=}O^+$
Short-chain products				756.4	453.4	141.1 $R_2'C_8$=O
	596.4	455.4	143.1 $R_2'C_7OOH$	758.4	455.4	143.1 $R_2'C_7OOH$
				770.4	467.4	155.1 $R_2'C_9$=O
	610.4	469.4	157.1 $R_2'C_8OOH$	772.4	469.4	157.1 $R_2'C_8OOH$
	624.4	483.4	171.1 $R_2'C_9OOH$	786.4	483.4	171.1 $R_2'C_9OOH$
Long-chain products				892.5	589.5	277.2
	732.5	591.5	279.2	894.5	591.5	279.2
	746.5	605.5	293.2	908.5	605.5	293.2
				910.5	607.5	295.2
	764.5	623.5	311.2	926.5	623.5	311.2
				928.5	625.5	313.2

spectra of these ions show characteristic losses of glycated PE and fragmentation pathways typical of oxidation at *sn*-2 acyl chain, indicating oxidation at C-9 and C-12, like non-glycated PLPE reported before [10].

Interestingly, the oxidation product at *m*/*z*892.5 (glycated PLPE+14 Da) is formed by insertion of one oxygen atom with formation of one double bound. This product ion appears after the first day of oxidation, but the relative abundance then gradually decreases, disappearing at the third day (Fig. S5, see Electronic supplementary material). The reconstructed ion chromatogram shows a major isomer at 24.3 min and a minor isomer at 27 min (Fig. 3). The isomer eluting at 24.3 min is an oxidation product modified in the *sn-2* acyl chain (Fig. 3) confirmed in MS/MS spectrum due to a major product ion at *m*/*z*589.5 (loss of 303 Da) and the fragmentation pattern of glucose moiety (neutral loss of one to three water molecules and 84 Da). Minor ions at *m*/*z*467.4 and 507.4 indicate that the oxidation occurred at C-9 and C-12, respectively. The fragment ion at 507.4 is more abundant relatively to fragment ion at 467.4, which indicate that most probably C-12 is the first target to $OH^{\cdot}$ oxidation. However, the MS/MS spectrum (Fig. 3) of the isomer that eluted at 27 min show a major fragment ion at *m*/*z*575.5 due to the loss of oxidized glycated polar head (303+14 Da). Moreover, we also observe the fragment ion at *m*/*z*318.1 corresponding to the oxidized glycated polar head and the fragmentation pattern observed in the glycated PEs that allowed the assignment of the glucose moieties, namely ion due to neutral loss one, two, and three H_2O molecules, and of 84 Da (loss of three water molecules and H_2CO) is absent in this spectrum. In conclusion, we have detected oxidation in glycated polar head but only for the oxidation product formed by insertion of one oxygen molecule with formation of one double bound.

For deeper evaluation of oxidation on glycated polar head group, we studied the oxidation of glycated dPPE under the same conditions as for glycated PLPE. dPPE phospholipid was chosen because the absence of unsaturated fatty acyl chains avoids the oxidation of the fatty acyl chains [16, 17]. During the oxidation of glycated dPPE (data not shown), we did not find any oxidation product when analyzing by MS in the positive ion mode, up to 2 weeks of oxidation at 37 °C. The hypothesis to be tested was that oxidation occurs in the glycated polar head with formation of carboxylic and carbonylic groups. Due to possible loss of the nitrogen atom as result of oxidation, detecting the glycated polar head oxidation would be very difficult when analyzing the oxidation products in MS positive ion mode. Furthermore, the deprotonation of the phosphate group enables the detection in negative ion mode, particularly of species containing carbonyl groups. Given that, we analyze the glycated dPPE reaction mixtures in the negative ion mode, expecting that the glycated polar

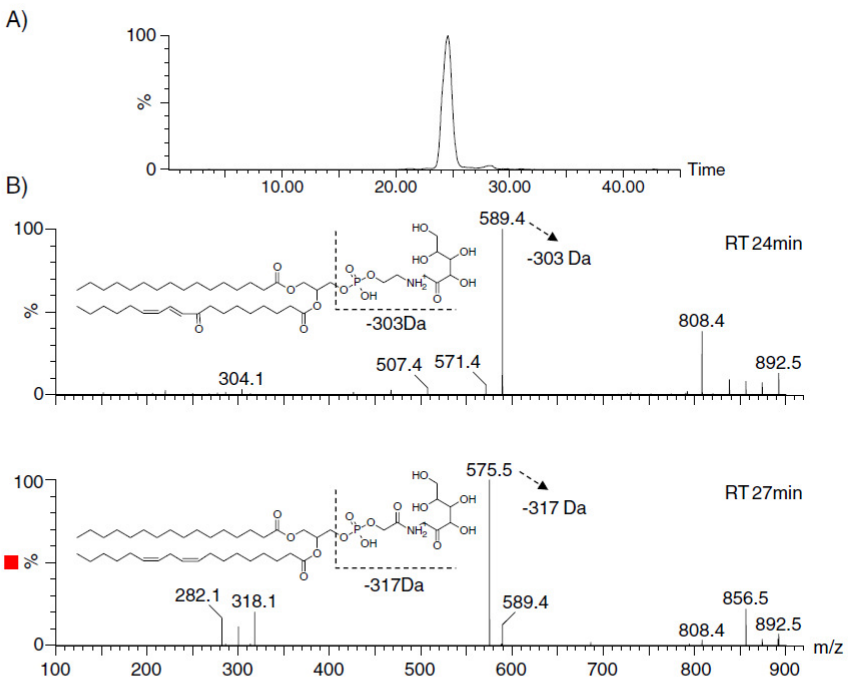


Fig. 3 **a** Reconstructed ion current chromatograms of $[M+H]^+$ ions of long-chain glycated PLPE oxidation product at *m*/*z*892.5 after 1 day of incubation and LC-MS/MS spectra (**b**) for the isomers that eluted at 24 min (B_1) and 27 min (B_2)

head oxidation products would be more easily detected. The analysis on the MS negative ion mode show species that were not observed in the positive ion mode, namely the $[M-H]^-$ ions at *m/z* 705.5, 718.5, 748.5, and 880.6. The most abundant oxidation product found was the ion at *m/z* 718.5, followed by ions at *m/z* 705.5, 748.5, and 880.6, successively. Analysis by LC-MS in negative mode revealed that each oxidation product elute in only one peak, thus allowing to infer that they correspond to only one product each (Fig. S6, see Electronic supplementary material). These species have been analyzed by tandem mass spectrometry, and the results suggesting that glycated PE could suffer oxidation in the polar head group are presented below:

- Unlike the other ions, the oxidation product ion at *m/z* 705.5 has odd *m/z* value, showing that during their oxidation, this product loses the amino group. Tandem mass spectrum of this ion shows the loss of 58 Da, which corresponds to loss of CH_2CO_2 group and also the loss of the fatty acyl chains, combined or not with the loss of CH_2CO_2 (Fig. 4). The product ions at *m/z* 137.0 ($C_3H_6O_4P$) and 153.0 ($C_3H_6O_5P$) correspond to the phosphate group linked to glycerol. And the ions at *m/z* 255.2 ($RCOO^-$), 391.2 ($[M-H-58-RCOOH]^-$), 409.2 ($[M-H-58-R{=}C{=}O]^-$), and 647.4 ($C_{35}H_{68}O_8P$) formed by cleavage of the bond between phosphate group and the modified polar head confirm that the fatty acyl chains did not suffer oxidation. Interestingly, all the other oxidation products indentified for the glycated dPPE show in their MS/MS spectra this ion at *m/z* 647.4, as well as the ions at *m/z* 255.2, 391.2, and 409.2, confirming that all are oxidation products formed due to oxidation in glycated polar head. The ions at *m/z* 718.5 and 748.5 also have lower *m/z* than the glycated dPPE, which indicates that oxidation reaction induces cleavage of the glycated dPPE.
- The MS/MS spectrum of the ion at *m/z* 718.5 (Fig. 5) shows the intact $RCOO^-$ product ion (*m/z* 255.2) and the ions formed by loss fatty acyl chains, which leads to the conclusion that oxidation did not involve the fatty acyl chains. Minor fragment ions, observed at *m/z* 224.0 ($C_6H_{11}NO_6P$) and 168.0 ($C_3H_7NO_5P$), correspond to modified polar head group, suggesting that this oxidation product is a –NHCHO derivative; the structure of these ions were confirmed by exact mass measurements that allow to identify their molecular formula with an error of −1.8 ppm for the ion at *m/z* 224.0 and an error of −5.9 ppm for the ion at *m/z* 168.0.
- The MS/MS spectrum of the ion at *m/z* 748.5 is shown in Fig. 6. We observe fragment ions that were attributed to non-oxidized $RCOO^-$ and $[M-H-RCOOH]^-$, confirming that the oxidation occurred in the glycated polar head. This ion is proposed to be a –NHCHOHCHO derivative since we observe losses of 30 Da ($H_2C{=}O$) group and 58 Da (CHOHCHO).
- The ion at *m/z* 880.6 (Fig. 7) has higher *m/z* than glycated dPPE, so the glycated polar head is oxidized without cleavage. The increase of 28 Da in relation to the non-oxidized glycated dPPE shows that this oxidation product has two keto groups. These two keto groups are proposed to be located at ethanolamine carbons because fragmentation pattern of glucose (losses of 60, 90, 120, and 162 Da) is observed and also because it is observed that there is a loss of glycated ethanolamine moiety plus 28 Da, combined or not with the loss of RCOOH.

After identifying oxidation of glycated dPPE, we studied the oxidation of glycated PLPE, in negative ion mode, trying to find a possible oxidation in the glycated polar head group. The negative ESI-MS spectra of glycated PLPE reactions after 3 days' incubation under non-oxidative and oxidative

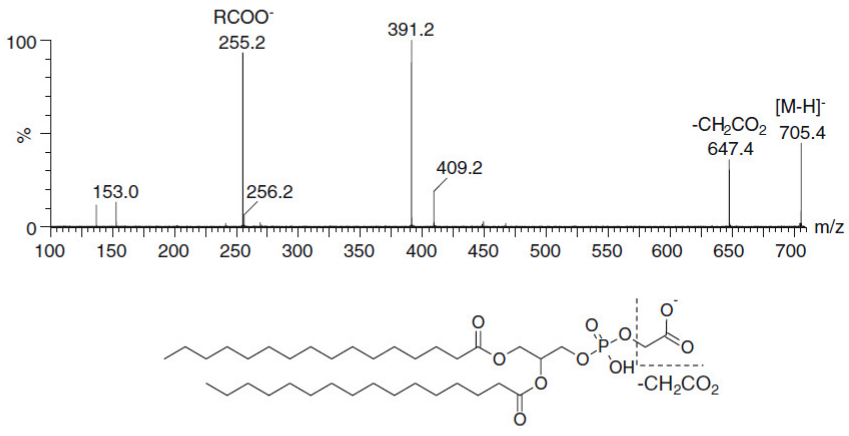


Fig. 4 Tandem mass spectrum and the schematic representation of $[M-H]^-$ ion at *m/z* 705.5, which is a glycated dPPE oxidation product

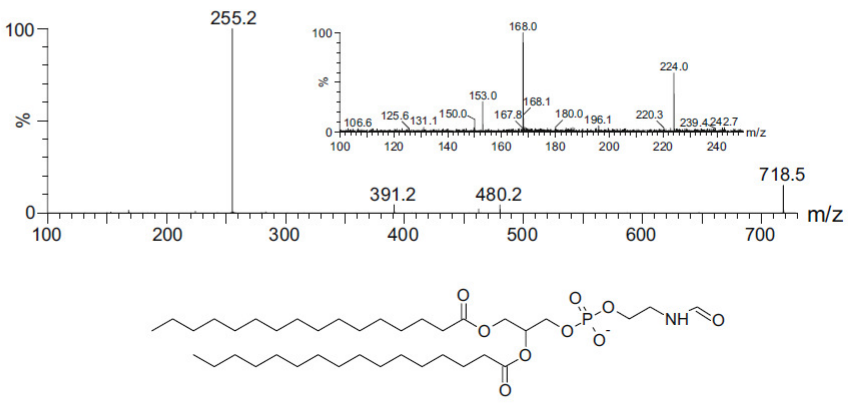


Fig. 5 Tandem mass spectrum and the schematic representation of $[M–H]^-$ ion at *m/z* 718.5, which is a glycated dPPE oxidation product

conditions, as described in the "Experimental" section, are plotted in Fig. S6 (see Electronic supplementary material). In the spectra, we found oxidation products already seen in the positive ion mode, but now negatively charged as $[M–H]^-$: (1) short-chain oxidation products without the glucose moiety (*m/z* 608.4, 622.4, and 594.4), (2) short-chain oxidation products with the glucose moiety (*m/z* 754.4, 756.4, 768.4, 770.4, and 784.4), (3) long-chain product ions without the glucose moiety (*m/z* 730.4, 744.4, 762.4, and 764.4), and (4) long-chain products with the glucose moiety (*m/z* 892.6, 906.6, 908.6, 924.7, and 926.7). Tandem mass spectrometry of the oxidation products has confirmed the identification previously made in positive ESI-MS/MS:

- Briefly, oxidation product at *m/z* 622.4 ($R_2'C_9OOH$) originated fragment ions 187.1 and 255.2 that correspond to *sn*-2 shortened fatty acid chain and *sn*-1 non-oxidized fatty acid chain, respectively (Fig. S7, see Electronic supplementary material). Other minor fragment ions were formed by loss of 141 Da and/or loss of the fatty acyl chains.
- Fragmentation of the long-chain oxidation products without glucose moiety (as an example, the spectrum of oxidation product at *m/z* 730.4 is shown in Fig. S7, see Electronic supplementary material) is similar to the fragmentation of short-chain oxidation products without glucose moiety. This spectrum shows major fragment ions of the *sn*-1 non-oxidized fatty acid chain and *sn*-2 oxidized fatty acid chain, and other minor fragment ions resulting from loss of fatty acyl chains with or without loss of polar head (141 Da). In long-chain oxidation products, the fragmentation of the *sn*-2 oxidized fatty acid reveals the site of oxidation. The most frequent oxidation sites were the same as previously described for the positive ion mode: C-13, C-12, and C-9.
- The tandem mass spectra of the short-chain oxidation products with the glucose moiety, at *m/z* 754.4 ($R_2'C_8$=O), 756.4 ($R_2'C_7OOH$), 758.4 ($R_2'C_9$=O), 770.4 ($R_2'C_8OOH$), and 784.4 ($R_2'C_9OOH$), show losses of 162, 120, and 90 Da, due to the glucose moiety. After losing the glucose moiety, the fragmentation pathway is similar to the correspondent fragment ions without

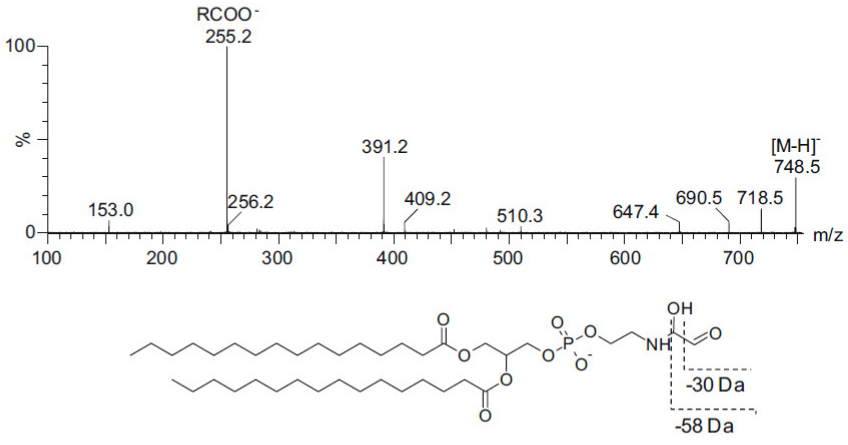


Fig. 6 Tandem mass spectrum and the schematic representation of $[M–H]^-$ ion at *m/z* 748.5, which is a glycated dPPE oxidation product

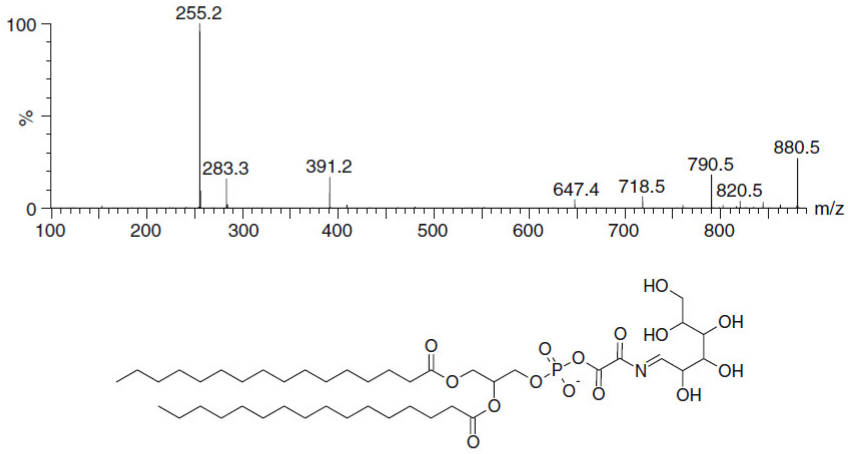


Fig. 7 Tandem mass spectrum and the schematic representation of $[M–H]^-$ ion at *m/z* 880.5, which is a glycated dPPE oxidation product

the glucose moiety at *m/z* 592.4, 594.4, 606.4, 608.4, and 622.4, respectively. The major observed fragment ions are the *sn*-2 oxidized fatty acid chain ($R_2'COO^-$) and *sn*-1 non-oxidized fatty acid chain (R_1COO^-), but fragment ions resulting from losses of fatty acyl chains with or without the loss of polar head are also observed (Fig. S7, see Electronic supplementary material).

– Long-chain product at *m/z* 890.5 (876.5+14 Da) was not seen in the negative ion mode, probably because it is easily degraded in other products, as mentioned before. The observed long-chain oxidation products with glucose moiety were at *m/z* 892.6 (glycated PLPE+16 Da), 906.6 (glycated PLPE+30 Da), 908.6 (glycated PLPE+32 Da), 924.7 (glycated PLPE+48 Da), and 926.7 (glycated PLPE+50 Da). The tandem mass spectra of these species show fragmentation due to loss of 162, 120, and 90 Da, confirming the glycated nature and that oxidation occurs at the *sn*-2 fatty acyl chain. The spectrum of the oxidation product at *m/z* 892.6 (Fig. S7, see Electronic supplementary material) shows loss of 18 Da from the $R_2'COO^-$ fragment ion, and fragmentation at C-9 of the fragment ion at $R_2'COO^-$. The oxidation product at *m/z* 906.6 is formed by insertion of two oxygen molecules, with formation of one double bond. Tandem mass spectrum shows loss of one water molecule from the $R_2'COO^-$ fragment ion and fragmentation at C-9 and C-12, thus identifying the sites of oxidation. The tandem mass spectrum of the oxidation product at *m/z* 908.6 reveals that *sn*-2 acyl chain is oxidized at C-9 and C-12, similar to other oxidation products. Oxidation products at *m/z* 924.7 and 926.7 were formed by insertion of three oxygen molecules, and the results are similar to the ones obtained for the other ions.

To investigate the oxidation in glycated polar head group, we looked, in the mass spectrum of the oxidized glycated PLPE, for the correspondent ions (*m/z* 729.5, 742.5, 772.5, and 904.6) found on glycated dPPE. However, among these oxidation products, we only found the ions at *m/z* 742.5 (–NHCHO derivative) and 772.5 (–NHCHOHCHO derivative). Since we found much more oxidation products from glycated PLPE than from glycated dPPE, it is possible that the other oxidation products (*m/z* 729.5 and 904.6) from glycated polar head were suppressed by the other ions, and so we were not able to detect them or they could be additionally oxidized in the *sn*-2 acyl chain.

The ion formed by oxidation of glycated polar head group at *m/z* 742.5 corresponds to the ion at *m/z* 718.5 (–NHCHO derivative) found in dPPE glycated oxidation. The tandem mass spectrum shows the fragment ions at *m/z* 224.0 and 168.0, from the oxidation of glycated polar head, and at *m/z* 255.2 and 279.2, corresponding to the *sn*-1 and *sn*-2 non-oxidized fatty acid chains, respectively. The observation of these fragment ions confirms that this oxidation product originated from glycated PLPE and also that oxidation occurs at the glycated polar head.

In the tandem mass spectra of the oxidation product at *m/z* 772.5 (attributed to –NHCHOHCHO derivative), we expected to observe, similarly for what was observed on glycated dPPE, the losses of 30 and 58 Da and also the non-oxidized *sn*-2 and *sn*-1 fragment ions. Among them, we only found the non-oxidized *sn*-2 and *sn*-1 fragment ions at *m/z* 255.2 and 279.2. Instead, we observed the fragment ion at *m/z* 309.2, which corresponds to $R_2'COO^-$ plus 30 Da, and the fragment ions at *m/z* 224.0 and 168.0, as observed for the product ion at *m/z* 718.5. This can be interpreted as the presence of isomers: The oxidation product at *m/z* 772.5 has an isomer that corresponds to –NHCHOHCHO derivative and another isomer that corresponds to the oxidation product 742.5 (–NHCHO derivative) with an increment of 30 Da due

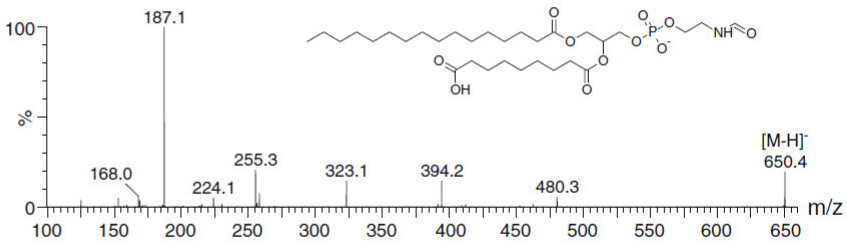
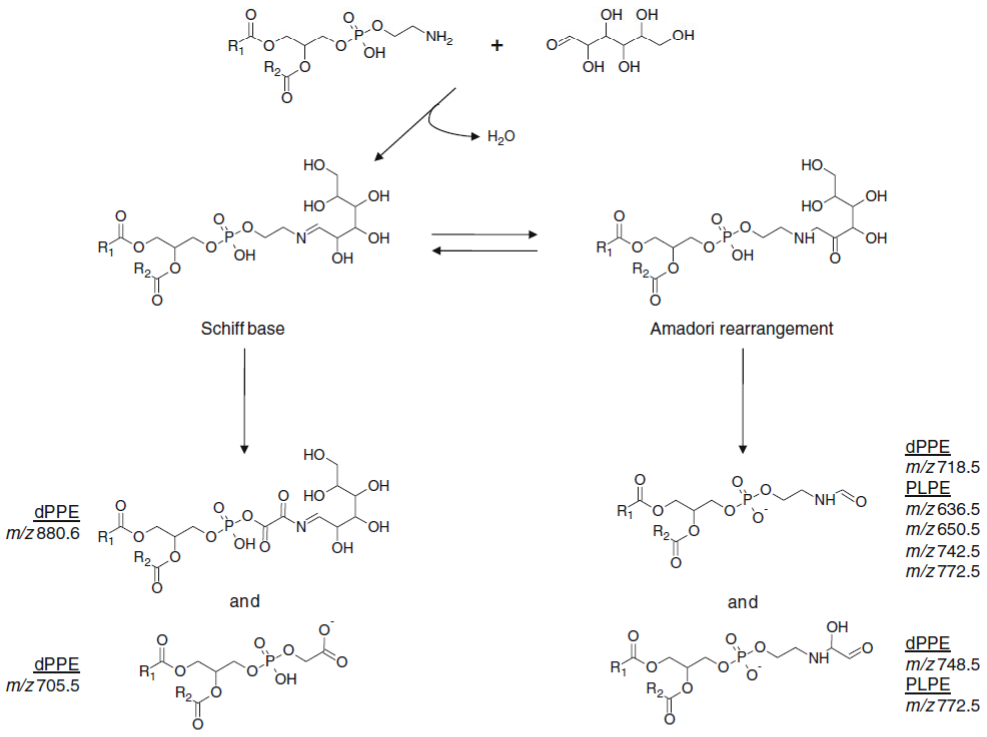


Fig. 8 MS/MS spectra of $[M–H]^-$ ion at *m/z*650.4, oxidation product from glycated PLPE

to insertion of two oxygen molecules and formation of one double bound in the *sn*-2 acyl chain.

In the mass spectrum of the oxidized glycated PLPE, we can see other oxidation products at *m/z*636.4 and 650.4. The tandem mass spectra of these ions show the product ions at *m/z*224.0 and 168.0. The analysis of these spectra allows to identify the oxidation products at *m/z*636.4 and 650.4 as –NHCHO derivatives that were oxidized in polar head group with shortened *sn*-2 acyl chain. The oxidation product at *m/z*636.4 has a *sn*-2 C8 dicarboxylic acid, as confirmed by $R_2'COO^-$ fragment ion at *m/z*173.1. The oxidation product at *m/z*650.4 (Fig. 8) has a *sn*-2 C9 dicarboxylic acid, as confirmed by $R_2'COO^-$ fragment ion at *m/z*187.1. This confirms that –NHCHO derivatives are the most stable and abundant oxidation products that show oxidation of phosphatidylethanolamines in the glycated polar head group.

Formation of oxidation products due to cleavage of glycated polar head resembles oxidation of peptides backbone with cleavage of peptidic chain with formation of short-chain product, as reviewed elsewhere [15, 18]. Considering the glycated PE as an Amadori product, there is a carbon atom between a nitrogen atom and a carbonyl moiety, such as the alpha-carbon of amino acids in peptides. It is known that during the oxidation induced by the hydroxyl radical, abstraction of hydrogen from the alpha-carbon is a common reaction leading to formation of alkoxyl radicals that can undergo further cleavage. Oxidation in this carbon leads to a radical that is stabilized by the neighboring electron-rich heteroatom (N atom) through



Scheme 1 Resume of the oxidation products formed from oxidation of glycated polar head

electron delocalization [18]. This mechanism justifies the formation of short-chain oxidation products in glycated polar head at *m/z* 718.5 and 748.5 for dPPE and at *m/z* 742.5, 772.5, 636.4, and 650.4 for PLPE (Scheme 1). The short-chain products formed with terminal carboxylic function at *m/z* 705.5 and the long-chain product at *m/z* 880.6 may be explained considering the Schiff glycated PE. In this case, oxidation occurs by abstraction of the hydrogen from the carbon vicinal to the imine bond, forming alkoxyl and/or peroxyl radicals. These radicals may undergo backbone cleavage (*m/z* 705.5) or forming carbonyl derivatives (*m/z* 880.6) [18]. Scheme 1 represents these two major mechanisms of cleavage.

It is important to notice that all reaction mixtures containing glycated PE oxidized more rapidly than the reaction mixtures that do not contain glycated products. Moreover, non-glycated PE oxidized more rapidly in the presence of glycated PE. This effect of glycated PE in the oxidation reaction was reported before [6–8] and can be explained with our assumption that glycated PE have additional oxidation targets in the glycated polar head group. Consequently, radical generation could be enhanced, the lipid peroxidation chain reaction could be accelerated, and the oxidation products would be formed more rapidly. The short-chain products formed during oxidation in glycated polar head could be valuable markers for monitoring diabetic patients and oxidative stress.

Conclusion

In this work, we identified oxidation products of glycated PE by ESI-MS/MS in both positive and negative ion modes. In the positive mode, oxidation products identified were similar to the products previously found in non-glycated PLPE. We found long-chain oxidation products, keto, hydroxy and peroxy derivatives, and short-chain products with terminal aldehyde and carboxylic moieties. The main targets of oxidation were primarily at C-9 but also at C-7, C-8, and C-12. Interestingly, we found both oxidation products with glucose moiety and without glucose moiety. When characterizing the oxidation products in the negative mode analysis, we found almost the same PLPE oxidation products that were found in the positive mode. Additionally, we were able to identify new oxidation products of glycated PE, formed by initial hydrogen abstraction in glycated polar head. Oxidation in glycated polar head was confirmed by studying the oxidation of glycated dPPE. Glycated polar head oxidation products comprise both short- and long-chain products. Short-chain products have lower *m/z* values than non-oxidized glycated PE and are formed by cleavage in glycated polar head and, in some cases, combined with oxidation and cleavage of unsaturated fatty acyl chains. Oxidation products with higher *m/z* values were observed particularly when the phospholipid had saturated fatty acyl chains. This leads us to conclude that glycated PEs have one more site for oxidation, located in polar head group, which could be responsible for the increase of oxidative stress modifications particularly important in hyperglycemic patients.

Acknowledgments The authors thank the financial support provided to Cláudia Simões (PhD grant, SFRH/BD/46293/2008), project PTDC/QUI-BIOQ/104968/2008, QOPNA, and RNEM by the Foundation for Science and Technology (FCT).

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Oxidation of glycated phosphatidylethanolamine's: evidence of oxidation in glycated polar head identified by LC-MS/MS

Cláudia Simões, Vanda Simões, Ana Reis, Pedro Domingues, M. Rosário M. Domingues

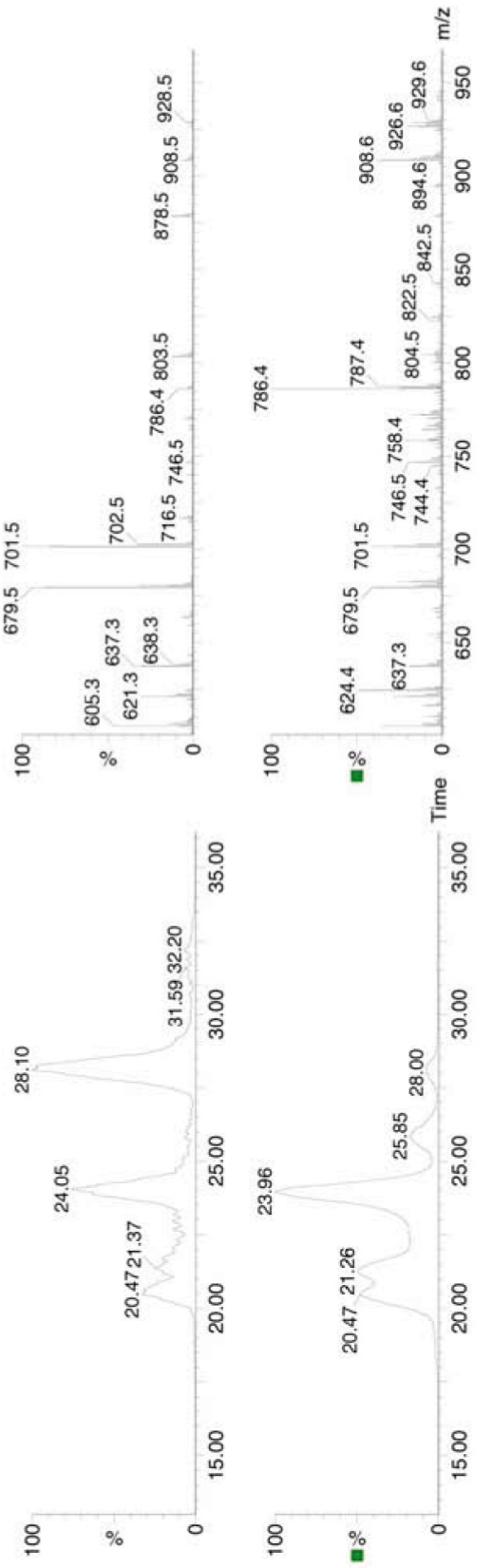

Figure S1 – LC-MS spectra (on the left) and ESI-MS (on the right), in positive ion mode, of glycated PLPE under non-oxidative conditions (in the top) and under oxidative conditions (in the bottom) after 3 days of incubation.

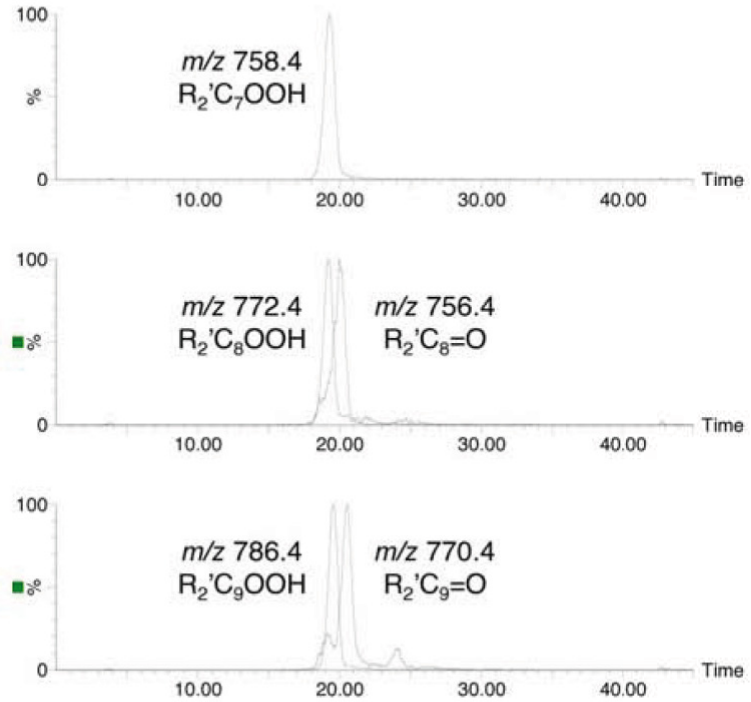

Figure S2 – Reconstructed Ion Current (RIC) chromatograms of $[M+H]^+$ ions of short chain glycated PLPE oxidation products at m/z 756.4, 758.4, 770.4, 772.4 and 786.4.

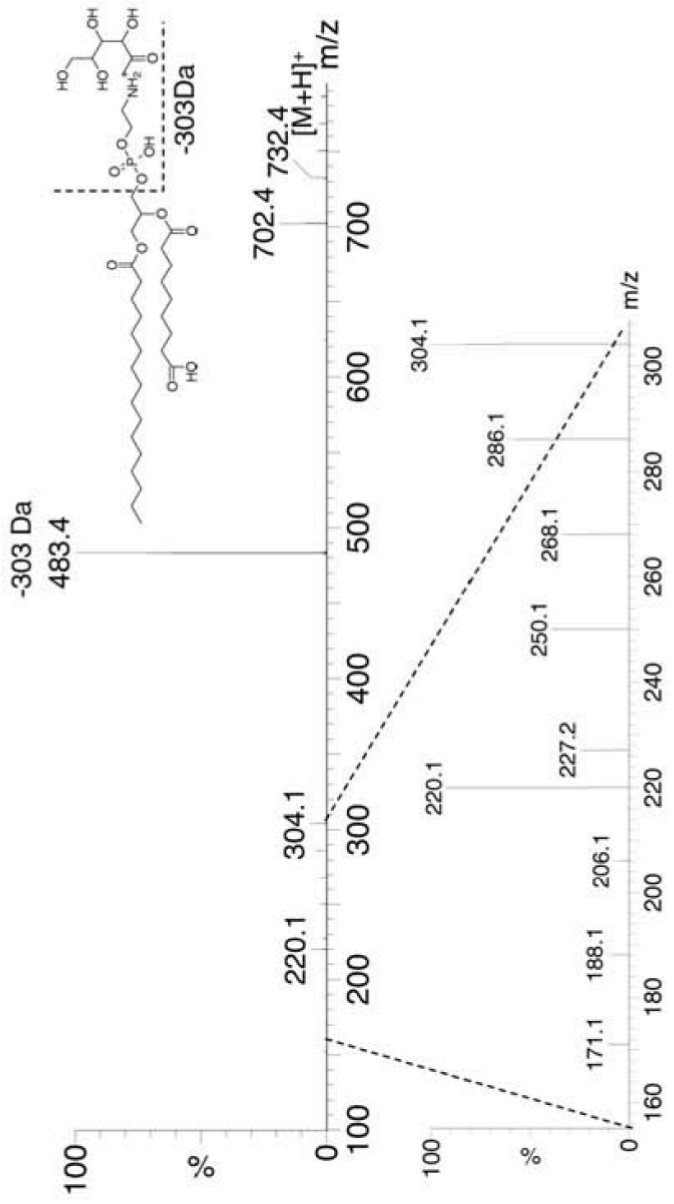

Figure S3 – LC-MS/MS spectra of $[M+H]^+$ ions of glycated PLPE short chain product at m/z 786.4 that eluted at 19 minutes.

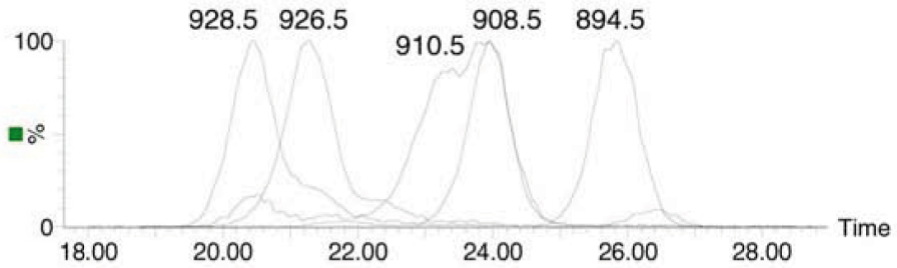

Figure S4 – Reconstructed Ion Current (RIC) chromatograms of $[M+H]^+$ ions of long chain glycated PLPE oxidation products at m/z 894.5, 908.5, 910.5, 928.5 and 926.5. This chromatogram shows that the oxidation product at m/z 910.5 has a peak which contains the isotopic contribution of the ion at m/z 908.5 due to co-elution of both compounds.

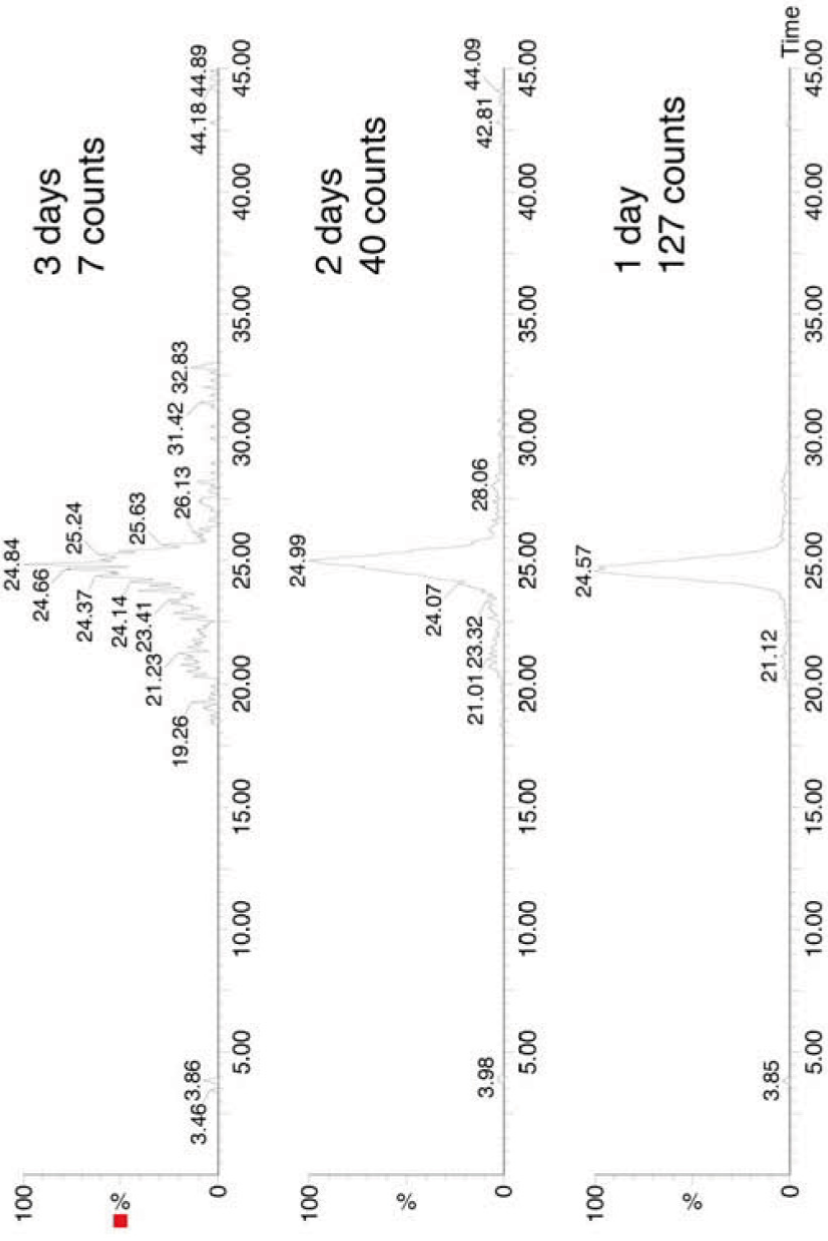

Figure S5 – Reconstructed Ion Current (RIC) chromatograms of $[M+H]^+$ ion of long chain glycated PLPE oxidation product at *m/z* 892.5, after 1, 2 and 3 days of oxidation.

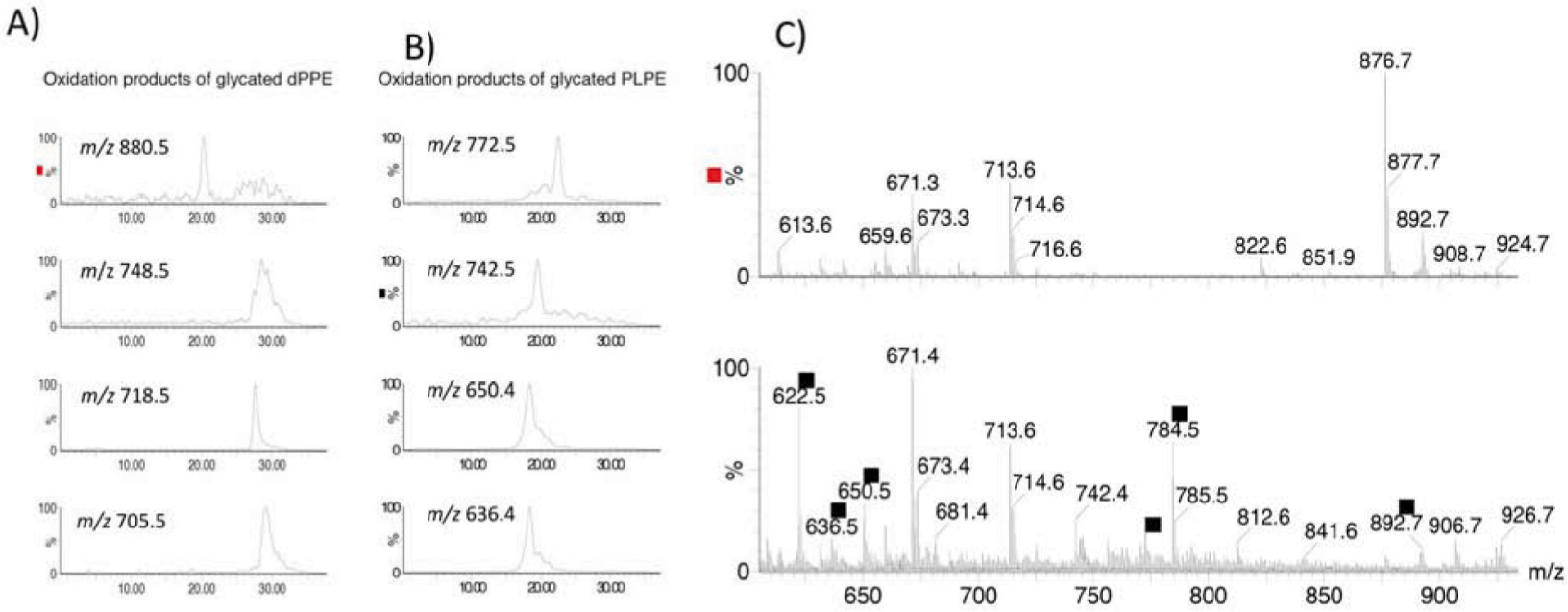

Figure S6 – Reconstructed ions chromatograms of the $[M+H]^+$ ions of the oxidation products formed by oxidation on the polar head of glycated dPPE(A) and glycated PLPE(B) and (C) ESI-MS in negative ion mode, of glycated PLPE under non-oxidative conditions (in the top) and under oxidative conditions (in the botton) after 3 days of incubation. ▪ indicates some oxidation products found.

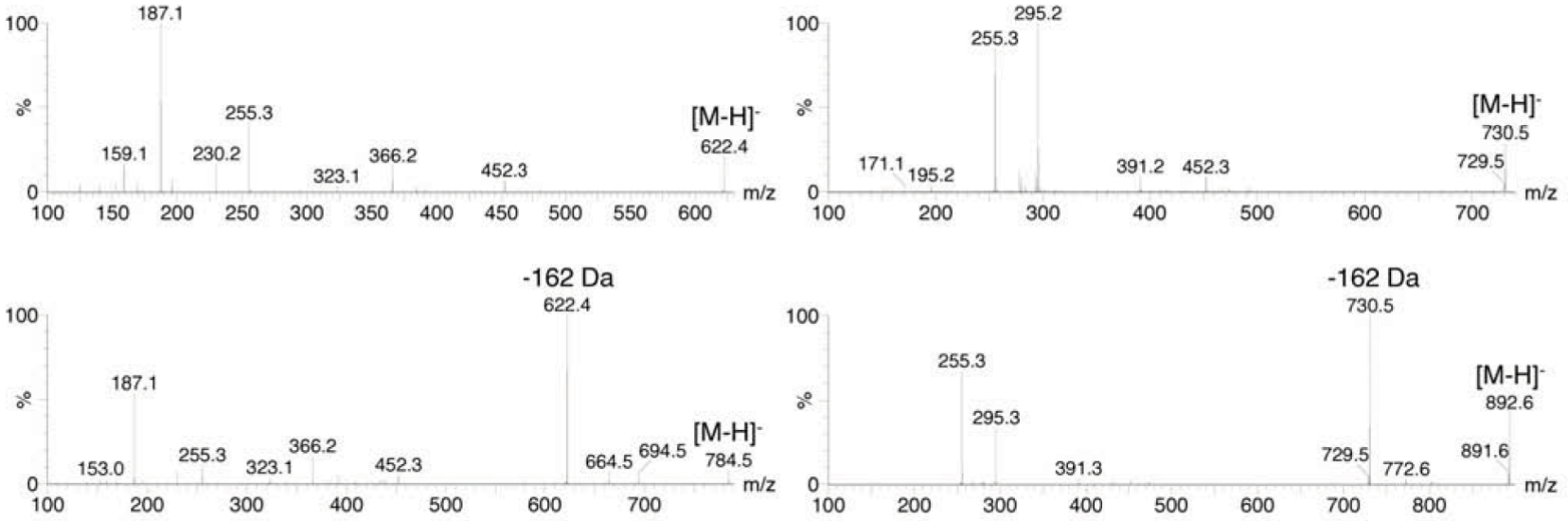

Figure S7 – MS/MS spectra of $[M-H]^-$ ions at *m/z* 622.4, 730.4, 784.4 and 892.6, oxidation products from glycated PLPE oxidation in the *sn*-2 acyl chain.

Research Article

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Identification of free radicals in oxidized and glycoxidized phosphatidylethanolamines by spin trapping combined with tandem mass spectrometry

Cláudia Simões, Pedro Domingues and Maria Rosario M. Domingues*

Mass Spectrometry Center, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro Portugal

RATIONALE: Nonenzymatic glycation of phosphatidylethanolamines (PEs) seems to a have a role in angiogenesis and atherosclerosis. Glycated PEs are more easily oxidized, enhancing oxidative stress. This study aims to evaluate the influence of glycation on the formation of intermediate radical species during oxidation of glycated PEs.
METHODS: In the present study, the radical intermediaries formed during the oxidation of palmitoyl-lineoyl phosphatidylethanolamine (PLPE) and glycated PLPE (gPLPE) were trapped using a spin trap (DMPO) and the radical adducts were analyzed by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS). Mass spectra were acquired using a electrospray Q-TOF 2 mass spectrometer.
RESULTS: Several spin adducts of PLPE and gPLPE were identified, corresponding to carbon- and oxygen-centered radicals. Interpretation of the MS/MS spectra showed the existence of different sites where radical formation occurred, at the *sn*-2 acyl chain, ethanolamine moiety (particularly in C-1) and, in the case of glycated derivatives, also in the glucose moiety (particularly in C-3, C-4 and C-5).
CONCLUSIONS: These results suggested the presence of more sites susceptible to oxidation in glycated PLPE, which may be responsible for the increase in the oxidative reaction rate occurring in glycated compounds. Copyright © 2012 John Wiley & Sons, Ltd.

Nonenzymatic glycation is a ubiquitous reaction between reducing sugars and polypeptides, proteins and lipids, leading to the irreversible formation of advanced glycation end products (AGEs). These compounds may have deleterious effects and may contribute to the onset and progression of various diseases such as angiopathy, atherosclerosis, renal, eye and neurological diseases.[1] Phosphatidylethanolamines (PEs) and phosphatidylserines (PSs) contain a polar domain that includes an amino group and, consequently, are susceptible to nonenzymatic glycation.[2–4] Glycated PEs have been detected *in vivo*, in erythrocytes,[2,3] plasma,[3,5] and in atherosclerotic plaques.[4] A significant positive correlation was observed between glycated hemoglobin and the presence of glycated PEs,[2] although the biological significance of glycated PEs is not known. Glycated PEs seem to have a role in angiogenesis[6] and could be correlated with complications of atherosclerosis-associated hyperglycemia.[4] Also, glycated PEs seem to be more easily oxidized and to induce oxidative modifications in other molecules.[2,4,7]

The oxidation of phospholipids has received considerable attention, since this process leads to the generation of biologically active phospholipids and key mediators of inflammation.[8] The oxidation of PEs is less well studied, although PE oxidation products have been detected in rat retinas during *in vitro* oxidation and in *in vivo* experiments[9] and in activated monocytes,[10] suggesting that they could play a role as signaling mediators in immune regulation and inflammation. *In vitro* studies of 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE) oxidation have shown that due to the modification of unsaturated fatty acyl chain, long- and short-chain oxidation products are produced.[11] So far, only one study has reported the identification of *in vitro* oxidative modifications of glycated PEs,[12] allowing the identification of oxidation products both in the unsaturated fatty acyl chain and in the glycated polar head. The products generated from oxidation of glycated polar head groups were formed by addition of oxygen atoms with formation of keto groups and by cleavage of the glycosidic moiety, leading to the formation of $-CH_2COOH$, $-C_2H_4NHCHO$ and $-C_2H_4NHCHOHCHO$ in the polar head group. Previously, the oxidized species gPLPE + 2O, correspondent to the formation of a keto derivative in the glycated polar head, was found to be a unstable oxidation product,[12] while the keto derivative of lineoly fatty acyl chain was considered to be a stable oxidation product.[11] In fact, the keto oxidation product in the glycated polar head was found to be short-lived, showing a decrease in the relative abundance in the first 24 h of oxidation, contrary to that observed for the corresponding ions in nonglycated PLPE oxidation. However, the role of glucose and oxidized glucose in the oxidation of glycated PEs is still not clear.

Further insight into the oxidation mechanism of biomolecules may be obtained through the identification of intermediate radical species formed during oxidation. The study

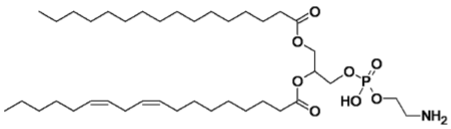
* *Correspondence to:* M. R. M. Domingues, Department of Chemistry, University of Aveiro, 3810–193 Aveiro, Portugal. E-mail: mrd@ua.pt

of the unstable intermediates generated from oxidation of biomolecules is important, as it can provide important information on the mechanisms of oxidation.[7] It is known that spin-trapping techniques can provide direct evidence of the presence of intermediate radical species in the reaction system. The combined use of spin trapping and mass spectrometry (MS) is a sensitive technique that has been used by a number of groups for identifying radical species of amino acids,[13] peptides,[14–16] proteins,[17–22] lipids,[23–26] and phosphatidylcholines.[14,15,27–29] In the present work, radical intermediaries formed during the oxidation of palmitoyl-lineoyl phosphatidylethanolmine (PLPE) and glycated PLPE (gPLPE) (Scheme 1) were trapped using a spin trap (DMPO) and the radical adducts formed were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS). By using a combination of a spin trap and tandem mass spectrometry, we evaluated the influence of glycation on the formation of intermediate radical species during oxidation.

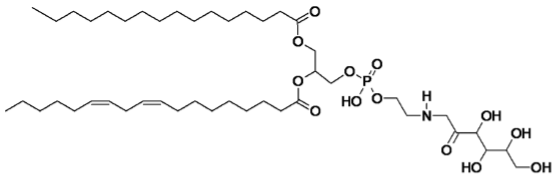
EXPERIMENTAL

Chemicals

2-Linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE) and 5,5-dimethyl-1-pyrrolidine-*N*-oxide (DMPO) were purchased from Sigma-Aldrich (Madrid, Spain). $FeCl_2$ and H_2O_2 (30%, w/v) used for the peroxidation reaction were acquired from Merck (Darmstadt, Germany). The water was of MilliQ purity filtered through a 0.22-μm filter (Millipore, USA), and all solvents used were HPLC grade.



(A) PLPE



(B) Glycated PLPE

Scheme 1. Structures of 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine: (A) PLPE and (B) glycated 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine (glycated PLPE).

Preparation of glycated PEs

PLPE was glycated in a methanol/water solution, as reported previously.[12] Briefly, were prepared two solutions, one of PLPE in methanol and the other of glucose in water. The two solutions were mixed and allowed to react in boiling water during 30 min. Then, the glycated PLPE was extracted from the reaction mixture using a modified Folch method,[30] and the organic phases were collected. Glycated PLPE was then purified by high-performance liquid chromatography (HPLC), as reported previously.[12] Chromatographic separations were performed on a model 2690 HPLC system (Waters Alliance, Milford, USA) using a C4 column (25 cm × 4.6 mm, 7 μm; Jones Chromatography, Llanbradach, UK). Mobile phase A consisted of 97.5% water and 2.5% acetonitrile with 0.1% (v/v) of formic acid. Mobile phase B consisted of acetonitrile with formic acid (0.1%, v/v). A volume of 10 μL of diluted (1:8 (v/v), in 75% methanol) glycation mixture was introduced into the column and the solvent gradient was programmed as follows: gradient started with 55% of B and held isocratically for 5 min before being increased to 70% of B during 15 min, followed by another linear increase to 100% B over 10 min, and then returned to initial conditions (55% of B) during 10 min. Glucose PLPE eluted from 16.03 to 16.73 min and PLPE eluted from 19.30 to 20.13 min. The collected fractions were dried and stored for further analysis.

Preparation of oxidized GPE vesicles

The phospholipid vesicles (nonglycated PLPE and glycated PLPE) were prepared from stock solutions of 1 mg/mL dried under a stream of nitrogen. Ammonium bicarbonate buffer (5 mM, pH 7.4) was added to give a final concentration of 50 mM of phospholipid and the mixture was vortexed.[31] Oxidation was initiated by adding $FeCl_2$ to a final concentration of 0.5 mM and H_2O_2 to a final concentration of 50 mM. Controls were performed by replacing hydrogen peroxide with water. The mixture was incubated at 37 °C in the dark for different periods of time. For spin-trapping experiments, DMPO (final concentration 20 mM) was added to the reaction, after 30 min incubation. At different time points, the phospholipid oxidation products were extracted using a modification of the Fölch method[30] with chloroform/methanol (2:1, v/v) and the oxidation products were monitored by mass spectrometry.

Mass spectrometry

All ESI mass spectra were acquired in the positive-ion mode on a Waters (Micromass) Q-TOF2 (Manchester, UK) equipped with a Z-spray ESI source. Conditions were as follows: flow rate, 10 $\mu L.min^{-1}$; cone voltage, 30 V; capillary voltage, 3 kV, source temperature, 80 °C; desolvation temperature, 150 °C, resolution was set to about 9000 (FWHM). Tandem mass spectra (MS/MS) were acquired by collision-induced decomposition (CID), using argon as the collision gas (measured pressure in the Penning gauge ~6 × 10–5 mbar). The collision energy ranged from 25 to 35 eV. Data acquisition was carried out with a MassLynx 4.0 data system.

Rapid Communications in Mass Spectrometry

RESULTS AND DISCUSSION

In a previous study, oxidation products formed by radical oxidation of the glucose moiety of the glycated PLPE polar head[12] were identified by LC/MS. Some of these oxidation products were found to be short-lived, with a decline of the relative abundance in the first 24 h of oxidation, contrary to that observed for the corresponding ions in nonglycated PLPE oxidation. Oxidation products formed by cleavage of the glucose moiety were also identified in that study, suggesting a susceptibility of glucose in the oxidation of glycated aminophospholipids. In this work, we have studied the influence of glycation of PLPE on the oxidation reaction and the radical species contributing to the formation of oxidation products involving the glycated polar head. In the following sections we will describe the results obtained with a combination of spin-trapping experiments with DMPO and tandem mass spectrometry, aiming to detect radical intermediates in the oxidation reaction of 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine, PLPE (Scheme 1, structure A) and glycated 2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine, glycated PLPE (Scheme 1, structure B).

DMPO spin adducts of PLPE

We incubated PLPE with hydrogen peroxide and $FeCl_2$ to generate the hydroxyl radical, in the presence or absence of DMPO, and determined the structures of the DMPO adducts of the oxidized products by using MS, as described in the Experimental section. Analysis of the oxidative mixture with DMPO by ESI-MS showed two sodiated molecules, $[M + Na]^+$ at *m/z* 867.6 ($[PLPE + DMPO + O + Na]^+$) and 883.6 ($[PLPE + DMPO + OO + Na]^+$) (Fig. 1(B)). These products presumably represent oxygen-centered spin adducts of alkoxyl and peroxyl radicals, respectively. Ionization of DMPO adducts as $[M + Na]^+$ during direct infusion and analysis by ESI-MS was observed in previous studies of spin-trapping experiments.[13,28] Analysis of the oxidative mixture without DMPO by ESI-MS (Fig. 1(A)) showed a wide range of oxidation products of PLPE, as described previously.[11]

Upon collisional activation (ion trap MS/MS), the ions at *m/z* 867.6 (Fig. 2(A), Table 1) and 883.6 (Fig. 2(B), Table 1) decomposed to an abundant product ion at *m/z* 754.4 and 770.4, respectively, which arise by loss of DMPO (−113 Da),

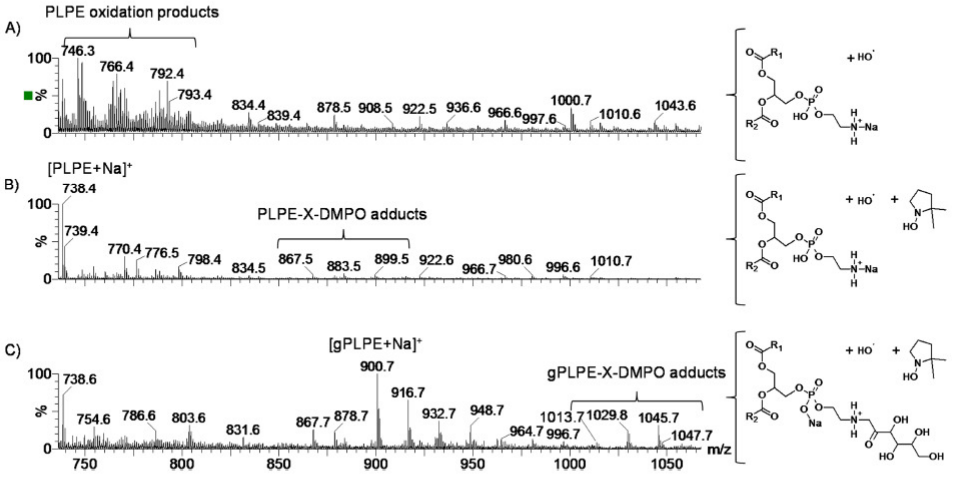


Figure 1. ESI-MS spectra of (A) PLPE under oxidative conditions, (B) PLPE under oxidative conditions in the presence of DMPO, and (C) glycated PLPE (gPLPE) under oxidative conditions in the presence of DMPO.

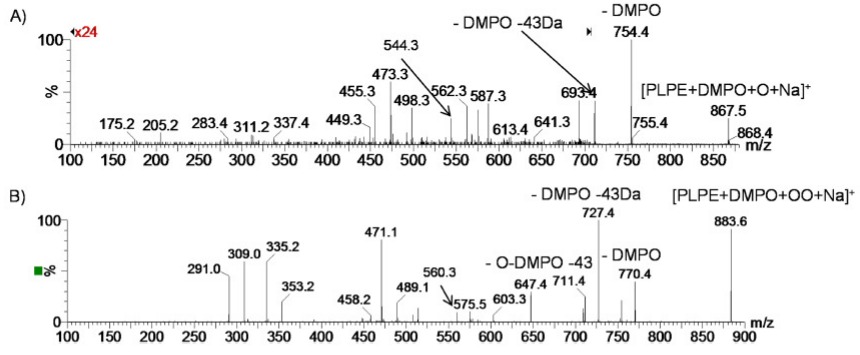


Figure 2. ESI-MS/MS spectra of $[M + Na]^+$ ions of PLPE oxygen-centered DMPO adducts ions: (A) $[PLPE + DMPO + O + Na]^+$ ion at *m/z* 867.5 and (B) $[PLPE + DMPO + OO + Na]^+$ ion at *m/z* 883.5.

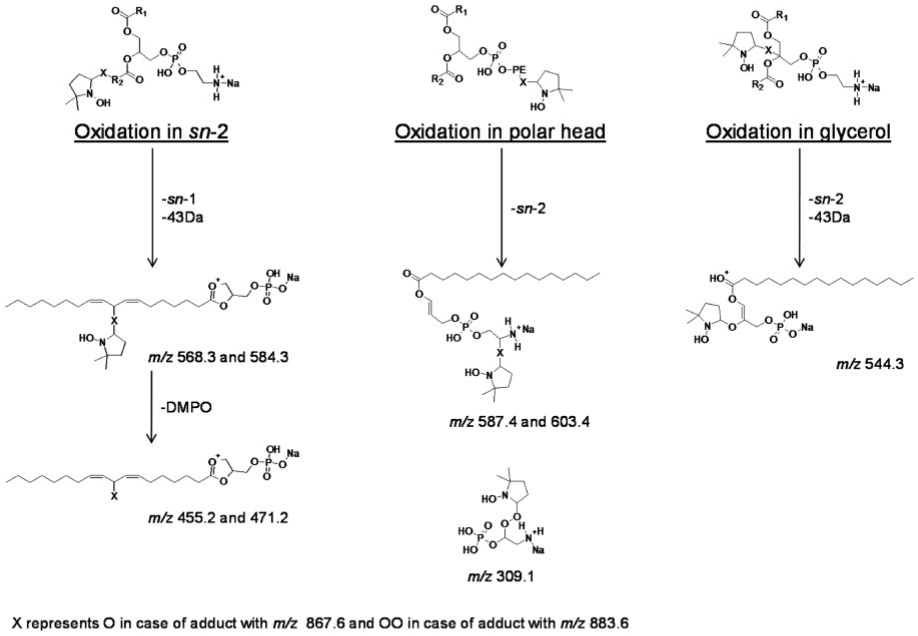
Table 1. Product ions observed in the ESI-MS/MS spectra of the $[M + Na]^+$ ions of the DMPO adducts of oxidized PLPE

$[M + Na]^+$	PLPE + O + DMPO	gPLPE + OO + DMPO
m/z	867.6	883.6
-DMPO (−113 Da)	754.4	770.4
-DMPO-OH (−129 Da)	738.5	754.5
-(DMPO + 43 Da[a])	695.55	711.5
-(DMPO-OH + 43 Da)	780.5	796.5
-(DMPO-OH + 43 Da + R_1COOH)	455.2	471.2
-R_2COOH	587.3	587.3
-(R_2COOH + 43 Da)	544.3	560.3
Polar head + OO + DMPO	–	309.1

[a]–43 Da corresponds to the neutral loss of aziridine.

consistent with the assigned structure.[28] Minor ions arising by loss of DMPO + O (−129 Da) were also observed in both spectra, respectively at *m/z* 738.5 and 754.5, consistent with the presence of the DMPO linked to oxygen atoms. Also, both spectra show ions arising by combined loss of DMPO and aziridine (loss of 43 Da is a typical neutral loss from PE sodiated molecules) at *m/z* 711.5 and 727.5, and combined loss of aziridine plus DMPO + O, at *m/z* 695.5 and 711.5, respectively. All these product ions confirm the presence of DMPO adducts and the formation of oxygen-centered radicals.

The tandem mass spectra of the sodiated molecules $[M + Na]^+$ at *m/z* 867.6 and 883.6 can be used to identify the site of oxidation and the presence of free radicals in the PLPE molecule. The product ions at *m/z* 455.2 (Fig. 2(A)) and 471.2 (Fig. 2(B)) arise from combined loss of fatty acid from *sn*-1 palmitoyl chain + DMPO + aziridine, consistent with the presence of alkoxyl and peroxyl radicals in the *sn*-2 lineoyl chain (Scheme 2). This is in agreement with previous LC/MS results showing that the oxidative modification takes place in the lineoyl fatty acyl chain.[11] However, the presence of minor product ions arising from loss of the unmodified *sn*-2 lineoyl chain (−R_2COOH), observed at *m/z* 587.3 (Fig. 2(A)) and 603.3 (Fig. 2(B)), show that oxidation also takes place in other sites of the molecule. Figure 2(B) yields a product ion at *m/z* 309.1, corresponding to DMPO linked to a peroxyl radical plus the PE polar head, which is consistent with the oxidation process also taking place in the PE polar head



Scheme 2. Proposed structures for product ions observed in the ESI-MS/MS spectra of PLPE oxygen-centered DMPO adducts: $[PLPE + O + DMPO + Na]^+$ at m/z 867.5 and $[PLPE + 2O + DMPO + Na]^+$ at *m/z* 883.5. These product ions suggest the presence of different sites of oxidative modifications: *sn*-2 fatty acyl chain, polar head moiety and glycerol.

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(Scheme 2). The site of oxidation is, most probably, in the alpha-carbon of the ethanolamine moiety (Scheme 2). The presence of a free amino group may favor radical abstraction from the carbon of the ethanolamine group attached to oxygen, similarly to the observed during peptide oxidation.[32] Figures 2(A) and 2(B) also show a minor product ion at *m/z* 544.3 and 560.3, respectively, arising by combined loss of the unmodified *sn*-2 fatty acyl chain ($-R_2COOH$) and aziridine ($-CH_2CHNH_2$, –43 Da), which is consistent with DMPO addiction of the glycerol backbone (Scheme 2). The adduct formation should take place on C-2 of the glycerol backbone, due to the generation of the more stable tertiary radical after hydrogen abstraction.

Overall, the evidence from this study suggests that during PLPE oxidation induced by the Fenton reaction, the initial hydrogen abstraction takes place preferentially in the *sn*-2 fatty acyl chain, although it can also take place in the polar head group or in the glycerol backbone.

DMPO spin adducts of glycated PLPE

We have also incubated glycated PLPE, with hydrogen peroxide and $FeCl_2$, to generate the hydroxyl radical, in the presence and absence of DMPO. Analysis of the oxidative mixture with DMPO by ESI-MS showed three new sodiated molecules $[M + Na]^+$ at *m/z* 1013.6 [gPLPE + DMPO + $Na]^+$, at *m/z* 1029.6 [gPLPE + DMPO + O + $Na]^+$ and at *m/z* 1045.6 [gPLPE + DMPO + OO + $Na]^+$ (Fig. 1(C)). These products presumably represent carbon- and oxygen-centered radical adducts, as described earlier for nonglycated PLPE.

In the following sections we will describe the tandem mass spectra of the spin adducts of DMPO with oxidized glycated PLPE. Since the ESI-MS/MS fragmentation pattern of sodiated glycated PE has not been reported before, we have also assessed the fragmentation pattern of sodiated glycated PLPE using ESI-MS/MS (Fig. 3(B)). Upon collisional activation (ion trap MS/MS), the ion $[M + Na]^+$ at *m/z* 900.6 (sodiated glycated PLPE molecules) decomposed to the product ions at *m/z* 882.5 and 864.5, which arise by loss of one and two molecules of H_2O molecules, respectively. Other product ions arise by cross-ring fragmentation of glucose and loss of $C_4H_8O_4$ (−120 Da, *m/z* 780.5), loss of the glucose residue (−162 Da, *m/z* 738.5), loss of glycated aziridine (−205 Da, *m/z* 695.5) and loss glycated polar head (−303 Da, *m/z* 597.5). The glycated PLPE sodiated molecule decomposed also to a major fragment ion at *m/z* 326.1, attributed to the sodium adduct of the glycated polar head, which can further decompose by loss of one and two H_2O molecules (Fig. 3(B)).

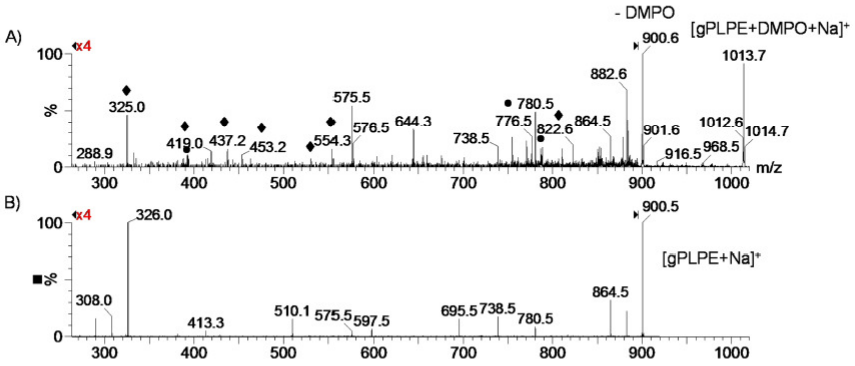


Figure 3. ESI-MS/MS spectra of $[M + Na]^+$ ions of: (A) glycated PLPE carbon-centered DMPO adduct at *m/z* 1013.7 and (B) glycated PLPE at *m/z* 900.5. The symbol ♦ assigns product ions which were used to identify the presence of oxidation in the glucose moiety. The symbol • assigns product ions which were used to identify the presence of oxidation in *sn*-2 fatty acyl chain.

Table 2. Product ions observed in the ESI-MS/MS spectra of the $[M + Na]^+$ ions of the DMPO adducts of oxidized glycated PLPE

$[M + Na]^+$	gPLPE-DMPO	gPLPE-O-DMPO	gPLPE-OO-DMPO
m/z	1013.6	1029.6	1045.6
-DMPO	900.6	916.6	932.6
-(DMPO + H_2O)	882.5	898.5	–
-(DMPO + $2H_2O$)	864.5	880.5	896.5
-(DMPO + 120 Da)	780.5	796.5	812.5
-(DMPO + glucose)	738.5	754.5	770.5
-(DMPO + glucose +43 Da)	695.5	711.5	727.5
-(DMPO + R_1COOH)	644.3	660.3	676.3
-(DMPO + R_1COOH + $C_3H_6O_3$)	554.3	570.3	586.3
R_2COOH + nO + DMPO	392.3 (n = 0)	408.3 (n = 1)	424.3 (n = 2)

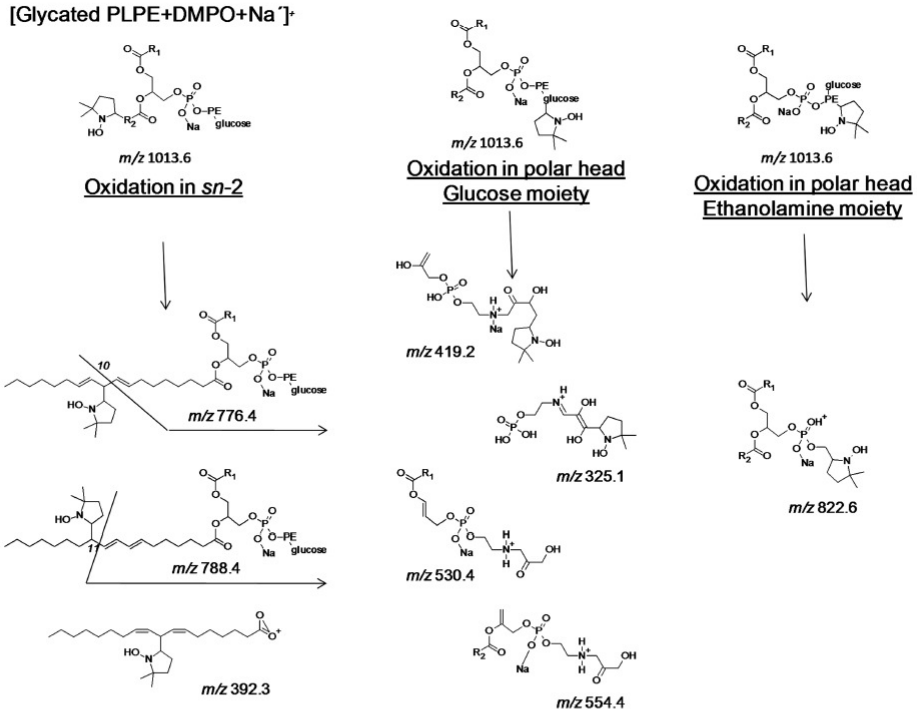
Carbon-centered adducts DMPO – oxidized glycated PLPE

Upon collisional activation, the ion at *m/z* 1013.6, assigned as the glycated PLPE carbon-centered radical adduct [gPLPE + DMPO + Na]$^+$, decomposed to a major ion at *m/z* 900.6, due to loss of DMPO (−113 Da) (Fig. 3(A), Table 2). Other product ions arise from combined losses of DMPO and glycated aziridine (*m/z* 695.5), loss of one and two water molecules plus DMPO (*m/z* 882.5 and 864.5, respectively), loss of 120 Da plus DMPO (*m/z* 780.5) and 162 Da plus DMPO (*m/z* 738.5). The product ions at *m/z* 392.3 [R_2COOH + DMPO + H]$^+$, and the ions at *m/z* 776.4 and 788.4, which arise from cleavage of *sn*-2 acyl chain + DMPO in the vicinity of C-10 and C-11, linked to DMPO, respectively (Scheme 3), are consistent with the assigned structure in Scheme 3. This finding is in agreement with previous studies which showed oxidation in C-10 and C-11 of the linoleoyl fatty acyl chain.[12,29] Altogether, these fragments are consistent with the presence of DMPO adducts of free radicals on the unmodified polar head, and oxidation of the unsaturated *sn*-2 fatty acyl chain (Scheme 3).

In the MS/MS spectrum of the glycated PLPE-DMPO adduct (Fig. 3(A), Table 2) the product ion at *m/z* 326.1, attributed to the glycated polar head, is absent, while it was a major product ion in the MS/MS spectrum of glycated PLPE (Fig. 3(B)). Nevertheless, the MS/MS spectrum of the sodiated molecules [gPLPE + DMPO + Na]$^+$ at *m/z* 1013.6 (Fig. 3(A)) can be used to identify the sites of oxidation both at the glycated polar head and/or the fatty acyl chain. The MS/MS spectrum of the ion at *m/z* 1013.6 decomposed to the product ions at *m/z* 325.1 and 419.2, which arise, respectively, by cleavage of the C3–C4 and C4–C5 bonds of the glucose moiety, suggesting linkage of DMPO to C-3 and C-4 of the glucose moiety (Scheme 3).

Also, the product ion at *m/z* 554.3, which arises by combined loss of the fatty acid from *sn*-1 and cleavage of the C3–C4 bond, suggests linkage of DMPO to C-4 of the glucose moiety (Scheme 3). This assignment is also supported by the presence of the product ion at *m/z* 530.3 which arises by combined loss of *sn*-2 and cleavage of C3–C4.

Overall, these fragments are consistent with the presence of an oxidation product formed by hydrogen abstraction from the glucose molecule. In the MS/MS spectrum of the glycated PLPE-DMPO adduct there is also some evidence of oxidation of the ethanolamine moiety, namely, the product ion at *m/z* 822.6 which arises by cleavage of C1–C2 of the ethanolamine moiety (Scheme 3). As observed before for DMPO-phosphatidylcholine adducts, main cleavages occurred in the vicinity of the DMPO linkage, allowing the identification of the initial hydrogen abstraction due to hydroxyl radical oxidation.[28,29]



Scheme 3. Proposed structures for the product ions observed in the ESI-MS/MS spectrum of glycated PLPE-DMPO carbon-centered adduct ([M + Na]$^+$ ion at *m/z* 1013.6). These product ions suggest the presence of different sites of oxidative modifications: *sn*-2 fatty acyl chain, polar head moiety and glycerol.

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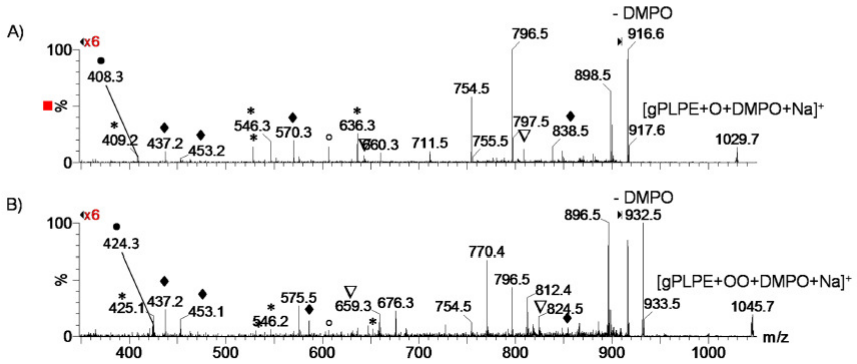
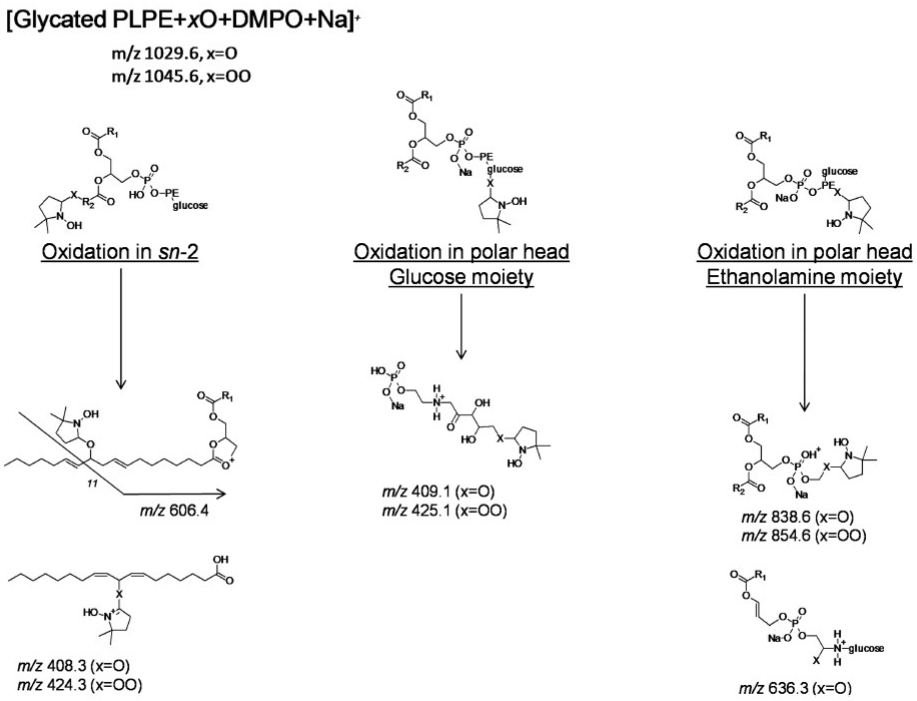


Figure 4. ESI-MS/MS of glycated PLPE oxygen-centered DMPO adducts ([M + Na]$^+$ ions: (A) [gPLPE + DMPO + O + Na]$^+$ ion at *m/z* 1029.7 and (B) [gPLPE + DMPO + OO + Na]$^+$ ion at *m/z* 1045.7. The symbol ♦ assigns product ions which were used to identify the presence of oxidation in the glucose moiety. The symbol * assigns product ions which were used to identify the presence of simultaneous oxidation in the glucose moiety and *sn*-2 fatty acyl chain. The symbol • assigns product ions which were used to identify the presence of oxidation in the *sn*-2 fatty acyl chain.



Scheme 4. Proposed structures for the product ions observed in the ESI-MS/MS spectra of glycated PLPE-DMPO oxygen-centered adducts, [glycatedPLPE + O + DMPO + Na]$^+$ at *m/z* 1029.6 and [glycatedPLPE + 2O + DMPO + Na]$^+$ at *m/z* 1045.6. These product ions suggest the presence of different sites of oxidative modifications: *sn*-2 fatty acyl chain, polar head moiety and glycerol.

Oxygen-centered adducts DMPO – oxidized glycated PLPE

Upon collisional activation, the ion at *m/z* 1029.6, assigned as the alkoxyl glycated PLPE oxygen-centered radical adduct [gPLPE + DMPO + O + Na]$^+$ (Fig. 4(A), Table 2), and the ion at *m/z* 1045.6, assigned as the peroxyl/hydroxyalkoxyl glycated PLPE oxygen-centered radical adduct [gPLPE + DMPO + OO + Na]$^+$ (Fig. 4(B)), decomposed respectively to:

- The product ions at *m/z* 408.3 and 424.3, assigned as DMPO adducts on the oxidized *sn*-2 fatty acid (Scheme 4);
- The product ions at *m/z* 711.5 and 727.5, which arise by combined loss of glycated ethanolamine plus DMPO, confirming the presence of the oxygen atoms in the *sn*-2 acyl chain;

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fragmentation pattern of sodiated glycated PE) combined with loss of DMPO molecule and one of the oxygens, evidencing the glucose moiety and the *sn*-2 fatty acyl chain as the oxidation sites.

Altogether, the MS/MS spectra of glycated PLPE spin adducts provide suggestive evidence of the existence of different sites for radical formation at the *sn*-2 acyl chain, the ethanolamine moiety (particularly in C-1) and, in the case of glycated derivatives, also in the glucose moiety (particularly C-3, C-4 and C-5). These results confirm that oxidation of the glucose moiety in glycated PEs is a favorable process in the presence of strong oxidants produced with the Fe^{2+}/H_2O_2 system. A recent study also described oxidation products of glycated PLPE which have arisen by oxidative cleavage of glucose at the C1–C2 and C2–C3 bonds, and the later could arise from radical formation in C-3.[12] It was expected that oxidation in the presence of DMPO would be associated with a *lower* reaction rate. Due to this fact, we were able to locate other sites of modification in glycated ethanolamine, which were not reported before. Oxidation in the glycated polar head group PLPE gives additional sites of oxidation, due to the presence of a glucose moiety. However, we have not identified adducts with two DMPO molecules. In this study, we have also shown evidence that, in gPLPE, simultaneous oxidation of the *sn*-2 acyl chain and glucose can also occur.

CONCLUSIONS

In this work, we have studied the influence of glycation of PLPE on the oxidation reaction using a combination of spin-trapping experiments with DMPO and tandem mass spectrometry (MS/MS). This approach allows the capture of free radicals generated in the oxidative process then subjected to further analysis by MS and MS/MS. One of the more significant findings to emerge from this study is that oxidation can occur in the PE polar head group, as well as in the *sn*-2 fatty acyl chain. Nonglycated PLPE undergoes major structural changes upon exposure to oxidants that result in oxidation of ethanolamine, glycerol and *sn*-2 moieties, while glycated PLPE is further oxidized at the glucose moiety. In this study, C- and O-centred radicals were detected in C-3, C-4 and C-5 of the glucose moiety. The presence of more sites susceptible to oxidation in glycated PLPE can be the factor responsible for the increase in the oxidative reaction rate occurring in glycated compounds. In this study C- and O-centred radicals were also detected on the ethanolamine and in the glycerol moieties. The current findings add substantially to our understanding of the mechanism of glycated and nonglycated phosphatidylethanolamine oxidation.

Acknowledgements

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CHAPTER IV

BIOLOGICAL EFFECTS OF THE OXIDATION PRODUCTS OF GLYCATED PES

INTRODUCTION

PE glycation was found to occur *in vivo* under hyperglycemic conditions and has been associated to increased oxidative stress. In turn, oxidative stress and oxidative modifications of PL (mainly PC) have been related with inflammatory processes and both pro-inflammatory and anti-inflammatory effects have been attributed to them. Besides protein glycation and advanced glycated end products (AGEs) of proteins are known to interfere with vessel wall homeostasis and contribute to a chronic inflammatory state in diabetes. However, the role of glycated, glycoxidized and oxidized phosphatidylethanolamines in the inflammatory responses had never been previously studied.

In this work, we aimed to study the influence of glycated, glycoxidized and oxidized phosphatidylethanolamines in the stimulation of antigen presenting cells, specifically monocytes and myeloid dendritic cells. To do so, peripheral blood was incubated with three different modified PEs, with different propensity to oxidation. Cell stimulation was evaluated using flow cytometry, after incubation and labeling with monoclonal antibodies directed to different cytokines (IL-1β, IL-6, IL-8, MIP-1β and TNF-α), and to cellular antigens (CD 33 HLA-DR, CD14, CD19, CD56 and CD3). Monocytes and dendritic cells were indentified and the expression of each cytokine under study was evaluated by frequency of cytokine-expressing cells and mean fluorescence intensity.

The results demonstrated, for the first time that glycated, glycoxidized and oxidized phosphatidylethanolamines produced an increase of the levels of cells expressing cytokines in comparison to the basal state. However, that increase was lower than the one obtained with LPS stimulus (positive control). In general, the results showed that oxidative modifications produced lower levels of cytokine-expressing cells when compared to the respective non-oxidized compounds (OxPE < PE and GluOxPE < GluPE). Additionally, the glycated compounds showed higher levels of cytokine-expressing cells when compared with non-glycated compounds (GluPE > PE and GluOxPE > OxPE). No variations were found the mean fluorescence intensity.

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PHOSPHATIDYLETHANOLAMINES GLYCATION, OXIDATION AND GLYCOXIDATION: EFFECTS ON MONOCYTE AND DENDRITIC CELL STIMULATION

Cláudia Simões[1], Ana Cristina Silva[1,2], Pedro Domingues[1], Paula Laranjeira[2], Artur Paiva[2], M. Rosário M. Domingues[1]*

[1]Mass Spectrometry Center, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Centro de Histocompatibilidade do Centro, 3001-301 Coimbra, Portugal

* To whom correspondence should be addressed: Maria R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

E-mail: mrd@ua.pt, telephone number: + 351-234-370698, fax number: +351-234-370084

ABSTRACT

Lipid glycation is a non-enzymatic reaction between glucose and the free amino group of aminophospholipids, particularly in chronic hyperglycemia. Glycated phosphatidylethanolamine have been found in plasma and atherosclerotic plaques of diabetic patients and was correlated with increased oxidative and inflammatory stress in diabetes. However, the biological roles of glycated lipids are not fully understood. In this study, we evaluated the effect of palmitoyl-oleolyl-phosphatidylethanolamine (POPE) oxidation, glycation and glycoxidation products on monocyte and myeloid dendritic cell stimulation. Flow cytometry analysis was used to evaluate the capability of each modified PE to induce the expression of different cytokines (IL-1β, IL-6, IL-8, MIP-1β and TNF-α) in monocytes or myeloid dendritic cells (mDC). Our results showed that PE modifications induced different effect on the stimulation of cells producing cytokines. All PE modifications induced higher frequencies of cytokine producing cells than basal state. Higher stimulation levels were obtained with glycated POPE, followed by glycoxidized POPE. In contrast, oxidized POPE negatively regulated the frequency of monocytes and mDC producing cytokines, when compared with non-modified POPE. In conclusion, we verified that PE glycation, compared with oxidation and glycation plus oxidation, had higher ability to stimulate monocytes and mDC. Thus detection of increased levels of PE glycation in diabetes could be considered a predictor of an inflammatory state.

KEYWORDS: phosphatidylethanolamine; glycation, oxidation; phospholipids, glycoxidation, cytokines, flow cytometry, inflammation and diabetes.

INTRODUCTION

Glycation is a well-known non enzymatic reaction between glucose (or other reducing sugars) and free amino groups of biomolecules, and is particularly relevant in diabetic patients with chronic hyperglycemia. Glycation of proteins and the effect of protein glycation in the development of diabetic complications have been thoroughly studied, but not the lipid glycation. Protein glycation and advanced glycated end products (AGEs) of proteins have pro-atherogenic action, interfering with vessel wall homeostasis and contributing to a chronic inflammation state that occurs in diabetes (1). Protein glycation has also been associated with angiopathy and the development of atherosclerosis, renal, eye and neurological diseases (2). Aminophospholipids, such as phosphatidylethanolamines and phosphatidylserines, have a free amino group in the phospholipid polar head group that can participate in glycation reactions. In fact, glycated phosphatidylethanolamines (GluPEs) were detected *in vivo*, in plasma and red blood cells of diabetic patients and in plasma of rats treated with *streptozotocin*, having a positive correlation with glycated hemoglobin (3-6). Glycated PE are major LDL lipid glycation products, and were associated with atherosclerotic complications of diabetic patients with associated hyperglycemia, as a result of an increase in lipid peroxidation (6). GluPEs were found to be more easily oxidized than non glycated PE, and were associated with an increase of ROS production, enhancing lipid peroxidation and cross-oxidative modification of other molecules (7). It was also suggested that GluPEs could have a role in angiogenesis, atherosclerosis, inflammation and other diabetic complications (6, 8). Glycation of PE induced major alterations on the polar head group (9), increasing the size of the polar group and the polarity. These changes in the molecular structure may be responsible for distinct molecular properties and biological effects (10, 11).

Oxidation reactions are also responsible for structural changes in PEs. Oxidation of PE was studied using mass spectrometry (MS), *in vivo* and *in vitro* systems (12). This study showed the presence of oxidative modification in unsaturated fatty acyl chains similar to the oxidation of phosphatidylcholine (OxPC) (10). Some studies reported that phospholipid oxidation products accumulate in tissues and atherosclerotic plaques mediating inflammation and immune response

(13-15). Different effects in inflammation have been described for oxidized phospholipids, varying from pro-inflammatory and anti-inflammatory effects or modulation of adaptive immunity, as reviewed by Bochkov *et al*. (10). The pro-inflammatory effects of oxidized phosphatidylcholines (OxPC) were attributed to promotion of monocyte binding to endothelial cells, through induction of expression of various adhesion molecules in endothelial cells, and also induction of growth factors and cytokines (10, 16). Anti-inflammatory effects OxPCs were attributed to different mechanisms, like induction of drug metabolism phase II genes and activation of antioxidant enzymes (10, 16). Furthermore, OxPLs could also disturb the endothelial barrier function, by increasing endothelial permeability or promoting endothelial protection (10, 16). The biological activity of oxidized phospholipids was proposed to be dependent on the nature of the *sn*-2 fatty acyl chain, thus not depending on the *sn*-3 fatty acyl chain or the phospholipid class (14, 17). However, there is not enough evidence to support this claim. Also, oxidation of glycated PE induces the simultaneous oxidation of the glucose moiety, the fatty acyl chains and the glycated polar head with formation of distinct polar head moieties (9) but the biological significance of these products were not evaluated. The limited number of studies is not enough to guaranty that this proposal is applicable to all the products formed during oxidation of the distinct phospholipids, bearing some of them so distinct structural properties. Still, many biological effects of glycated phospholipids and derived glycoxidized PE remain uncertain and their biological role is not fully understood.

The objectives of this research were to determine whether glycation and oxidation of PE can be associated with a pro-inflammatory effect that could be associated to diabetic inflammatory state that underlies progression of diabetic complications. To do so, we evaluated the ability of native palmitoyl-oleoyl-phosphatidylethanolamine (POPE), oxidized POPE, glycated POPE and glycoxidized PE products to stimulate peripheral blood monocytes and myeloid dendritic cells. For that purpose, expression of cytokines IL-1β, IL-6, IL-8, MIP-1β and TNF-α were determined by flow cytometry. Oxidized POPE, glycated POPE and glycoxidized POPE were also characterized using electrospray mass spectrometry.

EXPERIMENTAL

Chemicals

The 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE) was purchased from Sigma-Aldrich (Madrid, Spain). $FeCl_2$ and H_2O_2 (30%, w/v) used for the peroxidation reaction were acquired from Merck (Darmstadt, Germany). The water was of MilliQ purity filtered through a 0.22-µm filter (Millipore, USA), and all solvents used were HPLC grade. For stimulation of monocytes and dendritic cells RPMI-1640 medium was purchased from Gibco; Paisley, Scotland, UK, Brefeldin-A and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5) from Sigma, St. Louis, MO and interferon-γ (IFN-γ) from Promega, Madison, WI. Monoclonal antibodies: anti-HLA-DR—peridin chlorophyll protein (PerCPcy 5.5) (clone L243), anti-CD33—allophycocyanin (APC) (clone P67.6), anti-interleukin (IL)-1β (clone AS10), anti-IL-8 (clone AS14A) were purchased from BDB, San Jose, CA, USA; anti-IL-6 (clone MQ2- 6A3), MIP-1β (clone D21-1351) and anti-tumour necrosis factor (TNF)-α (clone Mab11) were purchased from BD Pharmingen, San Diego, CA, USA and Dendritic Cell Exclusion Kit—flourescein isothiocyanate (FITC) was purchased from Cytognos, Salamanca, Spain. Phosphate-buffered saline (PBS) was purchased from Dulbecco 1x, Biochrom AG, Berlin, Germany and permeabilization kit, INTRACELL was purchased from Immunostep, Salamanca, Spain.

Preparation of Glycated PE

POPE was glycated in a methanol/water solution with a final concentration of 3 µM for POPE and 0.5 mM of glucose, reacting in boiling water during 30 minutes, according with a previous optimized procedure (9). Then, the glycated POPE was extracted from the reaction mixture using a modified Folch method (18), and the organic phases were collected. Glycated POPE was then purified by HPLC, on a Waters Alliance HPLC system (Model 2690, Milford, USA) using a C4 column (25 cm x 4.6 mm, 7 µm) (Jones Chromatography, Llanbradach, UK). The mobile phase A consisted of 97.5% water and 2.5% acetonitrile with 0.1% (v/v) of formic acid. The

mobile phase B consisted of acetonitrile with formic acid (0.1%, v/v). 10µL of diluted (1:8 (v/v), in 75% methanol) glycation mixture was introduced into column and the solvent gradient was programmed as follows: gradient started with 55% of B and held isocratically for 5 minutes before increased to 70% of B during 15 minutes, followed by other linear increase to 100% B over 10 minutes, and then returned to initial conditions (55% of B) during 10 minutes. Glycated POPE eluted from 16.0 to 16.7 minutes and POPE eluted from 19.3 to 20.1 minutes. The collected fractions were dried and oxidized under the following conditions.

Preparation of Oxidation Mixtures

The phospholipid vesicles (non-glycated POPE and glycated POPE) were prepared from stock solutions of 1 mg/mL dried under nitrogen stream. Ammonium bicarbonate buffer (5 mM, pH 7.4) was added to give final concentration of 50 mM of phospholipid and the mixture was vortexed (19). Oxidation was initiated by adding $FeCl_2$ to final concentration of 0.5mM and H_2O_2 to a final concentration of 50mM. Non-oxidized POPE and glycated POPE were performed using water instead of hydrogen peroxide. The mixture was incubated at 37°C in the dark for 5 days, after that the compounds were extracted using a modification of the Folch method (18) with chloroform-methanol (2:1, v/v) and re-suspended in same volume of PBS. Phospholipid vesicles were prepared by the addition of PBS to phospholipids and glycated phospholipid extracts. The mixtures were shaken mechanically on a vortex mixer for 10 min and then sonicated for 1 minute in a sonicator.

Mass Spectrometry

ESI mass spectra were acquired on a Waters (Micromass) Q-TOF2 (Manchester, U.K.) equipped with a Z-spray ESI source and on a linear ion trap LXQ (ThermoFinnigan, San Jose, CA, USA) mass spectrometers. Q-TOF2 conditions in positive mode were as follow: flow rate, 10 $\mu L.min^{-1}$; cone voltage, 30 V; capillary voltage, 3 kV, source temperature, 80ºC; desolvation temperature, 150ºC, resolution was set to about 9,000 (FWHM). Tandem mass spectra (MS/MS)

were acquired by collision-induced decomposition (CID), using argon as the collision gas (measured pressure in the Penning gauge ~ 6x10-5 mBar). The collision energy ranged from 20 to 30 eV. Data acquisition was carried out with a MassLynx 4.0 data system. The LXQ conditions in negative mode were as follows: electrospray voltage was - 4.7 kV; capillary temperature was 275 ºC and the sheath gas flow was 8 U. Normalized collision energy^{TM} (CE) was varied between 17 and 20 (arbitrary units) for MS/MS. Data acquisition was carried out on a Xcalibur data system (V2.0).

Samples

A total of 10 peripheral blood (PB) samples were collected from healthy adult subjects. The mean age of the healthy individuals whose peripheral blood was used to monocyte and mDC cultures were 29+/- 7 years. From these individuals, 4 (40%) were males and 6 (60%) were females. Informed consent, according to local ethics committee, was obtained from all individuals enrolled in this study. All PB samples were collected in heparin as anticoagulant and immediately processed for the analysis of cytokine production.

***In vitro* Stimulation of Cytokine Production by mDCs and Monocytes**

For better results, we stimulated monocytes and myeloid dendritic cells (mDC) in separated cultures. For stimulation of mDCs, we diluted 500 µl of PB sample 1/1 (vol/vol), in RPMI-1640 medium, supplemented with 2 mM L-glutamine. Additionally, we added 100 ng/ml of lipopolysaccharide (LPS) plus 100 U/ml of interferon-γ to one tube to stimulate the cells (positive control), in other tube we added 20 µg of POPE plus 100 U/ml of IFN-γ, while the other tube was incubated without stimulation and used as negative control. To the study of cytokine production, we added 10 µg/ml of Brefeldin-A to all tubes to prevent the release of cytokines outside the cells. In all cases, incubation was performed at 37℃ in a 5% CO_2 humid atmosphere, in a sterile environment for 6h.

The protocol for monocyte stimulation was the same as mDC stimulation, but in the case of monocytes without the addition of IFN-γ.

Flow Cytometric Analysis of Intracytoplasmic Cytokine Production

Immediately after the incubation period, all samples were distributed in different tubes (200 µl/tube) in order to analyze the expression of each cytokine under study, by monocytes and mDCs subset.

In order to identify mDC subset, a 10 µl of Dendritic Cell Exclusion Kit combined with 10 µl of HLA-DR-PerCPcy 5.5 and 5 µl of CD33-APC were added to each tube. After gentle mixing, cells were sequentially incubated for 10 min at room temperature, in the darkness and washed once in with 2 ml of PBS (5 min at 540x g). After discarding the supernatant, cells were fixed and permeabilized with INTRACELL, according to manufacturer's instructions and stained with PE-conjugated mAb directed against different human intracytoplasmic cytokines: IL-1β (10 µl), IL-6 (10 µl), IL-8 (10 µl), MIP-1β (1.5 µl) and TNF-α (1.5 µl). Each anti-cytokine mAb reagent was placed in a separate tube containing either the stimulated or the unstimulated sample. The tubes were incubated for 10 min at room temperature in the darkness. Then, cells were washed twice with 2 ml of PBS (5 min at 540x g) and resuspended in 250 µl of PBS. In monocytes cultures, 10 µl of anti-HLA-DR-PerCP, 5 µl of anti-CD33-APC and the cytokines reported before were added to each tube. In this case, the Dendritic Cell Exclusion Kit was not added to the sample. Incubation and washing steps were the same preformed for mDC.

Flow Cytometry Data Acquisition and Analysis

Data acquisition for mDC was performed in two consecutive steps in a FACSCalibur flow cytometer (BDB, San Jose, USA) equipped with an argon ion laser and a red diode laser. In a first step, 2 x10^4 events, corresponding to all nucleated cells present in the sample, were collected and information on them stored. In a second step, information was stored exclusively for those cells

included in a live gate containing HLA-DR^{+} events, in a minimum of 2x10^{5} events. For data acquisition and analysis, the CellQuest (BDB, San Jose, USA) and INFINICYT (Cytognos, Salamanca, Spain) software programs were used.

mDCs were identified by the combined expression of CD33^{bright} and HLA-DR^{bright} and absence of CD14, CD19, CD56 and CD3 with intermediate forward (FSC) and side scatter (SSC) between those of lymphocytes and monocytes.

For monocytes cultures, the data acquisition was performed in one step in a FACSCalibur flow cytometer (BDB, San Jose, USA). For this, 5 x10^{5} events, corresponding to all nucleated cells present in the sample, were collected and information on them stored. We identified monocytes as expressing CD14, CD33 and HLA-DR and by their FSC and SSC characteristics.

Statistical Methods

Mean values and their standard deviations, as well as median values and range, were calculated for each variable under study by using the SPSS software program (SPSS 17.0, Chicago, USA). The statistical significance of the differences observed between groups was assessed using the nonparametric Mann-Whitney U test. *P* values< 0.05 were considered to be associated with statistically significant differences between groups.

RESULTS

The capability of phosphatidylethanolamine oxidation, glycation and glycoxidation products to enhance cytokine production was evaluated using POPE. Although POPE is not the most abundant PE specie in plasma, the glycated POPE was already detected in plasma and in atherosclerotic plaques from diabetic patients (4, 6). Glycated POPE (previously synthesized and purified) and native POPE were oxidized by the hydroxyl radical under Fenton conditions to obtain

OxPOPE and GluOxPOPE, respectively, that were characterized by electrospray mass spectrometry (ESI-MS and ESI-MS/MS).

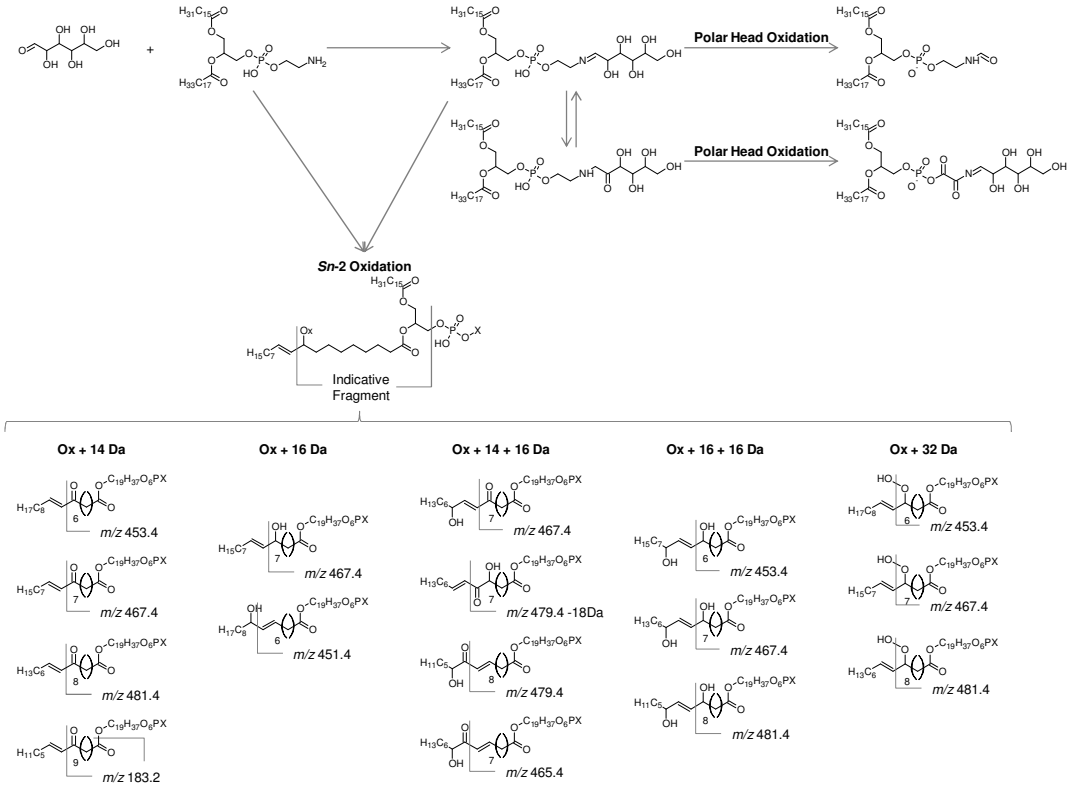
Characterization of POPE and glycated POPE oxidation products by MS

Oxidation products of POPE and GluPOPE formed in the presence of hydroxyl radical generated by Fenton reaction conditions were characterized by ESI-MS and MS/MS. Oxidation of other PE and glycated PE species induced by the hydroxyl radical were already studied using mass spectrometry in our lab (9, 20), and by other groups, utilizing different methods for induce oxidation (21, 22). POPE (*m/z* 718.5) oxidation leaded to the formation of long chain oxidation products, namely keto (*m/z* 732.5), hydroxy (*m/z* 734.5), keto-hydroxy (*m/z* 748.5), dihydroxy and peroxy (*m/z* 750.5) derivatives, with modifications localized on carbons 8, 9, 10 and 11, of *sn-2* acyl chain. It were not detected short chain products, due to cleavage of long chain *sn-2* oxidation products, similar to what was observed previously for oxidized 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylcholine (POPC) (23). Table 1 summarizes the oxidation products of POPE identified by MS and MS/MS. Oxidation of GluPOPE led to the formation of different oxidation products. Glycated long chain products obtained from oxidation of GluPOPE (*m/z* 880.5) were products with keto (*m/z* 894.5), hydroxy (*m/z* 896.5), dihydroxy (*m/z* 912.5) and peroxy (*m/z* 912.5) derivatives in *sn-2* acyl chain, as summarized in Table 1. Additionally, oxidation products of POPE (*m/z* 732.5, 734.5 and 750.5) were also formed, due to oxidative cleavage of GluPOPE with loss of glucose, as observed previously in GluPLPE oxidation (9). Furthermore, the negative ion mass spectra allowed to identify oxidation products formed due to oxidative modification in glucose moiety present in the glycated polar head. The ions at *m/z* 906.5, identified as glycated POPE+2O-4Da, suggested the formation of two keto-groups in glycated ethanolamine (which corresponds to *m/z* 880.5 observed in di-palmitoyl-phosphatidylethanolamine, dPPE). The ion at *m/z* 744.5, was identified as product with a polar head group $–OCH_2CH_2NHCOH$ (formed due to oxidative cleavage of glucose, similarly to the ion *m/z* 718.5 observed previously in dPPE) (9). The structures of the oxidation products are represented in the Scheme 1.

TABLE 1 – POPE oxidation products and GluPOPE oxidation products found in positive ion mode, and their modifications on *sn-2* acyl chain.

Ion (*m/z*)	Retention time (minutes)	Modification	Modified Carbon	Indicative Fragments (*m/z*)	Structure Reference
732.5 (718.5+14)	26	Keto-derivative	C-8 C-9 C-10 C-11	453.4 467.4 481.4, 169.1 183.2	(20)
734.5 (718.5+16)	27	Hydroxy-derivative	C-9 C-10	467.4 451.4	
748.5 (718.5+30)	25	Hydroxy-Keto-derivative	C-9 C-10 C-11	467.5 479.5 465.5	
750.5 (718.5+32)	23	di-Hydroxy-derivative	C-8 C-9 C-10	453.4 467.5 481.5	
	25	Peroxy-derivative	C-8 C-9 C-10	453.4 467.5 481.5	
894.5 (880.5+14)	25	Keto-derivative	C-8 C-9 C-10 C-11	453.4 467.4 481.4 183.2	
896.5 (880.5+16)	26	Hydroxy-derivative	C-9 C-10	467.4 451.4	
912.5 (880.5+32)	23	di-Hydroxy-derivative	C-8 C-9 C-10	455.4 467.5 481.5	
	25	Peroxy-derivative	C-8 C-9 C-10	453.4 467.5 481.5	

The non-modified POPE, glycated POPE, oxidized POPE (with insertion of oxygen molecules in *sn-2* acyl chain) and glycoxidized POPE (with insertion of oxygen molecules in *sn-2* acyl chain and/or oxidation of in glycated polar head) were evaluated for their effects on cytokine production in cells of the immune system. Glycated POPE was modified in the polar head group, oxidized POPE contained oxidative modifications in the unsaturated fatty acyl chain of *sn-2* position, while glycoxidized POPE had oxidative modifications in polar head group and in the fatty acyl chain.



SCHEME 1 – Structures of the oxidation products formed from POPE and GluPOPE oxidation. The oxidation products formed by *Sn*-2 oxidation were the same for POPE and GluPOPE, and were formed by insertion oxygen molecules (+ 14 Da, + 16 Da, + 30 Da, or + 32 Da). In such products, X represents $–(CH_2)_2NH_2$ or $–(CH_2)_2NC_6H_{12}O_5$ if they are formed from oxidation of POPE or GluPOPE, respectively. The oxidation of GluPOPE also yields products with modifications in polar head.

Cytokine produced by monocytes and mDC

The capability of native POPE and modified POPE (GluPOPE, OxPOPE and GluOxPOPE) to induce the production of different cytokines by peripheral blood monocytes and myeloid dendritic cells (mDCs) were studied. The frequencies of cytokine-producing cells (monocytes or mDC), as well as the total amount of cytokines produced *per* cell, expressed as mean fluorescent intensity (MFI), were evaluated in this study, in attempt to establish the relation between PE oxidation and/or glycation and the level of activation of antigen-presenting cells, specifically monocytes and myeloid dendritic cells.

First of all, we tested different conditions of stimulation with POPE (different POPE quantity in the presence and absence of recombinant IFN-γ) in order to obtain a maximum of cells

producing cytokines. Different amounts of POPE were tested (10, 20, 30, 40 and 50 µg), and the best results were obtained with 20 µg of POPE. IFN-γ was only added to stimulate mDCs, since monocytes did not need IFN-γ to respond to lipid stimulation.

After procedure optimization, we studied the production of IL-1β, IL-6, IL-8, MIP-1β and TNF-α by monocytes and mDC at basal level (negative control), and upon stimulation with LPS for monocytes and LPS plus IFN-γ for mDC (positive control), with non-modified POPE, GluPOPE, OxPOPE and GluOxPOPE. The results of stimulation with non-modified POPE were first compared with positive and negative controls. Subsequently, the results obtained from the stimulation with the different modified POPE were compared with each other, in order to determinate the modification that induced a higher effect on stimulation of these cells, and thus on cytokine production.

For all studied cytokines, we found a significant increase in the frequencies of monocytes and mDC expressing cytokines after stimulation with POPE compared with negative control ($p<0.05$; Table 2; Figure 1). This means that POPE stimulated both cell populations, increasing the frequency of cytokine producing cells. POPE stimulation did not affect the amount of each cytokine produced *per* cell, given by the mean fluorescent intensity (MFI), for cytokines IL-1β, IL-6 and IL-8, while the amount of TNF-α and MIP-1β *per* monocytes (not *per* mDC), tended to be higher than those observed in negative control (Table 2). These results showed that the stimulation with POPE induced an increase in the percentage of monocytes and DC producing cytokines, but did not affect, significantly, the amount of cytokines *per cell*, in both cell types, when compared to the negative control. Native POPE induced cells to produce cytokines; however, this stimulus was lower than the one obtained with LPS, reflecting either lower number of cells producing cytokines and lower amounts of cytokines produced *per* cell.

TABLE 2 – Frequency of cytokine producing monocyte and mDC and amount of each cytokine *per* cell (MFI), after LPS plus IFN-γ (positive control); after POPE plus IFN-γ stimulation in the case of mDC cultures; after POPE stimulation in monocytes cultures and without stimulation (negative control).

		Monocyte		mDC	
		%	MFI	%	MFI
TNF-α	Control	5.7 ± 2.5	68 ± 53	6.6 ± 4.1	22 ± 8
	LPS	83.3 ± 14.9	275 ± 172	82.1 ± 19.4	56 ± 52
	POPE	21.6 ± 5.3[e]	197 ± 113	23.8 ± 3.2[e]	20 ± 4
MIP-1β	Control	12.7 ± 4.8	194 ± 133	8.3 ± 6.2	33 ± 14
	LPS	90.0 ± 10.8	294 ± 196	88.3 ± 20.3	98 ± 56
	POPE	37.9 ± 4.4[e]	353 ± 91	40.0 ± 11.0[e]	29 ± 9
IL-6	Control	6.2 ± 3.7	23 ± 8	7.1 ± 4.4	16 ± 5
	LPS	88.1 ± 13.0	50 ± 13	81.7 ± 9.4	43 ± 22
	POPE	22.2 ± 14.7[e]	24 ± 5	21.0 ± 7.3[e]	13 ± 3
IL-8	Control	6.8 ± 3.1	22 ± 6	5.7 ± 2.9	16 ± 5
	LPS	83.6 ± 16.7	39 ± 19	89.5 ± 9.5	32 ± 17
	POPE	21.1 ± 6.7[e]	25 ± 5	15.3 ± 2.5[e]	16 ± 5
IL-1β	Control	8.6 ± 3.7	22 ± 6	4.5 ± 2.1	17 ± 4
	LPS	88.2 ± 15.6	49 ± 16	87.0 ± 8.2	33 ± 11
	POPE	31.7 ± 6.4[e]	24 ± 4	17.6 ± 8.8[e]	17 ± 6

Results are expressed as mean ± standard deviation. %, percentage of positive cells; MFI, mean fluorescence intensity of positive cells. Statistically significant differences were considered when $p<0.05$ (Mann–Whitney U test). [e POPE *versus* Negative Control].

Stimulation with PE modifications

After the evidence that POPE was able to stimulate the production of cytokines, the next step was to determine the effects of OxPOPE, GluPOPE and GluOxPOPE on cytokine production (IL-1β, IL-6, IL-8, MIP-1β and TNF-α) by monocytes and mDC. The dot-plots with levels of TNF-α obtained with different POPE stimulation are present in figure 2.

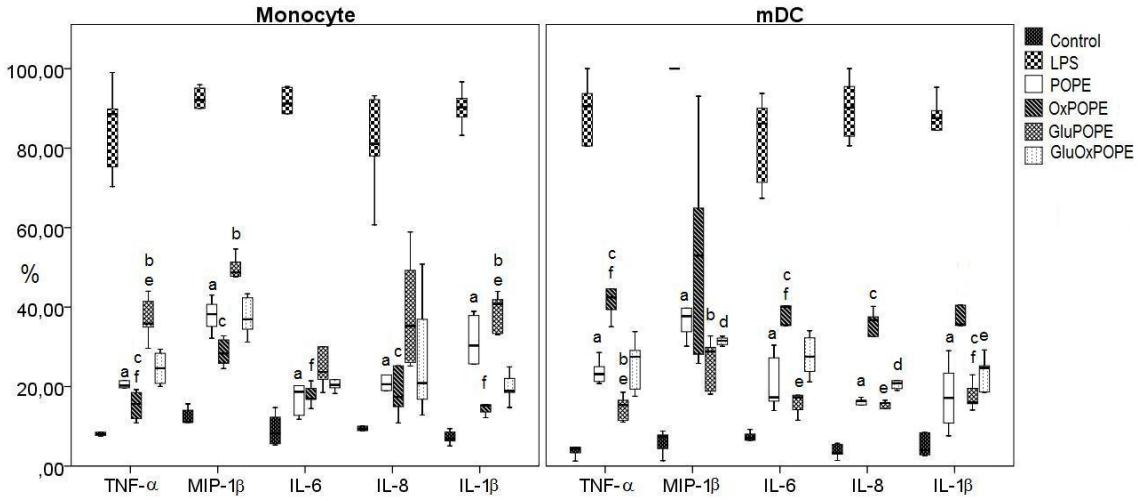


FIGURE 1 – Frequency of cytokine producing monocyte and mDC after stimulation with LPS (positive control), POPE, GluPOPE, OxPOPE, GluOxPOPE and without stimulation (negative control). In mDC cultures the stimulation with POPE and their glycation and/or oxidation products were carried out in the presence of IFN-γ. Statistically significant differences were considered when $p<0.05$ (Mann–Whitney U test). [a POPE *versus* Negative Control] [b POPE *versus* GluPOPE] [c POPE *versus* OxPOPE] [d POPE *versus* GluOxPOPE] [e GluOxPOPE *versus* GluPOPE] [f GluOxPOPE *versus* OxPOPE].

After stimulation with OxPOPE (compared with POPE) we observed a statistically significant decrease in the frequency of monocytes producing TNFα, MIP-1β and IL-1β and in the frequency of mDC producing TNF-α and MIP-1β ($p<0.05$; Table 3; Figure 1). Despite the decrease in the percentage of cells producing cytokines after stimulation with OxPOPE (compared with POPE), the amount of cytokines at single cell level revealed no statistically significant differences (Table 3).

When monocytes and mDC were stimulated with GluPOPE, the frequency of cells that produce cytokines was increased, when compared to stimulation with POPE. The observed increase was statistically significant for TNF-α, MIP-1β and IL-8 in monocytes and for TNF-α, IL-6, IL-8 and IL-1β in mDC ($p<0.05$; Table 3; Figure 1). However, the amount of cytokines produced *per* cell (MFI) was not altered. Thus, GluPOPE positively regulated the frequency of monocytes and mDC producing cytokines, although the amount of cytokines produced *per* cell tend to be similar with those obtained with POPE.

TABLE 3 – Frequency of cytokine producing monocyte and mDC, and amount of each cytokine *per* cell (MFI), following POPE, GluPOPE, OxPOPE, GluOxPOPE stimulation. In mDC cultures the stimulation with POPE and their glycation and/or oxidation products were carried out in the presence of IFN-γ.

		Monocyte		mDC	
		%	MFI	%	MFI
TNF-α	POPE	21.6 ± 5.3	197 ± 113	23.8 ± 3.2	20 ± 4
	GluPOPE	35.5 ± 4.8[b]	170 ± 128	44.6 ± 10.2[b]	19 ± 5
	OxPOPE	14.4 ± 3.8[a,d]	188 ± 49	14.6 ± 3.3[a,d]	23 ± 1
	GluOxPOPE	24.6 ± 4.3[e]	225 ± 64[e]	25.5 ± 6.8[e]	20 ± 7
MIP-1β	POPE	37.9 ± 4.4	353 ± 91	40.0 ± 11.0	29 ± 9
	GluPOPE	50.0 ± 3.6[b]	352 ± 67	53.0 ± 27.9	37 ± 8
	OxPOPE	28.7 ± 3.6[a,d]	412 ± 80[d]	25.7 ± 6.7[a]	42 ± 14
	GluOxPOPE	37.6 ± 5.2[e]	395 ± 114[e]	31.4 ± 1.1[c]	36 ± 13
IL-6	POPE	22.2 ± 14.7	24 ± 5	21.0 ± 7.3	13 ± 3
	GluPOPE	29.1 ± 13.2	35 ± 18	40.2 ± 6.0[b]	12 ± 4
	OxPOPE	17.8 ± 2.7[d]	26 ± 8[d]	17.2 ± 5.1[d]	14 ± 6
	GluOxPOPE	22.3 ± 5.2[e]	28 ± 7	27.8 ± 5.5[e]	14 ± 4
IL-8	POPE	21.1 ± 6.7	25 ± 5	15.3 ± 2.5	16 ± 5
	GluPOPE	38.9 ± 14.8[b]	27 ± 12	32.0 ± 10.9[b]	15 ± 5
	OxPOPE	22.8 ± 13.8	26 ± 8	14.6 ± 2.6[d]	20 ± 6
	GluOxPOPE	27.7 ± 15.9	27 ± 9	21.2 ± 2.5[c]	15 ± 5
IL-1β	POPE	31.7 ± 6.4	24 ± 4	17.6 ± 8.8	17 ± 6
	GluPOPE	38.6 ± 5.0	32 ± 8[b]	38.4 ± 10.8[b]	16 ± 7
	OxPOPE	15.5 ± 3.3[a,d]	29 ± 8	17.7 ± 3.6[d]	19 ± 7
	GluOxPOPE	19.8 ± 3.9[c, e]	27 ± 6	23.3 ± 4.6[e]	17 ± 10

Results are expressed as mean ± standard deviation. %, percentage of positive cells; MFI, mean fluorescence intensity of positive cells. Statistically significant differences were considered when $p<0.05$ (Mann–Whitney U test). [a POPE *versus* OxPOPE] [b POPE *versus* GluPOPE] [c POPE *versus* GluOxPOPE] [d GluOxPOPE *versus* OxPOPE] [e GluOxPOPE *versus* GluPOPE].

The GluOxPOPE (oxidized GluPOPE), induced a lower cell stimulation in comparison with GluPOPE, and similar, for the majority of the studied cytokines, to those stimulation obtained with OxPOPE related to POPE. The lower frequency of cells producing cytokines after GluOxPOPE stimulation (in comparison with GluPOPE) was observed for almost all studied cytokines in both monocytes and mDC, reaching statistical significance for IL-1β, IL-6, TNF-α and MIP-1β in monocytes ($p<0.05$; Table 3; Figure 1). However, the amount of cytokines produced (MFI) *per* mDC was not altered and *per* monocytes we only observed a significant increase in the amount of TNF-α and MIP-1β ($p<0.05$) when compared with GluPOPE.

Regarding GluOxPOPE stimulation, in relation to OxPOPE activation, the GluOxPOPE presented higher cell stimulation, with a higher frequency of monocytes/mDC producing cytokines, in the same way of results from GluPOPE stimulation compared with POPE stimulation. This difference is statistically significant in monocytes for MIP-1β, IL-6, IL-1β and TNF-α, and in mDC for all cytokines with exception of MIP-1β ($p<0.05$; Table 3; Figure 1).

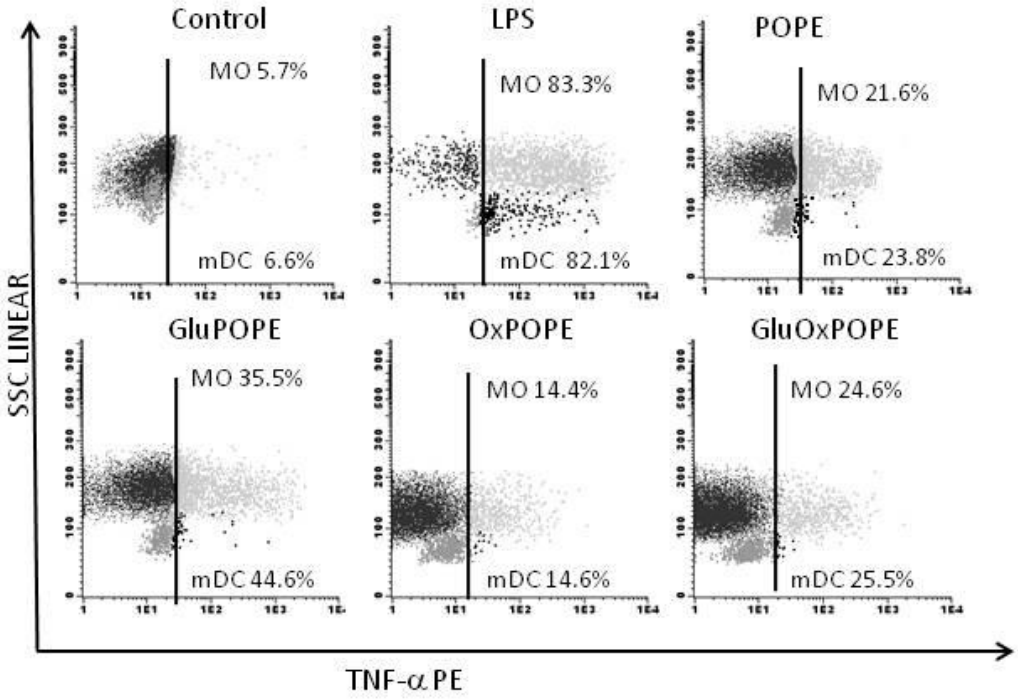


FIGURE 2 – Expression of TNF-α in monocyte and mDC after stimulation with LPS (positive control), POPE, GluPOPE, OxPOPE, GluOxPOPE and without stimulation (negative control).

In summary, our results demonstrated that glycation and/or oxidation of PE produced different effects on monocyte and mDC stimulation. Furthermore their effect on the frequency of monocytes/mDC producing cytokines was not necessary the same on the amount of cytokine produce *per* monocytes/mDC. In fact, although the amount of cytokine *per cell* was, in most cases, did not change, the total of cytokines production was enhanced due to the increase number of cell that produce cytokines.

DISCUSSION

Previous studies reported elevated plasma levels of some of the cytokines studied, IL-6 (24-27), IL-8 (28, 29) and TNF-α (24, 25), in diabetic patients with associated hyperglycemia. IL-1β and MIP-1β were not reported to be increased in diabetic patients, but it is probable that they would be also increased in inflammatory conditions with associated hyperglycemia. In fact IL-1β, together with TNF-α and IL-6, are pro-inflammatory cytokines that are involved in mediating acute inflammatory responses (30). In addition, MIP-1β, like IL-8, is a chemotactic cytokine, involved in acute and chronic inflammatory responses mainly by recruiting pro-inflammatory cells (monocytes, dendritic, NK and T cells) (30, 31).

Hyperglycemia is usually associated with an increase of glycation levels of proteins and lipids (aminophospholipids) and is also associated with an increase of oxidative status of cells and plasma, concomitant with an increase of plasma levels of cytokines (IL-6 and TNF-α) (25). In the present work we aimed to evaluate if distinct modifications of PE structure, derived from hyperglycemia, could have influence on monocyte stimulation, and so, be related to elevated plasma levels of cytokines in diabetic patients.

In this study, the monocytes and mDCs were stimulated directly by addition of free phospholipids to blood and cytokine production was evaluated by flow cytometry. In the majority of cases, the amount of cytokine produced *per cell* was not changed, but an increase in the number of cell that produce cytokines leads to an unregulated cytokine production. In fact, Pickup *et al.* hypothesized before that the increased serum levels of cytokines in diabetic patients was not due to circulating cells but most probably to endothelial cells or adipocytes (32). Our results, demonstrate that monocytes and dendritic cells respond to stimulation with PE and PE modifications, but do not alter their cytokine production *per* cell. We observed that oxidized compounds induce lower stimulation of monocytes and mDCs when compared with non-modified PE (OxPOPE < POPE and GluOxPOPE < GluPOPE) and the opposite was expected, since oxidized lipid are, in general considered pro-inflammatory agents. Previous studies on the effect of

oxidized phospholipids either using endothelial cells or low density lipoprotein (LDL), revealed that oxidized phospholipids activate the endothelium binding of monocytes and macrophage proliferation (13, 33-35). Endothelial cells treated with OxPAPC presented different protein phosphorylation levels, including high levels of β1-integrin phosphorylation (among others), a protein that is known to be involved in monocyte binding to endothelial cells (36). These findings suggested that OxPAPC modulate endothelial cell migration and barrier function (36). In this study, phospholipids were added directly to blood, thus the stimulation was not mediated by LDL or endothelial cells, and that probably leaded to lower stimulation of monocytes and mDCs. So we could suppose that endothelial cells play an important role on monocyte stimulation induced by oxidized phospholipids and without them, the effects of oxidized phospholipids, were not so prominent. On the other side, GluPOPE showed higher effects on mDCs/monocytes, revealing that profound alterations on PE structure, attributable to glycation, could induce cell stimulation. In fact, previous studies with AGE-LDL demonstrated an increased inflammatory state due to irreversible glycation products, with increasing of IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1) production (37-40). Although these studies used AGE-LDL without knowing whose specific compounds were responsible for the production of the elevated inflammatory levels. Since LDL have both proteins and lipids, and both of them can undergo glycation and oxidation, it seems reasonable to separate both lipidic and proteic fractions of LDL to evaluate their effects. Regarding that, Sonoki *et al*. correlated the lyso-phosphatidylcholine (formed by oxidation of PC) content in LDL with MCP-1 mRNA expression in human umbilical vein endothelial cells (HUVEC) (41). Besides the studies with AGE-LDL, studies with AGE-albumin revealed induction of expression of VEGF, MCP-1, TNF-α, IL-8, IL-1β, MIP-1β, macrophage-colony stimulating factor (M-CSF) and expression of tissue factor in macrophages due to different AGE-albumin (42-49) and also induction of DCs maturation, which enlarge their capacity to stimulate T-cell (50). To our knowledge no studies were done with glycated phospholipids. Here we stimulated mDCs/monocytes with free GluPE and we demonstrated that glycated phospholipids alone can induce stimulation of cells. So we supposed that PE glycation is the major feature, above other structural modifications of phospholipids, involved in increasing the inflammatory state.

Overall for both cell populations, it was observed that after stimulation with non-modified POPE and modified POPE, there were a higher percentage of cells producing cytokines due to stimulation with glycated compounds (either oxidized or not), in comparison to the correspondent (oxidized or non-oxidized) non-glycated compounds. Interestingly oxidized compounds (glycated and non-glycated) had lower effect on cell stimulation than the correspondent non-oxidized compounds. However, these effects were not accompanied by the same results in the amount of cytokine produced *per* cell that are not affected. Even so, it was possible to infer that PE glycation increase the stimulation of circulating monocytes and mDC and this increase in the stimulation of cells appear to be impaired by oxidation of glycated PE.

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DIFFERENT CYTOKINE EXPRESSION IN MONOCYTES AND DENDRITIC CELLS AFTER STIMULATION WITH MODIFIED PHOSPHATIDYLETHANOLAMINES

Cláudia Simões[1], Ana Cristina Silva[1,2], Pedro Domingues[1], Artur Paiva[2], M. Rosário M. Domingues[1]*

[1]Mass Spectrometry Center, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Centro de Histocompatibilidade do Centro, 3000- Coimbra, Portugal

* To whom correspondence should be addressed: Maria R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

E-mail: mrd@ua.pt, telephone number: + 351-234-370698, fax number: +351-234-370084

ABSTRACT

Glycation of phosphatidylethanolamine (PE) is a reaction that is known to occur under the chronic hyperglycemic conditions found in diabetic patients. Glycated phosphatidylethanolamines were found in plasma and atherosclerotic plaques of diabetic patients, and its presence was correlated with increased oxidative stress. Oxidative stress can also promote the formation of new glycoxidized PE species. Although it is recognized that glycated proteins have a role in the onset of inflammation and diabetes related diseases. Moreover, upregulation of cytokines and other inflammatory mediators can be observed not only in diabetes, but also under oxidized phosphatidylcholine stimulation. In this study, we evaluated the effect of dipalmitoyl-phosphatidylethanolamine (DPPE) and lineolyl-palmitoyl-phosphatidylethanolamine (PLPE) structural oxidation (OxPE), glycation (GluPE) and glycoxidation (GluOxPE), on monocyte and myeloid dendritic cell (mDC) stimulation. Expression of cytokines, IL-1β, IL-6, IL-8, MIP-1β and TNF-α, were determined, using flow cytometry after stimulation of monocytes and myeloid dendritic cells with native PEs, oxidized PEs, glycated PEs and glycoxidized PEs. Native PE, PLPE and DPPE, and all modified PEs were able to increase the stimulation levels of monocytes and mDCs in relation to basal state. Variations in stimulation levels induced by the different PE modifications were observed in the frequency of cytokine-producing cells, but not in the amount of cytokine produced *per* cell. Generally, in monocytes and mDCs stimulation, GluOxPLPE and GluDPPE were the PLPE/DPPE modifications that induced the most pronounced increase in cytokine production. However the GluOxDPPE was the DPPE modification that produced the lowest stimulation levels of mDCs and monocytes. Our results indicate that glycated PE and glycoxidized PE may have an important role in contributing to the low-grade systemic inflammation associated with diabetes and to the development of diabetic complications.

KEYWORDS: phospholipids, glycoxidation, glycation, oxidation, flow cytometry, atherosclerosis, diabetes, inflammation.

INTRODUCTION

Phosphatidylethanolamine (PE) is the second most abundant phospholipid class in mammalian cell membranes and lipoproteins (1). Modifications in PE structure can have deleterious effects on the properties of cellular membranes, altering their function and conditioning cell homeostasis. PE can be modified in consequence of oxidation in the unsaturated fatty acyl chains and/ or due to glycation in the polar head group, particularly in hyperglycemia (2, 3). PE oxidation products (4-7) and glycated PE (8-11) were found *in vivo* in retina, in plasma and erythrocytes, and have been associated with retinopathy (7), blood coagulation (12) and diabetic complications, such as atherosclerosis (3). In general, the oxidation of other phospholipids has been associated with other pathophysiological conditions, including inflammation (2, 4, 13). Nevertheless, the biological effects in inflammation and immune response of modified PE, namely oxidized PE, glycated PE and glycoxidized PE were not been comprehensively studied.

It is well known that oxidized lipids are important signaling molecules in inflammation and immune response and can play distinct biological roles, depending on their specific structure (4). Oxidized phosphatidylcholines, and particularly oxidized 2-arachidonoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylcholine (oxPAPC), have been the most intensely studied oxidized phospholipids. So far, oxPAPC is recognized to induce distinct inflammatory effects, ranging from anti-inflammatory to pro-inflammatory properties, effects on blood coagulation, on endothelial barrier and angiogenic activity, as recently reviewed (4). The oxidized phospholipids are recognized by innate and adaptive immune system through antibodies (IgG and IgM) directed to oxidized LDL, binding to scavenger receptors (like CD36) and to C-reactive protein, leading to activation of various intracellular signaling mechanisms. Pro-inflammatory effects of oxidized phospholipids include the ability to stimulate the production of chemokines, activation of adhesion molecules and adhesion of monocytes to endothelial cells. Those are important mechanisms involved in foam cell formation and development of atherosclerotic lesions, in which oxPAPC can play a role (4).

Immunological changes, including altered levels of specific cytokines, as well as changes in the number and activation state of different leukocyte populations and increased cell apoptosis, were also found in diabetes mellitus (14-16). The upregulation of cytokines and other inflammatory mediators, observed during diabetes, leads to persistent low-grade inflammation and endothelial dysfunction that ultimately leads to diabetic vascular complications, including atherosclerosis (14, 17, 18). However, the mediators involved in the inflammation processes and their role in the development of diabetic microvascular diseases is still unclear (17, 18). Additionally, it is well known that oxidative stress is enhanced under chronic hyperglycemia conditions like those found in diabetes mellitus. Glycated PE increases oxidative stress and promote oxidation of other biomolecules (11, 19). Also, the oxidation of glycated PE species generates advanced glycated end products (AGES) (20, 21) and other oxidation products (22). Lipid AGEs and lipid oxidation was found to be increased in low density lipoprotein (LDL) of diabetic patients (23) and AGEs-PE species were detected in erythrocytes and plasma of diabetic patients (24). This suggests that the presence of oxidized, glycated and/or glycoxidized PE could have an active role in the upregulation of cytokines and in the development of the inflammatory status associated with diabetes.

In this study, we analyzed the potential of oxidized PE, glycated PE and glycoxidized PE to stimulate peripheral blood monocytes and dendritic cells. Two PE species, bearing different *sn*-2 fatty acyl chains, namely 2-linoleoyl-1-palmitoyl-phosphatidylethanolamine (PLPE) and 1,2-dipalmitoyl-phosphatidylethanolamine (DPPE) were used. PLPE is susceptible to oxidation in acyl chain, but in contrary, DPPE is not, because contains two saturated fatty acids. These PE were selected to evaluate the oxidative modifications of acyl chain and polar head group in the stimulation of monocytes and dendritic cells. Expression of various cytokines was determined by flow cytometry after stimulation of the immune cells with native PE, oxidized PE, glycated PE and glycoxidized PE.

EXPERIMENTAL

Chemicals

2-linoleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), were purchased from Sigma-Aldrich (Madrid, Spain). $FeCl_2$ and H_2O_2 (30%, w/v) used for the peroxidation reaction were acquired from Merck (Darmstadt, Germany). The water was of MilliQ purity filtered through a 0.22-µm filter (Millipore, USA), and all solvents used were HPLC grade. For cell stimulation, RPMI-1640 medium was purchased from Gibco (Paisley, Scotland, UK) Brefeldin-A and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5) from Sigma (St. Louis, MO, USA) and interferon-γ (IFN-γ) from Promega (Madison, WI, USA). The conjugated monoclonal antibodies: anti-HLA-DR peridin chlorophyll protein (PerCPcy 5.5) (clone L243), anti-CD33 allophycocyanin (APC) (clone P67.6), anti-interleukin (IL)-1β phycoerythin (PE) (clone AS10) and anti-IL-8 PE (clone AS14A) were purchased from BDB (San Jose, CA, USA); anti-IL-6 PE (clone MQ2- 6A3), macrophage inflammatory protein (MIP)-1β PE (clone D21-1351) and anti-tumor necrosis factor (TNF)-α PE (clone Mab11) was purchased from BD Pharmingen (San Diego, CA, USA) and Dendritic Cell Exclusion Kit flourescein isothiocyanate (FITC) were purchased from Cytognos (Salamanca, Spain). Phosphate-buffered saline (PBS) was purchased from Dulbecco 1x, Biochrom AG (Berlin, Germany) and permeabilization kit, INTRACELL, was purchased from Immunostep (Salamanca, Spain).

Preparation of Glycated PE and Oxidation Mixtures

Phospholipids (PLPE and DPPE) (final concentration of 3 µM) were glycated in a methanol/water solution supplemented with glucose (0.5 mM), during 30 minutes in boiling water. Then, glycated PE was extracted from the reaction mixture using a modified Folch method (25),

and purified by HPLC as reported before (22). The glycated and non-glycated fractions were collected, dried and oxidized under the following conditions:

The phospholipid vesicles (non-glycated PE and glycated PE) were prepared from stock solutions of 1 mg/mL dried under nitrogen stream. Ammonium bicarbonate buffer (5 mM, pH 7.4) was added to 50 mM of phospholipid and the mixture was vortexed (26). Oxidation was initiated by adding $FeCl_2$ to final concentration of 0.5 mM and H_2O_2 to a final concentration of 50mM. Non-oxidized PE and glycated PE were prepared the same way but using water instead of hydrogen peroxide. The mixture was incubated at 37°C in the dark for 5 days, after that the compounds were extracted using a modification of the Folch method (25) with chloroform-methanol (2:1, v/v) and re-suspended in same volume of PBS. Phospholipid vesicles were prepared by the addition of PBS to dried phospholipids and glycated phospholipid extracts and vortexed for 10 minutes.

Reactions of glycation and oxidation were controlled by ESI mass spectra, acquired on a Waters (Micromass, Manchester, UK) Q-TOF2 (Micromass, Manchester, UK) equipped with a Z-spray ESI source and/or on a linear ion trap LXQ (ThermoFinnigan, San Jose, CA, USA) mass spectrometers.

Samples

A total of 10 peripheral blood (PB) samples from healthy adult subjects (4 males and 6 females) were analyzed. Informed consent, according to local ethics committee, was obtained from all individuals enrolled in this study. Monocytes and myeloid dendritic cells (mDC) were stimulated in separated cultures. 5 samples were utilized for monocyte stimulation (mean age: 29+/- 7 years), and the other 5 samples were utilized for mDC cultures (mean age: 26+/- 2 years). All PB samples were collected in heparin as anticoagulant and immediately processed as follow.

In vitro Stimulation of Cytokine Production by mDCs and Monocytes

Stimulation of monocytes and mDCs were performed in PB samples that were previously diluted 1:1 (vol/vol) in RPMI 1640 complete culture medium, supplemented with 2 mM L-glutamine. To prevent the cellular release of cytokines, Brefeldin-A (10 µg/ml) was added to all tubes. Phospholipids mixtures (20 µg) were added alone (monocyte stimulation) or in combination with 100 U/ml of IFN-γ (mDC stimulation) to the respective tube. Positive control was performed by addition of LPS (100 ng/ml) alone (monocyte stimulation) or in combination with 100 U/ml of IFN-γ (mDC stimulation); and the negative control was performed in the absence of both LPS and IFN-γ. All samples were then incubated in a sterile environment and 5% CO_2 humid atmosphere, at 37ºC during 6 hours.

The phospholipid mixtures tested for cell stimulation were native PLPE, native DPPE, oxidized PLPE, oxidized DPPE, glycated PLPE, glycated DPPE, glycoxidized PLPE and glycoxidized DPPE.

Flow Cytometric Analysis of Intracytoplasmic Cytokine Production

After incubation, all samples (phospholipid stimulations, positive and negative controls) were distributed in different tubes (200 µl diluted PB/tube) and stained with monoclonal antibodies, in order to analyze the expression of the different cytokines under study. For that purpose, 10 µl of HLA-DR-PerCPcy 5.5 and 5 µl of CD33-APC were added to each tube. In case of mDC subset, 10 µl of Dendritic Cell Exclusion Kit were additionally added. Cells were sequentially incubated for 10 min at room temperature, in the darkness, washed once with 2 ml of PBS (5 min at 540x g) and the supernatant was discarded. Then cells were fixed and permeabilized with INTRACELL according to manufacturer's instructions and stained with PE-conjugated mAb directed against different human intracytoplasmatic cytokines: IL-1β (10 µl), IL-6 (10 µl), IL-8 (10 µl), MIP-1β (1.5 µl) and TNF-α (1.5 µl). Each anti-cytokine mAb was placed in a different tube containing either the stimulated or the unstimulated sample. The samples were incubated for 10 min at room temperature in the darkness,

washed twice with 2 ml of PBS (5 min at 540x g) and resuspended in 250 µl of PBS for flow cytometry acquisition.

Flow Cytometry Data Acquisition and Analysis

Data acquisition was performed in a FACSCalibur flow cytometer (BDB, San Jose, USA) equipped with an argon ion laser and a red diode laser, and was different for mDC and monocyte samples. Data from mDC samples were acquired in two consecutive steps. First, information of all nucleated cells ($2x10^4$ events), was collected and stored. Secondly, information of HLA-DR^+ events (minimum of $2x10^5$ events) was collected in a live gate and stored. The software programs used for data acquisition and analysis were, respectively, the CellQuest (BDB, San Jose, USA) and INFINICYT (Cytognos, Salamanca, Spain). mDCs were identified by the combined expression of CD33 and HLA-DR and absence of CD14, CD19, CD56 and CD3 with intermediate forward (FSC) and side scatter (SSC) between those of lymphocytes and monocytes. Data from monocytes were acquired in one step, where all nucleated cells ($5x10^5$ events) present in the sample, were collected and information on them stored. Monocytes were identified through the expression CD33 and HLA-DR and their FSC and SSC characteristics.

Statistical Methods

Mean values and standard deviations, as well as median values and range, were calculated for each variable under study by using the SPSS software program (SPSS 17.0, Chicago, USA). The statistical significance of the differences observed between groups was assessed using the nonparametric Mann-Whitney U test. p values < 0.05 were considered to be associated with statistically significant differences between groups.

RESULTS

The oxidation products of DPPE, PLPE and the oxidation products of their glycated derivatives were identified recently using LC-MS and MS/MS (22, 27). It was found that DPPE is hardly oxidized due to the presence of two saturated fatty acyl chains, although some oxidation may occur at polar head group. In turn, PLPE oxidation was more extensive and the oxidation products formed were generally due to oxidation at linoleoyl *sn*-2 acyl chain. Furthermore, it was found that glycated derivatives originated more oxidized species than the non-glycated compounds, as result of oxidation of the glucose moiety and oxidative cleavage of glycated polar head group. Thus, oxidation of glycated PLPE, can yield oxidation products with modifications simultaneously at glycated polar head and at *sn*-2 unsaturated acyl chain (22, 27, 28).

The capability of these native PE (DPPE and PLPE) and the correspondent modified PE: glycated PE (GluPE), oxidized PE (OxPE) and glycoxidized PE (GluOxPE), to induce the production of different cytokines (IL-1β, IL-6, IL-8, MIP-1β and TNF-α) in peripheral blood monocytes and mDCs were studied. The frequency of cytokine-producing cells (monocytes or mDC), as well as the total amount of cytokines produced *per* cell, expressed as mean fluorescence intensity (MFI), were determined for each condition. The differences induced in the activation of antigen-presenting cells due to fatty acyl composition/oxidation and/or glycation were also evaluated.

Cytokine-producing monocytes and mDCs after stimulation with native PE (DPPE and PLPE)

The conditions used for stimulation of monocytes and mDCs were optimized with non-modified PLPE and DPPE. The parameters tested were the amount of PE and the presence or absence of IFN-γ. The amount of PLPE and DPPE that produced higher frequency of cytokine-producing cells (monocytes and mDCs) was found to be 20 µg. For higher (30, 40 and 50 µg) and

lower (10 µg) amounts of PE, lower frequency of cytokine-producing cells (monocytes and mDCs) were obtained. The addition of IFN-γ was not needed to obtain a response of monocytes to PE stimulus, however mDCs did not respond to PE stimulation in the absence of IFN-γ, thus IFN-γ was added to all mDCs stimulating experiments.

The percentage of cytokine-producing cells and the amount of cytokine produced *per* cell induced by PLPE and DPPE stimulation, in monocytes and mDC, were compared with basal production level (negative control) and LPS stimulus (positive control). Non-modified PLPE and DPPE were found to induce significant increase of the percentage of cells (monocytes and mDCs) that express the cytokines under study (IL-1β, IL-6, IL-8, MIP-1β and TNF-α) in comparison to negative control ($p<0.05$; Table 1). However those percentages were always lower than the ones obtained after LPS stimulation (positive control). With respect to the amount of cytokines produced *per* cell, given by mean fluorescent intensity (MFI), native PLPE and DPPE also induced higher values than negative control and lower than LPS stimulus, for all cytokines in study. Statistically significant increases were observed in TNF-α produced by monocytes and in IL-1β, IL-6, IL-8 produced by mDCs after stimulation with DPPE and in MIP-1β produced by mDCs after stimulation with PLPE. Interestingly, for mDC, either the amount of all cytokines and the frequency of mDC expressing cytokines, tend to be higher upon stimulation with DPPE than with PLPE.

TABLE 1 – Frequency of cytokine-producing monocytes and mDC, and amount of cytokine produced *per* cell (MFI), without stimulus (negative control) and after stimulation with LPS (positive control), DPPE and PLPE. mDC cultures were carried out in the presence of IFN-γ, while the monocyte cultures were preformed without IFN-γ.

		Monocyte		**mDC**	
		% positive cells	MFI	% positive cells	MFI
TNF-α	Control	5.7 ± 2.5	68 ± 53	6.6 ± 4.1	22 ± 8
	LPS	83.3 ± 14.9	275 ± 172	82.1 ± 19.4	56 ± 52
	DPPE	57.1 ± 24.6[a]	193 ± 116[a]	53.9 ± 19.8[a]	21 ± 4
	PLPE	60.1 ± 6.6[b]	101 ± 79	19.6 ± 9.0[b]	17 ± 4
MIP-1β	Control	12.7 ± 4.8	194 ± 133	8.3 ± 6.2	33 ± 14
	LPS	90.0 ± 10.8	294 ± 196	88.3 ± 20.3	98 ± 56
	DPPE	61.6 ± 36.4[a]	163 ± 88	47.5 ± 19.9[a]	39 ± 23
	PLPE	64.3 ± 30.3[b]	141 ± 93	23.2 ± 8.9[b]	55 ± 34[b]

IL-6	Control	6.2 ± 3.7	23 ± 8	7.1 ± 4.4	16 ± 5
	LPS	88.1 ± 13.0	50 ± 13	81.7 ± 9.4	43 ± 22
	DPPE	59.1 ± 27.5[a]	36 ± 22	61.2 ± 17.1[a]	27 ± 7[a]
	PLPE	50.1 ± 27.8[b]	31 ± 19	22.5 ± 12.2[b]	16 ± 3
IL-8	Control	6.8 ± 3.1	22 ± 6	5.7 ± 2.9	16 ± 5
	LPS	83.6 ± 16.7	39 ± 19	89.5 ± 9.5	32 ± 17
	DPPE	71.4 ± 20.8[a]	28 ± 16	64.2 ± 23.7[a]	25 ± 8[a]
	PLPE	37.9 ± 24.2[b]	26 ± 14	22.5 ± 10.7[b]	19 ± 5
IL-1β	Control	8.6 ± 3.7	22 ± 6	4.5 ± 2.1	17 ± 4
	LPS	88.2 ± 15.6	49 ± 16	87.0 ± 8.2	33 ± 11
	DPPE	56.3 ± 39.8[a]	32 ± 20	33.2 ± 21.0[a]	25 ± 4[a]
	PLPE	61.7 ± 26.8[b]	28 ± 12	16.2 ± 3.5[b]	17 ± 1

Results are expressed as mean ± standard deviation. MFI, mean fluorescence intensity of positive cells. Statistically significant differences were considered when $p < 0.05$ (Mann–Whitney U test). [a DPPE *versus* Negative Control] [b PLPE *versus* Negative Control].

Cytokine-producing monocytes and mDC after stimulation with modified PE

Knowing that PLPE and DPPE are able to stimulate monocytes and mDCs, the next goal was to evaluate how the modifications of PE (oxidation, glycation and glycoxidation) affect cytokine production (IL-1β, IL-6, IL-8, MIP-1β and TNF-α) in monocytes and mDC. It was found that all of those PE modifications were able to induce an increased frequency of cells producing cytokines relative to the negative control (Tables 2 and 3 and Figures 1 and 2). However variations in the stimulation levels were dependent on the PE modification, cell type and/or cytokine considered.

Stimulation with oxidized PE (OxDPPE and OxPLPE)

Stimulation of monocytes with oxidized DPPE (OxDPPE) showed frequencies of cells expressing cytokines similar to non-modified DPPE stimulation, probably due to the very low oxidation of this PE (Table 2 and Figure 1). Oxidized PLPE was found to decrease the frequency of monocytes expressing all cytokines when compared with native PLPE. This decrease in the frequency of monocytes producing cytokines treated with OxPLPE was statistically significant for IL-1β and TNF-α ($p < 0.05$). However, the frequency of monocytes expressing cytokines was

higher under OxPLPE stimulation than in the basal state. The number of cytokines produced *per* monocyte (MFI) upon OxDPPE/OxPLPE stimulation, when compared with DPPE/PLPE stimulation, was almost identical (Tables 2 and 3).

TABLE 2 – Frequency of cytokine producing monocyte and mDC and amount of each cytokine per cell (MFI), following DPPE, GluDPPE, OxDPPE, GluOxDPPE plus IFN-γ stimulation.

		Monocyte		mDC	
		% positive cells	MFI	% positive cells	MFI
TNF-α	DPPE	57.1 ± 24.6	193 ± 116	53.9 ± 19.8	21 ± 4
	OxDPPE	58.1 ± 37.8	237 ± 133[b]	25.1 ± 3.2[a]	19 ± 6
	GluDPPE	60.6 ± 31.0	190 ± 35[b]	32.8 ± 14.6	18 ± 5
	GluOxDPPE	39.6 ± 36.4[a]	30 ± 14	24.1 ± 14.3[a]	17 ± 5
MIP-1β	DPPE	61.6 ± 36.4	163 ± 88	47.5 ± 19.9	39 ± 23
	OxDPPE	67.9 ± 33.4	123 ± 78	55.4 ± 8.0[b]	44 ± 21
	GluDPPE	69.5 ± 38.7	158 ± 84	68.2 ± 28.8[b]	34 ± 13
	GluOxDPPE	53.5 ± 29.2	115 ± 80	18.3 ± 5.3[a]	29 ± 12
IL-6	DPPE	59.1 ± 27.5	36 ± 22	61.2 ± 17.1	27 ± 7
	OxDPPE	61.4 ± 32.8	41 ± 24	44.3 ± 12.6[b]	20 ± 6
	GluDPPE	76.0 ± 14.9	40 ± 20	35.4 ± 9.6[a,b]	19 ± 6[a]
	GluOxDPPE	34.5 ± 20.8	28 ± 13	18.9 ± 7.1[a]	16 ± 6[a]
IL-8	DPPE	71.4 ± 20.8	28 ± 16	64.2 ± 23.7	25 ± 8
	OxDPPE	56.1 ± 31.1	26 ± 14	62.2 ± 19.3[b]	18 ± 4
	GluDPPE	68.5 ± 22.3[b]	23 ± 7	26.0 ± 8.2[a]	18 ± 4
	GluOxDPPE	50.6 ± 27.1	19 ± 5	22.8 ± 11.2[a]	16 ± 5[a]
IL-1β	DPPE	56.3 ± 39.8	32 ± 20	33.2 ± 21.0	25 ± 4
	OxDPPE	66.3 ± 29.0	27 ± 16	26.4 ± 10.2[b]	20 ± 6
	GluDPPE	61.9 ± 35.4	31 ± 17	53.4 ± 27.3[b]	21 ± 4
	GluOxDPPE	42.4 ± 37.3	32 ± 26	15.4 ± 9.8[a]	18 ± 5

Results are expressed as mean ± standard deviation. MFI, mean fluorescence intensity of positive cells. Statistically significant differences were considered when $p < 0.05$ (Mann–Whitney U test). [a] shows statistical significance when compared with DPPE, and [b] statistical significance when compared with GluOxDPPE.

Stimulation of mDCs with OxDPPE induced a significant decrease of the frequency of TNF-α producing-cells related to stimulation with non-modified DPPE. Stimulation of mDCs with OxPLPE did not induce significant variations on the frequency of cytokine-producing cells when compared with non-modified PLPE. The number of cytokines produced *per* mDCs (MFI) upon

OxDPPE/OxPLPE stimulation, when compared with DPPE/PLPE stimulation, was almost the same, with exception of the MFI of TNF-α, that was increased under OxPLPE stimulation (Tables 2 and 3).

TABLE 3 – Frequency of cytokine producing monocyte and mDC and amount of each cytokine per cell (MFI), following PLPE, GluPLPE, OxPLPE, GluOxPLPE plus IFN-γ stimulation.

		Monocyte		mDC	
		% positive cells	MFI	% positive cells	MFI
TNF-α	PLPE	60.1 ± 6.6	101 ± 79	19.6 ± 9.0	17 ± 4
	OxPLPE	20.5 ± 17.2[a,b]	54 ± 22	22.9 ± 12.6	20 ± 9[a]
	GluPLPE	37.9 ± 26.1	88 ± 42	20.5 ± 6.9	15 ± 2[a]
	GluOxPLPE	66.8 ± 17.6	103 ± 63	26.6 ± 8.8	16 ± 2
MIP-1β	PLPE	64.3 ± 30.3	141 ± 93	23.2 ± 8.9	55 ± 34
	OxPLPE	56.1 ± 25.9	101 ± 58[b]	32.2 ± 23.3	33 ± 9
	GluPLPE	52.7 ± 26.9	120 ± 83[b]	13.5 ± 6.4[b]	28 ± 9
	GluOxPLPE	61.7 ± 35.9	241 ± 56	24.2 ± 6.5[a]	30 ± 6
IL-6	PLPE	50.1 ± 27.8	31 ± 19	22.5 ± 12.2	16 ± 3
	OxPLPE	21.6 ± 10.1	25 ± 14	42.6 ± 34.7	15 ± 2
	GluPLPE	30.8 ± 17.1	40 ± 30	21.3 ± 5.4	14 ± 3
	GluOxPLPE	50.2 ± 34.3	37 ± 26	19.9 ± 11.5	16 ± 4
IL-8	PLPE	37.9 ± 24.2	26 ± 14	22.5 ± 10.7	19 ± 5
	OxPLPE	19.2 ± 9.9	21 ± 6	25.9 ± 16.3	15 ± 2
	GluPLPE	41.9 ± 27.5	49 ± 37	15.8 ± 5.2[a]	15 ± 3[a]
	GluOxPLPE	56.4 ± 34.6	32 ± 20	18.3 ± 5.2	17 ± 3
IL-1β	PLPE	61.7 ± 26.8	28 ± 12	16.2 ± 3.5	17 ± 1
	OxPLPE	18.1 ± 12.0[a]	23 ± 6	16.4 ± 8.5	16 ± 1
	GluPLPE	40.9 ± 10.1	34 ± 24	42.6 ± 35.9	17 ± 3
	GluOxPLPE	49.2 ± 37.5	32 ± 19	18.6 ± 4.7	16 ± 1

Results are expressed as mean ± standard deviation. MFI, mean fluorescence intensity of positive cells. Statistically significant differences were considered when $p < 0.05$ (Mann–Whitney U test). [a] shows statistical significance when compared with DPPE, and [b] statistical significance when compared with GluOxDPPE.

Stimulation with glycated PE (GluDPPE and GluPLPE)

The effect of glycated PE on monocytes and mDCs stimulation levels was also evaluated, when compared with the effect of non-modified PE. In monocytes, GluDPPE and GluPLPE did not show any significant differences in the frequency of cells expressing cytokines. The number of

cytokines produced *per* monocyte (MFI) upon GluDPPE/GluPLPE stimulation, when compared with DPPE/PLPE stimulation, showed no significant variations (Tables 2 and 3). Besides GluDPPE and GluPLPE stimulation were similar to the DPPE/PLPE, they both induced higher frequency of monocytes expressing cytokines when compared with the basal state.

In mDCs, both GluDPPE and GluPLPE induced a decrease of the frequency of cells expressing several cytokines in comparison with non-modified PE. The effects of GluPE in mDCs were more evident than on monocytes, and some statistically significant decreases were found in mDCs. The frequency of cells expressing IL-8 was significantly reduced upon stimulation with GluDPPE or GluPLPE. Also, the frequency of mDCs expressing IL-6 was significantly reduced, but only upon stimulation with GluDPPE. The amount of cytokines produced *per* cell (MFI) did not change significantly with exposition to glycated PE. However the MFI of TNF-α and IL-8 that was significantly reduced under GluPLPE stimulation and MFI of IL-6 that was significantly reduced under GluDPPE stimulation.

Stimulation with glycoxidized PE (GluOxPLPE or GluOxDPPE)

Glycoxidized PE (GluOxPLPE or GluOxDPPE) effects on cell stimulation, when compared to glycated PE (GluPE), are different upon GluOxDPPE and GluOxPLPE, either in monocytes and mDCs. GluOxDPPE significantly decreased the frequency of monocytes expressing IL-8 and the frequency of mDCs expressing MIP-1β, IL-6 and IL-1β. In opposition, GluOxPLPE significantly increased the frequency of mDCs expressing MIP-1β. Also, the MFI of TNF-α was significantly decreased in monocytes stimulated with GluOxDPPE, while the MFI of MIP-1β was significantly increased in monocytes stimulated with GluOxPLPE.

Additionally Glycoxidized PE (GluOxPLPE or GluOxDPPE) effects on cell stimulation, when compared to oxidized PE (OxPE), are also different upon GluOxDPPE and GluOxPLPE. GluOxDPPE stimulation significantly reduced the frequency of mDCs expressing all cytokines in study, except TNF-α. While GluOxPLPE stimulation significantly increased the frequency of monocytes expressing TNF-α. Accordingly, the amount of TNF-α produced *per* monocyte, given by

MFI, was significantly reduced under GluOxDPPE and the amount of MIP-1β produced *per* monocyte, was significantly increased under GluOxPLPE.

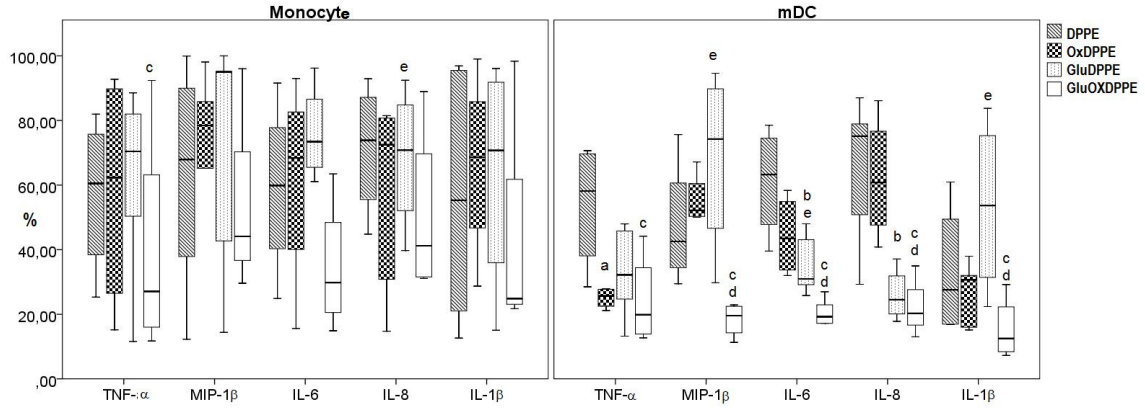

FIGURE 1 – Frequency of cytokine producing monocyte and mDC after stimulation with DPPE, GluDPPE, OxDPPE and GluOxDPPE. The mDC cultures were carried out in the presence of IFN-γ, while the monocyte cultures were performed without IFN-γ. Statistically significant differences were considered when $p<0.05$ (Mann–Whitney U test). [a DPPE versus OxDPPE] [b DPPE versus GluDPPE] [c DPPE versus GluOxDPPE] [d OxDPPE versus GluOxDPPE] [e GluOxDPPE versus GluDPPE].

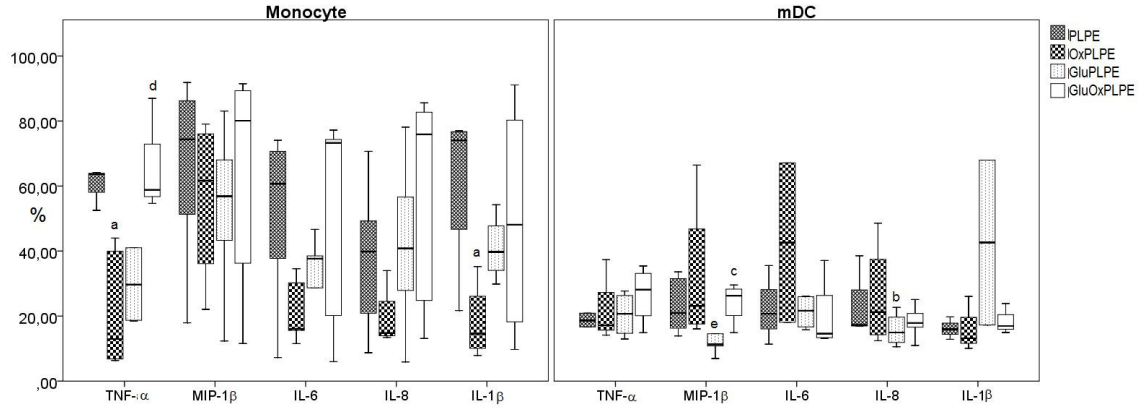

FIGURE 2 – Frequency of cytokine producing monocyte and mDC after stimulation with PLPE, GluPLPE, OxPLPE and GluOxPLPE. The mDC cultures were carried out in the presence of IFN-γ, while the monocyte cultures were performed without IFN-γ. Statistically significant differences were considered when $p<0.05$ (Mann–Whitney U test). [a PLPE versus OxPLPE] [b PLPE versus GluPLPE] [c PLPE versus GluOxPLPE] [d OxPLPE versus GluOxPLPE] [e GluOxPLPE versus GluPLPE].

In summary, our results demonstrated that different oxidative modifications (oxidation on polar head or oxidation on acyl chains) and glycation had different effects on cell stimulation and affect differentially monocytes and mDCs. Both native and modified PE had the ability to stimulate peripheral blood monocytes and mDC, as they raised the percentage of cytokine producing cells,

though to a lesser extent than LPS did. However, the effect on the frequency of monocytes/mDC producing cytokines was not the same observed on the amount of cytokine produce *per* monocytes/mDC. Although the amount of cytokine *per* cell did not change in most cases, the total amount of cytokine produced was enhanced or reduced due to the variations in the frequency of cell producing cytokines. These results also showed that in monocytes and mDCs, GluDPPE, was the DPPE modification that showed a more pronounced effect in enhancing the expression of cytokines. Among the modifications of PLPE studied, the more pronounced effect was observed when using GluOxPLPE. In contrast, GluOxDPPE was the DPPE modification that showed a less pronounced effect in increasing the expression of cytokines, either in monocytes and mDCs.

DISCUSSION

The effects of PAPC oxidation products on inflammation have been characterized and shown to stimulate several cell types and various signal-transducing pathways, as reviewed elsewhere (4). IL-8 is the best documented cytokine known to be increased due to OxPL stimulation (4, 29-31). Also, it has been reported that in human derived macrophages TNF-α, IL-6 and IL-1β production can be enhanced by oxidized PC (32, 33). In the present work, we aimed to evaluate the influence of different PE modifications (oxidation and glycation) on monocyte and mDCs stimulation. Particularly, the production of the pro-inflammatory cytokines, namely IL-1β, IL-6, IL-8, MIP-1β and TNF-α, were studied.

Our results showed that glycation and oxidative modifications of DPPE and PLPE had different effects on immune cells. Monocytes and mDCs responded differently to the same oxidized compounds, specifically in the case of PLPE. mDCs were more susceptible to PE stimulation, showing higher occurrence of statistically significant differences. Stimulation with glycated PE induced higher cell stimulation levels than with non-glycated compounds (GluDPPE > DPPE and GluOxPLPE > OxPLPE) similar to what was observed before with glycated POPE (34). However, oxidized compounds usually induced lower stimulation levels than the non-oxidized correspondent

compounds (OxDPPE < DPPE, GluOxDPPE < GluDPPE and OxPLPE < PLPE). Some exceptions were found, particularly when studying PLPE modifications, where GluPLPE and GluOxPLPE were respectively responsible for inducing the smallest and highest increase of monocytes and mDCs stimulation.

When comparing non-modified compounds, DPPE was found to induce higher frequency of cell expressing cytokines than PLPE, probably because of the presence of saturated fatty acid in the *sn*-2 position, which is unusual in living systems. The saturated fatty acid in the *sn*-2 position is resistant to oxidation, so the oxidation in DPPE is minimal and only occurs in the polar head. One reason for the levels of monocyte stimulation with OxDPPE incubation were not reduced, when compared native DPPE. Interestingly, in mDCs, OxDPPE incubation decreased the frequency of cells expressing cytokines, when compared with the non-modified DPPE. In accordance to what was observed with OxPOPE (34) and OxPLPE incubations, when compared with the respective native PE, that reduced the cell stimulation.

GluDPPE incubation increased the stimulation of monocytes when compared with DPPE incubation, as previously described for GluPOPE (34). However, GluDPPE decreased mDCs stimulation when compared with DPPE, the stimulation induced was the highest among all studied DPPE modifications. Additionally, GluPLPE decreased monocyte stimulation and mDCs stimulation when compared with native PLPE. The levels of monocytes and mDCs stimulation were also different upon incubation with GluOxDPPE and GluOxPLPE, when compared with GluDPPE and GluPLPE respectively. While GluOxDPPE was found to decrease monocytes and mDCs stimulation, GluOxPLPE was found to increase those cells stimulation. The decreased stimulation observed with GluOxDPPE incubation is in agreement with the previous findings when using GluPOPE stimulation (34). This behavior can be explained by the formation of oxidation products leading to the loss of glucose moiety and, consequently, to the reduction of the capability for cell stimulation. Still, the increase in monocyte and mDCs stimulation related to GluOxPLPE, can be due to the fact that glycated compounds produce higher amounts of oxidation products (22). In addition, GluOxPLPE can have oxidative modifications simultaneously in polar head and in the fatty acyl chain, which could be responsible for the enhanced cytokine production. This suggests that PL

which are difficult to be oxidized, as POPE and DPPE, or those with low oxidation levels, would produce lower stimulation levels of monocytes and mDCs. Whereas PL with higher oxidation levels, like those observed with GluPLPE would produce higher levels of monocytes and mDCs stimulation.

It is interesting to note that, in mDCs, MIP-1β was the cytokine that was most affected upon stimulation with GluOxPE. Thus, MIP-1β production could be more affected upon glycoxidized PE stimulation than the other cytokines. MIP-1β is a chemotactic cytokine, involved in acute and chronic inflammatory responses mainly by recruiting pro-inflammatory cells (monocytes, dendritic, NK and T cells) (35). Also the expression of this cytokine in OxPAPC-stimulated murine arteries has been reported (36). We have also observed that mDCs stimulations with GluDPPE and GluPLPE enhanced the frequency of cells producing IL-1β. IL-1β is a pro-inflammatory cytokine and an important signaling molecule in the immune response. Increased IL-1β levels were associated with type 2 diabetes mellitus related inflammatory state (37). This cytokine has also an important role in chronic inflammation, increasing the expression of adhesion molecules and is also an angiogenic factor (38).

The present results revealed that glycated PE and glycoxidized PE may have important inflammatory effect and may play a role in endothelial cell activation and monocyte adhesion, which are thought to be important in atheroma formation.

CONCLUSION

The results showed that native PE and all PE modifications studied in this work induced different levels of cell stimulation, but all to levels that were higher than basal state. Stimulation of monocytes with non-modified DPPE and modified DPPE leaded to a higher percentage of cells producing cytokines. The observed levels of induction could be ordered as follows: stimulation with glycated PE compounds > oxidized PE compounds > glycoxidized PE compounds. In mDCs, the

order of stimulation was non-modified DPPE > glycated > oxidized > glycoxidized compounds. After stimulation of monocytes with non-modified PLPE and modified PLPE the higher frequency of cells producing cytokines was observed when incubating cells with glycoxidized PLPE. Oxidized PLPE in monocyte stimulation and glycated PLPE in mDC stimulation were the found to have lower stimulation levels. These effects were not accompanied by the same results in the amount of cytokine produced *per* cell, which did not show major differences. The observations leaded to the hypothesis that cell stimulation is more responsive to modifications in PE polar head. Also, these results showed that that MIP-1β and IL-1β are the cytokines that are most affected by stimulation with glycated and glycoxidized compounds.

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CHAPTER V

ALTERATIONS OF PE SPECIES UNDER HYPERGLYCEMIA

INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disease characterized by hyperglycemia. The hyperglycemia conditions observed in diabetes mellitus are associated with perturbations in lipid homeostasis and increased oxidative stress. Furthermore, hyperglycemia is also associated with PE and protein glycation that, in turn, is associated to increased inflammatory state and oxidative stress. Such disturbances of lipid structure and homeostasis together with increased oxidative stress affect membrane properties and could impair cellular functions. Also, the liver can suffer the deleterious effect of the hyperglycemia and liver disease can arise with the time course of DM.

The present work aimed to evaluate changes in PE species of liver under uncontrolled hyperglycemia, found diabetic rats. The liver was chosen due to its involvement in glucose and lipid homeostasis. The livers from diabetic rats, streptozotocin-injected animals, were collected after four mouths of uncontrolled hyperglycemia and hepatic damage was confirmed by morphologic analysis of liver sections. The variations of phospholipid distribution between classes were determined using phosphorous assays and the variations in PL species were evaluated with HILIC LC-MS and LC-MS/MS. Variations in the fatty acids sterified into lipids were further confirmed by GC-FID.

The results showed a variation in the relative amount of liver phospholipid classes in T1DM rats and a remodeling in PL fatty acyl chains, concomitant with signs of hepatic damage. The PE class was found to be increased in liver of T1DM rats. A remodeling of acyl chains were observed in PE and other classes, and consisted of a reduction in the amount of 16:0 and an increase of 18:0 and 18:2 acyl chains.

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REMODELING OF LIVER PHOSPHOLIPIDOMIC PROFILE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

Cláudia Simões[1], Pedro Domingues[1], Rita Ferreira[1], Francisco Amado[1], José Alberto Duarte[2], Maria João Neuparth[2,3], Cláudia Rocha[4], Iola Duarte[4], M. Rosário M. Domingues[1]*

[1]Mass Spectrometry Center, QOPNA, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[2]CIAFEL, Faculty of Sport, University of Porto, Portugal

[3]CITS, IPSN, CESPU CRL, Famalicão, Portugal

[3]CICECO, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

*To whom correspondence should be addressed: Maria R. M. Domingues, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

E-mail: mrd@ua.pt, telephone number: + 351-234-370698, fax number: +351-234-370084

HIGHLIGHTS

- T1DM is associated with changes in liver lipid profile.
- Relative contents of PC, LPC and SM decrease, while the other PL classes increase.
- Remodeling of acyl chains comprises reduction of 16:0 and increase of 18:0 and 18:2.
- PL changes can have significance in liver dysfunction as a diabetic complication.

ABSTRACT

Background: Hepatic lipid homeostasis is known to be altered with diabetes mellitus, causing disturbances in cellular membrane properties, ultimately leading to liver damage and related complications. The present work aimed to evaluate changes in the liver phospholipid profile after four months of uncontrolled hyperglycemia.

Methods: Twenty Wister rats were divided in two groups: the control group and streptozotocin (T1DM) treated group. After four months, animals from both groups were sacrificed and morphological characterization of liver alterations was performed and related with serum markers of hepatic damage. Total lipid extracts were obtained from liver and phospholipid (PL) classes were quantified. Lipid molecular species were determined by HILIC LC-MS and LC-MS/MS, and lipid fatty acids by GC-MS.

Results: Concomitantly with signs of hepatic damage, we found a variation in the relative amount of liver phospholipid classes in T1DM rats, characterized by a decrease in all PL classes with choline head group, and by an increase in the relative content of other PL classes. A remodeling in PL fatty acyl chains was also observed in T1DM liver, with a similar pattern to all the PL classes. Remodeling of acyl chains consisted of a reduction in the amount of 16:0 and an increase of 18:0 and 18:2 acyl chains.

Conclusions: The observed changes in T1DM lipid content may contribute to altered membrane properties and changes in lipid signaling, which may impact the hepatic function, worsening the metabolic alterations that characterize the pathogenesis of type 1 diabetes mellitus.

KEYWORDS: Phospholipids, fatty acid remodeling, type 1 diabetes mellitus, mass spectrometry, liquid chromatography.

INTRODUCTION

Diabetes mellitus is a common metabolic disease characterized by hyperglycemia, due to insulin deficiency (type 1 Diabetes Mellitus, T1DM) or insulin resistance (type 2 Diabetes Mellitus, T2DM). Uncontrolled hyperglycemia is usually associated with the development of diabetic complications, increasing disease morbidity and mortality with significant impact on society and in economy (1, 2). Therefore, it is crucial to better understand the consequences of uncontrolled hyperglycemia to avoid and prevent the development of diabetes-related diseases.

Recently, different studies highlighted that uncontrolled hyperglycemia can have a deleterious impact in liver homeostasis (3-5). In fact, DM is known to be associated with an increased risk of chronic liver disease, including non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (6, 7). T2DM patients usually have high plasmatic levels of transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which is also not an uncommon feature in T1DM (6, 8, 9). Elevation of plasma ALT (as well as of other liver enzymes) is a signal of liver damage that is usually associated with NAFLD risk factors and with other diabetic complications, like retinopathy and neuropathy (5-8). This increase of liver enzymes in the plasma is correlated with elevated glycated hemoglobin, triglycerides and total cholesterol, supporting the hypothesis that liver disease can occur with the time course of T1DM (8, 9). The DM-related liver late changes are characterized by an altered liver metabolites profile (fatty acyl CoAs, amino acids and acylcarnitines), increased formation of advanced glycation end products (AGEs) and reactive oxygen species, DNA fragmentation, increased expression of inflammatory markers, and apoptotic cell death (3, 10).

In DM, insulin deficiency and hyperglycemia can disturb hepatic lipid homeostasis, causing abnormal lipid profile and, ultimately, diabetes-related complications. Furthermore, alterations in phospholipid profile could be responsible for disturbances in cellular membrane properties, such as regulation of membrane proteins and cellular transduction pathways, which can exacerbate the initial stimulus promoted by hyperglycemia. In fact, alterations in phospholipid profile due to STZ-induced diabetes were already observed in heart tissue (11, 12) and in retina (13). Also,

disturbance of lipids was observed in several liver diseases, including non-alcoholic fatty liver, non-alcoholic steatohepatitis (14), and alcoholic fatty liver diseases (15). Only few studies had focused on the lipid profile of diabetic liver, but some discrepancies were found on the results published so far. While some authors reported the increase of cholesterol, free fatty acids, triacylglycerols and phospholipid content in diabetic liver (16, 17) and liver microssomes (18, 19), others observed a decrease in such lipids (20). However, none of these studies reported the alterations of the phospholipid molecular species profile in T1DM liver.

The present work aimed to evaluate the changes in the liver phospholipid profile of long term hyperglycemia induced by streptozotocin-administration in Wistar rats (a well characterized animal model of T1DM). Phospholipid classes and their molecular species composition were quantified and the lipid profile was determined by LC-MS and LC-MS/MS, using a lipidomic approach. Alterations induced by diabetes were pointed in terms of potential effects in hepatic function leading to hepatopathy.

MATERIAL AND METHODS

Chemicals

Streptozotocin [N-(Methylnitrosocarbamoyl)-a-D-glucosamine] was obtained from Sigma Chemical Co. (St Louis, MO, USA), and prepared prior to use in 100 mM citrate, pH 4.5. Phospholipid internal standards (1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (CL), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (dMPA), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (dMPG), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (dMPS) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (dPPI)) were purchased to Avanti polar lipids, Inc (Alabaster, AL, USA). Malachite green and primuline were purchased from Sigma (St Louis, MO, USA), triethylamine and potassium hydroxide were purchased from Merck (Darmstadt, Germany) and perchloric acid from Panreac (Barcelona, Spain). Acetonitrile, chloroform, methanol and hexane from Fisher Scientific

(Leicestershire, UK) were of HPLC grade and were used without further purification. All other reagents and chemicals used were of highest grade of purity commercially available. The water was of MilliQ purity filtered through a 0.22-mm filter (Millipore, USA).

Animals

Male Wistar rats weighing 150 - 200 g at 6-8 weeks of age were used at this study (n=20). During the experimental protocol, the animals were housed in collective cages (4 rats *per cage*) in a room at normal environment (21–24 ℃; ~50–60% humidity) receiving food and water *ad libitum* in 12 h light/dark cycles. Housing and experimental treatment were in accordance with Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (21). The experimental procedures were complied with the current national laws and were approved by local Ethics Committee.

Induction of Experimental Diabetes

The animals were randomly divided into two groups (n=10 *per group*): type 1 diabetic group (T1DM) and control group (CONT). In T1DM, hyperglycemia was induced with a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg), after a 16-h fasting period. CONT animals were injected with citrate solution. Values of weight and blood glucose (from the tail vein with Glucocard (A. Menarini Diagnostics, Florence, Italy) were taken just before STZ administration and weekly until the end of the protocol. Animals were considered hyperglycemic when blood glucose exceeded 250 mg/dl. After 4 months, animals were with an anesthetic overdose (pentobarbital, 10 mg/100 g ip) and the liver was dissected out, divided in several portions for light microscopy and lipidomic analysis. Serum levels of transaminases, glucose, cholesterol and triglycerides were determined in duplicate on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ). Glycated hemoglobin HbA1c was measured in whole blood samples using a commercial kit (code 11045) from Biosystems (Biosystems Reagents and Instruments, Barcelona, Spain).

Light Microscopy

After excision, several pieces (2-4 mm^3) from each hepatic lobe were fixed in 4% formaldehyde, during 24 hours, dehydrated with graded ethanol and further included in paraffin blocks. Five µm thick sections were cut and individually collected to silane-prep slides. After deparaffinization with xylene, sections were rehydrated and stained with hematoxylin-eosin following standard procedures. Slides were observed with a light photomicroscope (Axio Imager A1, Carl Zeiss) and a qualitative analysis of tissue structure organization, including the sinusoidal dimensions, the hepatocyte morphology and arrangement, as well as the presence of necrotic areas or cellular infiltration was made.

Lipidomic Analysis

Phospholipid quantification by phosphorous assays

Liver tissue was homogeneized in phosphate buffer saline (PBS) pH 7.4. Total lipids were then extracted by Folch method (22) using a chloroform:methanol (2:1, v/v) solution. After extraction, total phospholipids (PL) were calculated by colorimetric determination of phosphorus, after digestion with perchloric acid at 180°C, as described before (23). The amount of PL within each class was measured by the same method after thin layer chromatography (TLC) separation. Total phospholipid content from liver were normalized to protein amount (12). Protein concentration of liver homogenates were determined by the biuret method using bovine serum as standard (24).

Separation of phospholipids classes by Thin Layer Chromatography

TLC was used for separate PL classes present in the total lipid extract (approximately 60 µg of total PL). Silica gel plates with a concentrating zone of 2.5x20cm (Merck, Darmstadt, Germany) were developed with chloroform/ethanol/water/triethylamine (35:30:7:35, v/v/v/v) (25) solvent mixture. Lipid spots were visualized with UV (λ=254 nm) after primuline spray and PL classes were identified by means of their *Rf* values.

Phospholipid quantification by ^{31}P NMR spectroscopy

Relative quantification of PC, PE and SM classes was also carried out by ^{31}P NMR. Extracts were dissolved in a ternary mixture of chloroform:methanol:water (5:4:1), forming a single homogeneous phase. ^{31}P NMR spectra were acquired on a Bruker Avance DRX-500 spectrometer (Bruker, Rheinstetten, Germany) operating at 202.5 MHz for ^{31}P observation, using a 5-mm QNP probe. ^{31}P NMR spectra were acquired at 298 K using a 1D sequence with proton decoupling ('zgdc' in Bruker library), by collecting 512 scans into 32768 data points, with a spectral width of 5000 Hz and a relaxation delay of 4 s. The spectra were processed with a line broadening of 2.00 Hz and a zero filling factor of 2, manually phased and baseline corrected. Chemical shifts were referenced to 85% H_3PO_4 external reference at 0.00 ppm. The assignment of resonances to different phospholipids was based on the ^{31}P spectra registered for solutions of standard compounds.

Phospholipid characterization by LC-MS

Characterization of individual phospholipid within each class was achieved by mass spectrometry (MS) after separation by liquid chromatography (LC). HPLC system (Waters Alliance 2690) were used with a Ascentis® Si column (15 cm × 1 mm, 3 µm) and a precolumn split (Acurate, LC Packings, USA) in order to obtain a flow rate of 20 $\mu L.min^{-1}$. The solvent system consisted in two mobile phases as follows: mobile phase A (Acetonitrile:Methanol:H_2O; 55:35:10 (v/v/v) with 1mM ammonium acetate) and mobile phase B (Acetonitrile:Methanol 60:40 (v/v) with 1mM ammonium acetate). Initially, 0% mobile phase A was held isocratically for 20 min followed by linear increase to 100% of A within 5 min and maintained for 40 min. Samples (10 µg of total phospholipid) were separated by HPLC, which was coupled to a linear ion trap (LXQ; ThermoFinnigan, San Jose, CA, USA) mass spectrometer. The LXQ were operated in both positive (electrospray voltage + 5 kV) and negative (electrospray voltage - 4.7 kV) with 275 ºC capillary temperature and the sheath gas flow of 8 U. Normalized collision energy™ (CE) varied between 20 and 27 (arbitrary units) for MS/MS. Data acquisition was carried out on the Xcalibur data system (V2.0). Relative quantification of individual phospholipid species were determined by the ratio between the area of

reconstructed ion chromatogram of a given *m/z* value against the area of the reconstructed ion chromatogram of the respective class.

Fatty acid analysis by GC-FID

Total fatty acyl substituents' were measured by GC after transesterification of lipid liver extracts (approximately 200 μg of total PL). Fatty acids were prepared using a methanolic solution of potassium hydroxide (2M) according to the previously described method (26). DMPE phospholipid (50 μg) and C17 (7.5 μg) fatty methyl ester were used as internal standards. 2 μl of the hexane solution containing methylated fatty acids was submitted to GC analysis. The GC injection port was programmed at 523.15 K and the detector at 543.15 K. Oven temperature was programmed as follows: initially stayed 3 min at 323.15 K, raised to 453.15 K (25 K.min-1), held isothermal for 6 min, with a subsequent increase to 533.15 K (40 K.min-1) and maintained there for 3 min, performing 19 min totally. The carrier gas was hydrogen flowing at 1.7 mL.min-1. The gas chromatograph (Clarus 400, PerkinElmer, Inc. USA) was equipped with DB-1 column with 30 m length, 0.25 mm internal diameter and 0.15 μm film thickness (J&W Scientific, Agilent Technologies, Folsom, CA, USA) and a flame ionization detector.

Statistical Methods

The results are presented as mean values and their standard deviations (mean ± SD) for each experimental group. The Kolmogorov–Smirnov test was used to test normality of distribution for all data. Since all variables were normal distributed, significant differences between groups were evaluated with the unpaired Student's t-test. Statistical Package for the Social Sciences (version 17.0; SPSS Inc, Chicago, IL, USA) was used for all analyses. *P* values <0.05 were considered to be statistically significant.

RESULTS

Streptozotocin-injected rats presented a significant body weight loss and a marked increase in blood glucose levels, which are signs of uncontrolled type 1 diabetes mellitus. Prolonged hyperglycemia, in this group, was confirmed by the significant increased levels of

hemoglobin A1c compared to the CONT group. During the experimental protocol, polyuria and polydipsia were noticed in STZ-injected rats, further supporting the diabetic condition. T1DM-related hepatic damage was given by serum hepatic enzyme activities, with increased levels of serum ALT (alanine aminotransferase) and AST (aspartate aminotransferase), relatively to control group, with statistically significant differences noticed for ALT. The overall status of T1DM and CONT groups is summarized in Table 1.

TABLE 4 – Characterization of animals' response to STZ administration. Values are expressed as mean ± standard deviation.

Experimental group	CONT	T1DM
Body weight (g)	437.1 ± 58.4	251.0 ± 43.1$^{\#}$
Glycemia (mg.dL^{-1})	66.3 ± 15.0	577.1 ± 234.0$^{\#}$
HbA1c (% total Hb)	3.96 ± 0.80	6.28 ± 1.12$^{\#}$
AST (U.L^{-1})	50.7 ± 5.8	66.0 ± 20.2
ALT (U.L^{-1})	25.1 ± 4.8	65.9 ± 31.3‡
Cholesterol (mg.dL^{-1})	57.3 ± 8.2	94.0 ± 17.7$^{\#}$
HDL (mg.dL^{-1})	20.0 ± 1.9	48.0 ± 15.8$^{\#}$
Triglycerides (mg.dL^{-1})	136.0 ± 40.6	168.9 ± 44.8
Liver Phospholipids (µg.mg protein^{-1})	190.0 ± 67.1	211.7 ± 41.7

(‡p<0.005, #p<0.001)

T1DM-related hepatic damage was confirmed by morphologic analysis of liver sections. Indeed, whereas in the liver from control animals it was observed several lobules with preserved architecture (Figure 1A), the liver from diabetic animals presented increased amount of connective tissue with cellular infiltration. Numerous Kupffer cells were apparent in the Disse space and disperse focal areas of necrosis were observed (Figure 1B-C). These alterations in the liver structure from T1DM rats were paralleled by a decreased content of phospholipids with phosphocholine head group (lysophosphatidylcholines, phosphatidylcholines and sphingomyelins) and by an increase in phosphatidylethanolamines and phosphatidylinositols, based on phosphorus amount *per* class after TLC separation (Figure 2). Complementary analysis by ^{31}P NMR was also used to quantify the PL content (Figure 2), allowing the relative quantification of PC, SM and PE. The tendency for PC and SM classes' decrease was confirmed and a significant increase of the PE

class in diabetic liver animals compared to controls was observed. The other phospholipid classes were not quantified by ^{31}P NMR analysis because of their lower abundance.

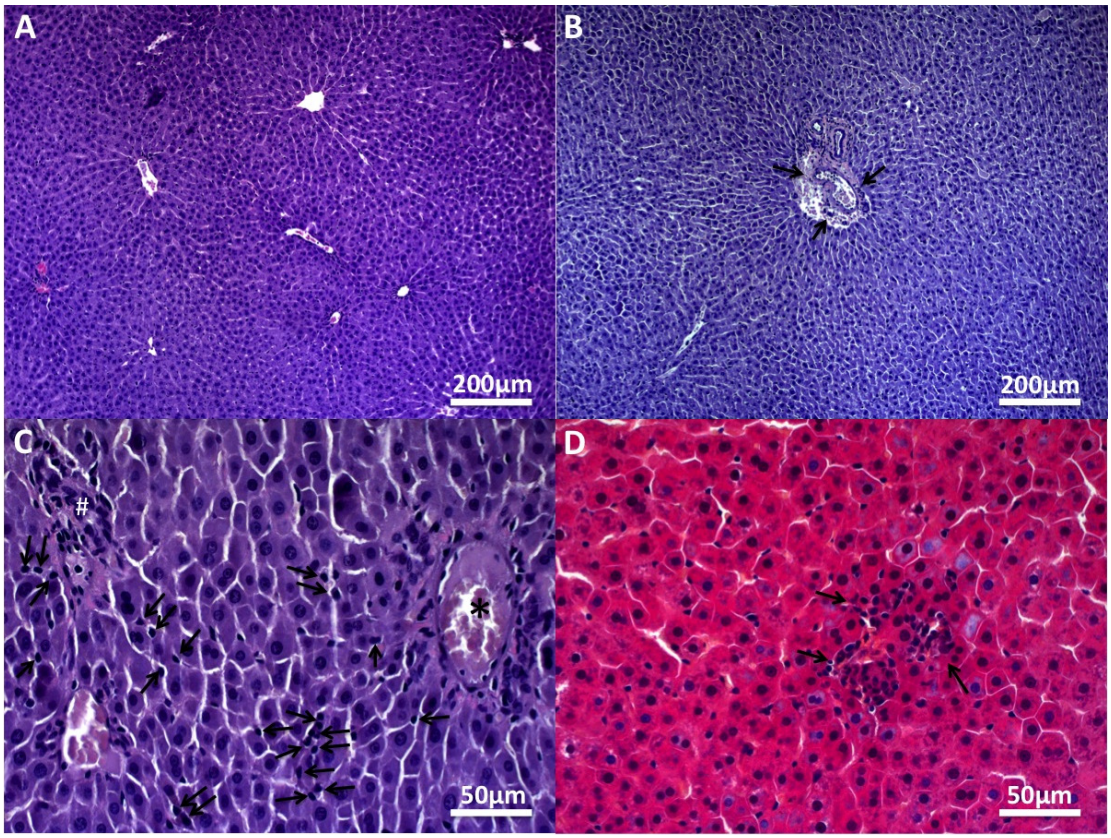


FIGURE 1 – Representative photomicrographs of liver from animals of control (A) and diabetes mellitus groups (B, C, and D). In A it can be observed several lobules with a preserved structure. A portal triad can be seen in B (arrows) surrounded by an increased amount of connective tissue with cellular infiltration. In C, it is depicted a portal triad (*) surrounded by connective tissue and an area of cellular necrosis, where some picnotic nucleus are notorious; numerous exuberant nucleus in the Disse space (arrows), suggestive of Kupffer cells, and a nodular cell infiltration (#) are also evident. Nodular cell infiltrations (arrows) are also observable in D.

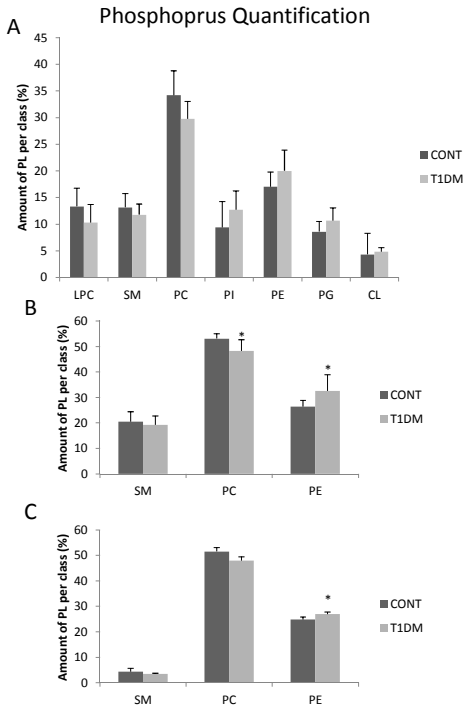


FIGURE 2 – Amount of liver phospholipid per class in control and diabetic rats. A and B corresponds to the data obtained by colorimetric assay preformed after TLC separation. A contains the results of all classes and B only contains the results of the classes that were analyzed by NMR. C shows the results obtained by NMR spectroscopy. (Values are means ± SD, *p<0.05).

Phospholipid species profile by LC-MS and LC-MS/MS

Individual phospholipid molecular species within each class were analyzed by LC-MS and LC-MS/MS and the chromatograms are presented in Figure 3. MS data are present by means of relative abundance *per* class and, depending on the phospholipid classes, the spectra were analyzed in positive or negative ion modes. Lysophosphatidylcholines (LPC), phosphatidylcholines (PC) and sphingomyelins (SM) were analyzed in the positive ion mode. Phosphatidylethanolamines (PE) and lysophosphatidylethanolamines (LPE) were analyzed in both positive and negative ion modes. Cardiolipins (CL), phosphatidylserines (PS) and phosphatidylinositols (PI) were analyzed exclusively in the negative ion mode. MS/MS was performed for each ion to identify and confirm their structure, according to the typical fragmentation pathways (27). Comparison of CONT and T1DM LC-MS data showed changes in lipid profile of PC, PI, PE and CL classes. LPC, SM (Figure S1) and PS did not show significant differences between control and diabetic groups, thus were not reported herein.

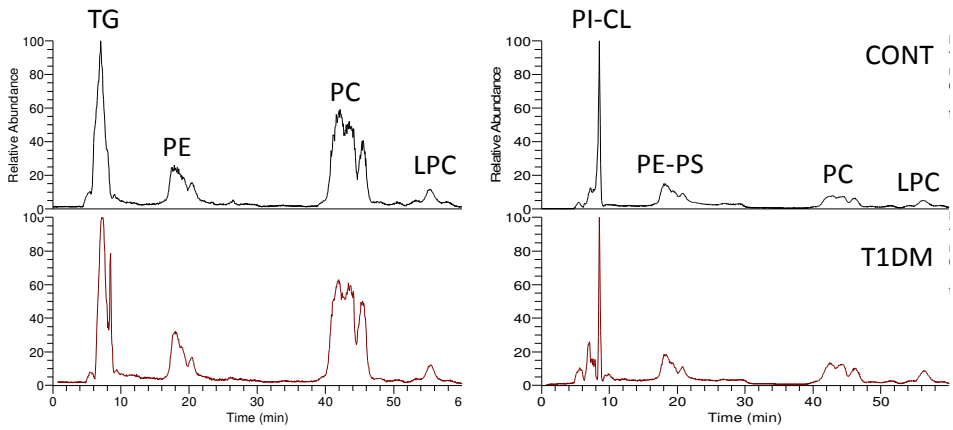


FIGURE 3 – Total ion chromatograms obtained by HILIC-LC-MS of phospholipids and triglycerides from the hepatic total lipid extracts of control and T1DM rats. The top chromatograms correspond to CONT hepatic lipid extracts and the bottom chromatograms correspond to T1DM hepatic lipid extracts. The left chromatograms were obtained in the positive mode for the detection of TG, PE, PC, SM and LPC classes. The right chromatograms were obtained in the negative mode for the detection of PI, CL; PE and PS classes.

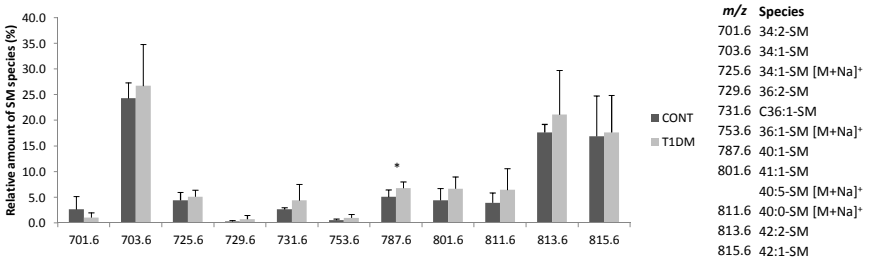


FIGURE S1 – SM molecular species relative composition in control and diabetic livers (Values are means ± SD, *p<0.05).

In PC classes, spectra of controls and diabetes showed the same PC $[MH]^+$ ions. The most abundant ions for both LC-MS spectra were observed at *m/z* 758.6 (16:0/18:2-PC), 782.6 (16:0/20:4-PC) and 810.6 (18:0/20:4-PC), as summarized in Figure 4. Comparison of control and diabetic liver revealed changes in the relative abundance of species with significant differences for almost all PC species identified. While some species showed higher relative abundance in T1DM, other species showed higher relative abundance in CONT. For instance, considering the most abundant PC species it was possible to observe that in T1DM liver, the 18:0/20:4-PC and 16:0/18:2-PC species were increased while the 16:0/20:4-PC specie was decreased. It seems that PC species bearing 18:0 acyl chains had a more pronounced increase in T1DM when compared

with those bearing 16:0 acyl chain, suggestive of PC fatty acyl substitution at *sn-1* position with accumulation of 18:0 acyl chain over 16:0 acyl chain.

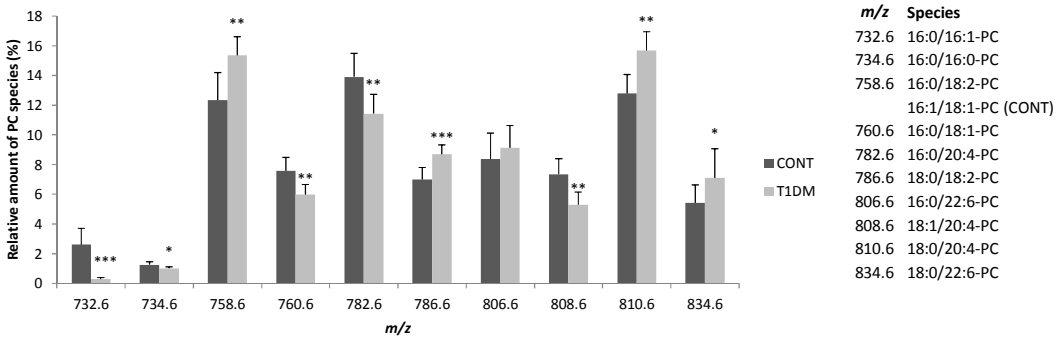


FIGURE 4 – PC molecular species relative composition in control and diabetic livers (Values are means ± SD, *p<0.05, **p<0.005 and ***p<0.001).

LC-MS analyses of the PE class were acquired in both positive and negative ion modes. Positive spectra, with formation of $[M+H]^+$ ions, showed the same molecular species of PE in CONT and T1DM (Figure 5). The major PE ions were observed at *m/z* 716.5 (16:0/18:2-PE), 740.5 (16:0/20:4-PE), 764.5 (18:0/18:2-PE) and 768.5 (18:0/20:4-PE); however, minor contribution of other isobaric species cannot be excluded. Besides the observation of the same molecular ions, significant differences in the relative abundance of the ions were notorious between CONT and T1DM (Figure 5). Comparing PE profile of liver extracts, it was noticed that 16:0/20:4-PE and 16:0/18:2-PE were more abundant in CONT than in T1DM, while the other ions were more abundant in T1DM group.

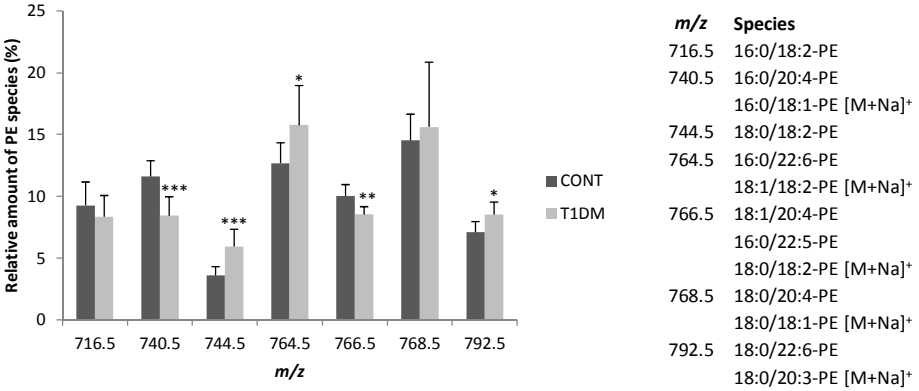


FIGURE 5 – PE molecular species relative composition in control and diabetic livers (Values are means ± SD, *p<0.05 and ***p<0.001).

Additionally, in LPE spectra, the phospholipid with 16:0 acyl chain (*m/z* 454.3) was more abundant in control, whereas phospholipids with longer acyl chains tended to be higher in T1DM, but only 18:2-LPE (*m/z* 482.3) showed a significant increase. Altogether, similarly to what was observed in PC spectra, the species containing the 16:0 acyl chains at the *sn-1* position seemed to be more abundant in the controls, while the species with 18:0 acyl chain at the *sn-1* position were more abundant in the diabetic animals.

LC-MS spectra of the PI class, in the negative ion mode with formation of the $[M-H]^-$, showed a major ion at *m/z* 885.5 corresponding to 18:0/20:4-PI, with no relative abundance variation between control and diabetic livers (Figure 6, top). Nevertheless, T1DM and CONT spectra showed minor ions with differences in their relative abundance, namely at *m/z* 857.5 (16:0/20:4-PI) and 861.5 (18:0/18:2-PI). The 16:0/20:4-PI (16:0 acyl chains at the *sn-1* position) and 18:0/18:2-PI (18:0 acyl chain at the *sn-1* position) were respectively decreased and increased in T1DM.

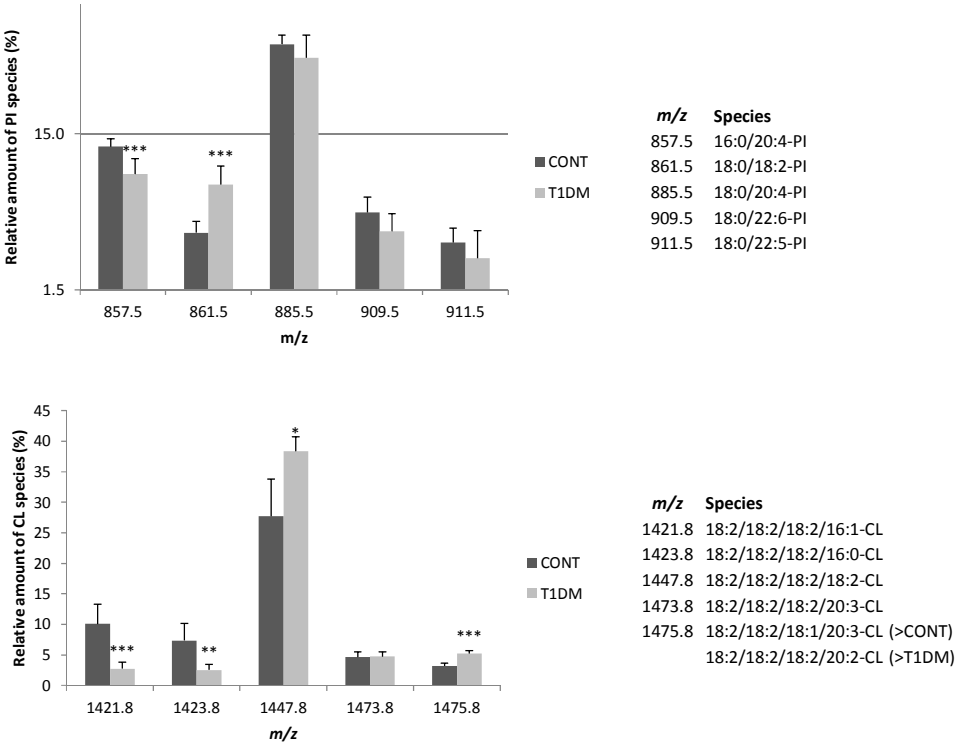


FIGURE 6 – PI (in the top) and CL (in the bottom) molecular species relative composition in control and diabetic livers (Values are means ± SD, *p<0.05, **p<0.005 and ***p<0.001).

Cardiolipin species were identified by LC-MS in negative mode as $[M-H]^-$ ions. By comparing the spectra of diabetic and control livers, we were able to see notorious differences in the cardiolipin molecular species profile. In both groups, the specie at *m/z* 1447.5 (corresponding to 18:2/18:2/18:2/18:2-CL) was the most abundant one, still was more abundant in the diabetic than in the control group (Figure 6, bottom). Moreover, the CL species at *m/z* 1421.5 (16:1/18:2/18:2/18:2-CL) and 1423.5 (16:0/18:2/18:2/18:2-CL) were more abundant in the control group. The CL specie at *m/z* 1475.5 (20:2/18:2/18:2/18:2-CL and 20:3/18:1/18:2/18:2-CL) was significantly increased in the liver from diabetic animals. This remodeling in the CL fatty acyl chains was similar to the observed for other PL classes, with 16:0 *sn*-1 acyl chain being more abundant in the control group.

Triacylglycerols species profile by LC-MS and LC-MS/MS

Triacylglycerol (TG) molecular ions present in lipid extracts from the liver of control and STZ-injected rats, were observed in positive ion mode as ammonium adducts ($[M+NH_4]^+$). Both LC-

MS spectra showed several molecular ions, which differ in their relative abundance (Figure 7). The most evident difference was observed for the abundant ion at *m/z* 874.7 (mainly 16:0/18:1/18:2-TG) in CONT and the ion at *m/z* 872.7 (mainly 16:0/18:2/18:2-TG) in T1DM. Moreover, the comparison of TG profile between groups demonstrated a decrease (over 50%) in the abundance of the species at *m/z* 848.7 (mainly 16:0/16:0/18:2-TG) and a significant increase in the abundance of the species at *m/z* 898.7 (mainly 16:0/20:4/18:2-TG), 898.7 (mainly 18:1/18:2/18:2-TG) and 920.7 (mainly 16:0/22:6/18:2-TG). Collectively data suggest that triacylglycerols are predisposed to alterations in molecular species related with the hyperglycemic status, which are expressed either by alterations in the relative abundance of compounds and by altered isobaric contribution for the same *m/z* value.

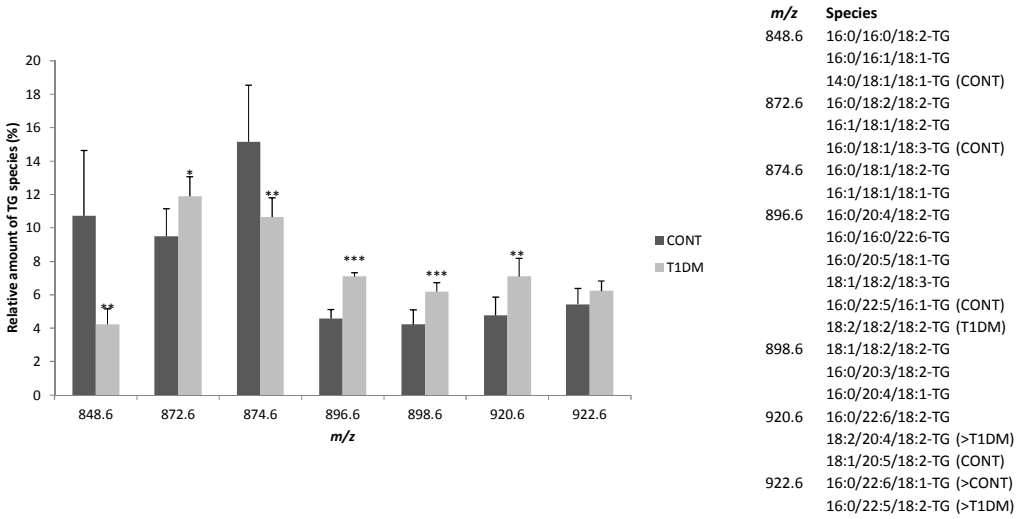
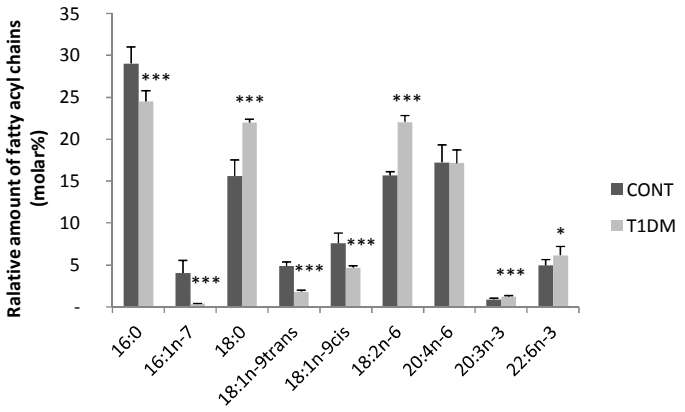


FIGURE 7 – TG molecular species relative composition in control and diabetic livers (Values are means ± SD, *p<0.05, **p<0.005 and ***p<0.001).

GC analysis of fatty acyl chains from the total lipid extracts confirmed the differences found by LC-MS for T1DM and CONT acyl chains (Figure 8). These results demonstrated a significant decrease of 16:0, 16:1 and 18:1 acyl chains in T1DM group. Simultaneously, there was a significant increase of 18:0, 18:2 and 22:6 acyl chains in T1DM compared to controls. Interestingly, 20:4 acyl chains did not show any variation between animals.



Experimental group	Saturated FA (molar%)	Unsaturated FA (molar%)	MUFA (molar%)	PUFA (molar%)	Double bond Index (molar%)	Unsaturated/ Saturated Ratio
CONT	44.7 ± 1.7	55.4 ± 1.8	16.6 ± 3.1	38.8 ± 2.8	149.4 ± 9.1	1.24 ± 0.09
T1DM	46.5 ± 1.5*	53.5 ± 1.5*	6.9 ± 0.4***	46.6 ± 1.2***	160.3 ± 7.0*	1.15 ± 0.07*

FIGURE 8 – Fatty acyl substituent's of total lipid extracts from control and diabetic livers, analyzed by GC. The table contains information about the sum of saturated, unsaturated, monoene (MUFA) and polyene (PUFA) fatty acids, the double bond index (number of double bonds for 100 moles fatty acids) and the unsaturated/saturated ratio. (Values are means ± SD, *p<0.05 and ***p<0.001).

DISCUSSION

The prevalence of hepatobiliary diseases is increased in patients with diabetes mellitus. Also, T1DM is associated with hemochromatosis and autoimmune hepatitis (28). To better understand the lipid alterations that occur in the liver from type 1 diabetic subjects, we determined the lipidomic profile of hepatic tissue from STZ-injected rats. This is a well-characterized animal model of T1DM. STZ has a half-life of 6 h being readily metabolized, and not exerting a direct effect on liver enzymes, such as in the activity of the antioxidant ones (29). In the present study, we observed that four months of hyperglycemia resulted in hepatic structural changes suggestive of fibrosis/inflammation and impaired metabolism underlined by alterations in the lipid profile, mainly characterized by an elevation in the amount of lipids bearing longer acyl chains.

In this study, we have noticed that the relative abundance of PL classes with choline head group (PC, LPC and SM) decreased in the liver from diabetic animals, while all the other PL

classes showed an increase (Figure 2). A decrease in the PC content was already been observed in alcoholic and non-alcoholic liver diseases (14, 15) and were generally associated with type 2 diabetes mellitus (28). This suggests that liver dysfunction is accompanied with changes in the phospholipid profile, with a typical decrease of PC and other phosphocholine lipids. Perturbation of phosphocholine metabolism might result from alterations in phospholipid biosynthesis and/or metabolism. Since PC is mainly synthesized in the liver, it is possible that liver damage may be related with impaired PC biosynthesis with a consequent decrease of PC content. Dysregulation of PC biosynthesis in the liver has been reported in T1DM (30) and T2DM (31) and it was suggest to be, at least partially, a result of alterations in transmethylation processes (32). Thus, PC synthesis via hepatic phosphatidylethanolamine *N*-mehyl transferase (PEMT) can be affected (30). Deficiency of PEMT would lead to decreased hepatic levels of choline-containing phospholipids. However, it was found that in T1DM, PEMT expression was increased, suggesting that an augmented requirement of PC during diabetes leaded a decrease on PC content (30). Van der Veen *et al*. (33) reported that a percentage of PC from liver cell membranes are converted into triglycerides. These authors found that when hepatic PC levels are reduced, VLDL secretion is diminished, resulting in hepatic accumulation of TG. This suggests that an additional requirement of PC for the TG biosynthesis in liver could exacerbate the decrease of PC content that was observed in the damaged liver of diabetics (33). No alterations in CL content were observed in the liver of T1DM in comparison to controls, in contrast to the decrease of CL levels previously reported in diabetic cardiomyopathy (12). This dissimilarity seems to reflect the functional specialization of tissues and organs response to hyperglycemia. The myocardium is specialized in energy production, showing more alterations in CL content, while the liver, important in regulating body homeostasis and metabolism, revealed changes that could affect membrane function and cell signaling. For instance, the decrease in PC/PE ratio is known to alter cell membrane function and was observed in non-alcoholic steatohepatitis (34).

Besides PL classes' alterations, a PL remodeling with fatty acyl substitution was also observed in lipids from T1DM liver. We found a similar pattern to all PL classes, which was characterized by a reduction in the amount of 16:0 acyl chains and an increase of 18:0 and 22:6 acyl chains. Similar results were reported by Douillet *et al*. (20), who demonstrated a T1DM-related

decrease in 16:0, 18:1, 18:2; 18:3 and 22:5 and an increase in 18:0, 20:3, 20:4 and 22:6 total fatty acids in the liver. Our results are also in accordance with Ghebremeskel *et al.* (35), that documented a decrease in saturated and monounsaturated free fatty acids and TG-acyl chains with a concomitant increase in n-6 and n-3 polyunsaturated free fatty acids and TG-acyl chains in the liver from STZ-injected animals. On the contrary, other authors reported a decrease in 20:4 (16, 18, 36) and an increase in 16:0 (16) fatty acids. These alterations in the fatty acid content related with diabetes mellitus seem to be due to alterations in the synthesis, desaturation, degradation, oxidation and/or incorporation (into phospholipids) of fatty acids. The increase of phospholipids containing 18:0 acyls chains, but not the decrease of those containing 16:0, can be explained by diabetes-related preferential synthesis of saturated fatty acids (16). Regarding the increase of 22:6 acyl chain, the dysregulation of the mechanism of 20:4 and 22:6 distribution between the TG and PL seems unlikely because a similar alteration in fatty acyl substitution was found in both PL and TG (35). This phenomenon seems to be universal so it is possible that it could be due to altered synthesis (including alteration of elongases), and/or altered phospholipase A2 or acyltransferases enzymatic activity (37). Changes in fatty acyl profile in retina and liver from T1DM animals were correlated with the down regulation of some elongases (13). These authors found a decrease in elongase 2 (Elovl2) and 6 (Elovl6) and a decrease in desaturases in the liver, which was associated with an increase in 18:2 acyl chain. No other changes were observed in liver fatty acyl profile. The reduction of elongases 4 (Elovl4) and 2 (Elovl2) expression levels observed in retina was correlated with a decrease in 22:6, and a decrease in the ratio of n-3 to n-6 polyunsaturated fatty acids (13). Our results reveal an increased 18:2 acyl chain that could be explained by a reduction of these enzymes activity/expression levels; however, other mechanisms must be involved since elongases activity cannot explain the increase of 22:6 acyl chain and the alteration of 18:0/16:0 ratio. The decrease of 16:0 acyl chains and the increase of 18:0 acyl chains at *sn*-1 position can be also explained by dysregulated hepatic PC metabolism, since one of PC biosynthetic pathways favors the presence of 16:0 acyl chains and the other favors the presence of 18:0 acyl chains. Therefore the dysregulation of hepatic PC metabolism could lead to the observed increased 18:0/16:0 ratio in T1DM, as already reported in T2DM (31). Additionally, the activity of palmitoyl-CoA chain elongation (PCE), an enzyme that catalyzes stearic acid (18:0) formation from

palmitic acid (16:0), increases in the liver upon glucose and fructose administration (38). Although the authors report a decrease of PCE activity in diabetic animals (38), our results clearly show an increase in the 18:0/16:0 ratio, suggesting that PCE activity could be increased.

In conclusion, our study indicates that the hepatic damage that occurs in T1DM, morphologically characterized by fibrosis, necrosis and cellular infiltration, is associated with the remodeling of liver lipid content. The observed changes in the lipid content may contribute to alteration of membrane properties and changes in lipid signaling, which impact liver function and consequently whole body homeostasis. Overall, data highlight the role of lipid profile remodeling on liver dysfunction, a hypothesis that needs to be further explored for the early diagnostic, monitoring and evaluation of liver disease in consequence of uncontrolled type 1 diabetes mellitus.

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CHAPTER VI

CONCLUSIONS

Comprehensive characterization of glycated and glycoxidized phosphatidylethanolamines was achieved using mass spectrometry strategies. Glycation of biomolecules is a well-known reaction that occurs *in vivo*, particularly in hyperglycemia. Glycation of aminophospholipids are still an underexplored field, however protein glycation has been extensively studied and is recognized as an important adverse outcome of diabetes mellitus. Glycated proteins are known to increase the oxidative stress and the inflammation that underlie diabetic complications, such as atherosclerosis.

Phosphatidylethanolamine is the most abundant class of phospholipids which has a free amino group, prone to undergo glycation. Glycated PE was analyzed by mass spectrometry, in positive and negative ion modes, using PE standards, and the major fragmentation pathways were identified under tandem mass spectrometry conditions (MS/MS). The typical fragmentation of glycated PE, in positive ion mode, corresponds to the loss glycated polar head, loss of 303 Da, while, in the negative ion mode, the main fragmentation is attributed to the loss of glucose, loss of 162 Da.

Since diabetes is associated with an increase of the oxidative stress status, we studied the modifications of glycated PE species induced by the hydroxyl radical. Using mass spectrometry, in positive and negative ion modes, it were identified the glycated PE radical intermediaries and the glycated PE oxidation products formed after reaction of glycated PE with hydroxyl radical, in the presence or absence of *spin traps*. These studies allowed the characterization of glycated PE oxidation, for the first time. Also, specific fragmentation patterns were assigned, essential for future detection of glycoxidation *in vivo*. This work contributed to understand the role of glycated PE on molecular susceptibility to oxidation and the interrelation between glycation and oxidation. It was demonstrated that besides oxidation of unsaturated *sn*-2 acyl chains, which is known to occur in phospholipids, other oxidation products were formed from glycated polar head oxidation. Oxidation of glycated polar head was confirmed by the study of a glycated PE with two fully saturated (palmitoyl) acyl chains. The identification by MS, of the radical intermediary adducts bearing the spin trap molecule linked to the glucose moiety reinforce the role of glucose in the oxidation of glycated species. All the glycated PE originated oxidation products by insertion of oxygen molecules in glycated polar head and/or in *sn*-2 acyl chain, by truncation of *sn*-2 and by truncation of glycated polar head. Also, glycated polar head oxidation was found to occur sooner than the oxidation of unsaturated acyl chains. Hence, the glycation of polar head increase the targets of oxidation and could be responsible for the exponential increase oxidative stress modifications that is known to occur under hyperglycemic conditions.

The stimulation of monocytes and dendritic cell was performed to determine the role of PE glycation in inflammation associated with diabetes. This study also revealed the inflammatory properties of glycated, glycoxidized and oxidized PE. All PE modifications were found to increase the frequency of monocytes and dendritic cells that express cytokines, when compared with basal level, but with an effect that was lower than the LPS. The different modifications showed to induce

different levels of cytokine-expressing cells. In general, the results showed that oxidative modifications produced lower levels of cytokine-expressing cells when compared to the correspondent non-oxidized compounds (OxPE < PE and GluOxPE < GluPE). Also the glycated compounds showed higher levels of cytokine-expressing cells when compared with non-glycated compounds (GluPE > PE and GluOxPE > OxPE). An exception to this was found with glycoxidized PLPE that increased the levels of cytokine-expressing cells when compared to glycated PLPE. This can be explained by the exponential increase of oxidative stress modifications, due to combined effect of glycation and linoleic acid oxidation, which is higher than oleic acid oxidation. In conclusion, cell stimulation and cytokine production was more pronounced when the PE polar head was modified. Additionally, low oxidation levels could reduce stimulation levels while higher oxidation levels could synergically increase those stimulation levels. Thus, glycated PE and glycoxidized PE may have important effect contributing to the low-grade systemic inflammation associated with diabetes, which could contribute to the development of diabetic complications.

The analysis of the livers of the T1DM rats showed that under prolonged hyperglycemia there were alterations of the phospholipidome that were associated with signs of hepatic damage, namely fibrosis, necrosis and cellular infiltration. Those alterations of phospholipidome included variations relative amount of liver phospholipid classes, and in the PL species with a remodeling in PL fatty acyl chains. A remodeling of acyl chains consisted of a reduction in the amount of 16:0 and an increase of 18:0 and 18:2 acyl chains. The alterations of PL species were found to occur in almost all PL classes, including PE. Unfortunately, no PE glycation and PE glycoxidation were found in diabetic liver, provably due to ion suppression phenomena, thus a directed approach for glycated PE would be necessary to specifically detected in biological samples. Also, the observed changes in the lipid content could be an indication that regulatory mechanisms were triggered, allowing the elimination of oxidized species. Such alteration on phospholipids could contribute to alteration of membrane properties and changes in lipid signaling, which may impact the liver function and consequently, whole body homeostasis. So the phospholipidome alterations that were found could be an important predictor of liver dysfunction in consequence of uncontrolled type 1 diabetes mellitus.

This study provided evidence on the role of hyperglycemia in alterations of PE structure, glycation and oxidation, in the PE content and PE species remodeling in the liver of diabetic rats (T1DM). It was explored the role glycation on the susceptibility of glycated PE to oxidation and the role of glycoxidized and glycated products in inducing cytokine production in monocytes and dendritic cells. Hence, the work contributed to better understanding of the intricate connections between hyperglycemia, oxidative stress, inflammation and diabetes that ultimately would lead to the development of diabetic complications.