Isolation and characterization of Low Density Lipoprotein lipid glycation products

by

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ABSTRACT

One of the pathological consequences of diabetes due to hyperglycaemia is the nonenzymatic glycation of free amino groups by glucose. Not only proteins and nucleic acids participate in this reaction but aminophospholipids also under go glycation.

This thesis describes the isolation and characterization of the products of the reaction between glucose and amino phospholipids *in vitro* and *in vivo* sources and their possible pathogenic effects. The major glycation products described here are the products of phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) with glucose which were identified by liquid chromatography with online electrospray mass spectrometry (LC/MS). The major product of the reaction was the glucosylated aminophospholipid which on the normal phase HPLC was resolved from the non glucosylated phospholipid. The glucosylated PtdEtn (Glc PtdEtn) and PtdSer (Glc PtdSer) were also identified in Red Blood Cells (RBC) incubated with various glucose concentrations. The pattern of glycation of the RBC aminophospholipids showed that the glycation reaction did not have any preference for specific molecular species or aminophospholipid class. Glycated PtdEtn was also identified in both plasma and RBC of control and diabetic individuals. There was a 10-fold increase in the amount of glycated PtdEtn in diabetic subjects when compared to the controls. Glycated PtdEtn was identified *in vitro* preparations of LDL in presence of various glucose concentrations. LDL PtdEtn glucosylation was concentration dependent and both diacyl and plasmalogenic species were glucosylated. The glycated PtdEtn when present in liposomal preparation showed an increased susceptibility to oxidation. Glc-PtdEtn also resulted in an increased oxidative susceptibility of other phospholipids, such as phosphatidylcholine

(PtdCho), when present in the liposomal mixture. This susceptibility was also observed for LDL specifically enriched in Glc-PtdEtn in presence of copper ions. As a result of Glc PtdEtn both LDL PtdCho hydroperoxides and PtdCho core aldehydes had 4 fold increase compared to control LDL during copper oxidation. Finally, it was established that Glc-PtdEtn present in LDL can result in increased LDL uptake by macrophages resulting in cholesteryl ester and triacylglycerol deposition in THP-1 macrophages. The results indicate that glucosylation of PtdEtn in LDL accounts for the entire effect of LDL glycation on macrophage uptake, and therefore the increased atherogenic potential of LDL in hyperglycemia.

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Abbreviations

AcLDL	-acetylated LDL
AGE	-advanced glycosylation end products
Apo	-apolipoprotein
BSA	-bovine serum albumin
DiI	-1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
Diradyl	-alkenylacyl, diacyl, alkylacyl
Cap Ex	-capillary exit
CE	-cholesteryl ester
EDTA	-ethylenediaminetetraacetate
Etn	-ethanolamine
FAME	-fatty acid methyl esters
Glc PtdEtn	-glucosylated PtdEtn
GLC	-gas liquid chromatography
GlyLDL	-glycated LDL
Gro	-glycerol
GroPEtn	-glycerophosphoethanolamine
h	-hours
HDL	-high density lipoprotein
HPLC	-high performance liquid chromatography
HSA	-horse serum albumin
IDDM	-insulin dependent diabetes mellitus
LC/ES/MS	-liquid chromatography with on-line electrospray mass spectrometry
LDL	-low density lipoprotein
Lyso PtdCho	-lyso phosphatidylcholine
MDA-LDL	-malondialdehyde treated LDL
NIDDM	-non insulin dependent diabetes mellitus
OxLDL	-oxidized LDL
PBS	-phosophate buffered saline
PL	-phospholipid

PtdCho	-phosphatidylcholine
PtdEtn	-phosphatidylethanolamine
PlsEtn	-alkenylacyl GroPEtn
PtdIns	-phosphatidylinositol
PtdSer	-phosphatidylserine
RBC	-red blood cells
SDS	-sodium dodecyl sulfate
SM	-sphingomyelin
TG	-triacylglycerol
TLC	-thin layer chromatography
TMS	-trimethylsilyl
VLDL	-very low density lipoprotein.

Chapter 1: Review of Literature

INTRODUCTION

Non-enzymatic glycosylation is a post-translational modification of a protein by a covalent attachment of a sugar residue, which results in a spontaneous amino-carbonyl bonding referred to as a Schiff base linkage. Specifically, the reaction leads to an addition of reducing sugars such as glucose to the primary amino groups of proteins. N-terminal amino acid residues and the ε–amino group of lysine participate in the first stage of the reaction in proteins by forming Schiff base adducts. Protein bound Schiff bases then undergo intramolecular rearrangement to produce Amadori products. These are more stable and achieve equilibrium *in vivo* over a period of two to three weeks. Over a time period of weeks and months, glucose derived Amadori products undergo further intra- and intermolecular reactions to produce a class of protein bound moieties called advanced glycosylation end products or AGEs. Within the last 10 years, due to advances in analytical instrumentation, several candidate compounds have been isolated and structurally characterized such as caboxymethyl lysine, pyrraline, and furoyl-furanyl imidazole. Although many more compounds have been identified, due to the complexity of the reactions and the diversity of the products generated, a general marker has not been recognized for *in vivo* glycation.

The glycation reactions have been shown to involve a spectrum of protein molecules. In addition to proteins with long half-lives, such as collagen, proteins with short half-life, such as lipoproteins, also have been shown to be affected by non enzymatic glycation in diabetes. This recognition has provided the basis for the hypothesis that the development of diabetic complications (specifically atherosclerosis), arises from increased glycation, resulting in structural alteration, that in turn leads to

functional abnormalities of proteins. For example, glycation could affect the action of enzymes, the affinity of receptors for ligands, or the rate of protein catabolism. AGE molecules themselves have been implicated in many of the processes that play a role in atherosclerosis. It is recognized that the highly reactive AGE molecules can cause thickening and rigidity of the vascular wall, interfere with nitric oxide (NO)-mediated vasodilation, and induce secretion of cytokines and growth factors, phenomena that are known to contribute to atherosclerosis.

This chapter reviews the non-enzymatic glycation reactions of proteins that take place *in vivo,* including those of lipoproteins. The discussion is focused on oxidative reactions resulting in glycoxidation and the pathological consequences of this process specifically in atherosclerosis.

NON-ENZYMATIC PROTEIN GLYCATION

Chemistry of non-enzymatic glycation

According to the nomenclature adopted by The International Union of Biochemistry, any reaction that links a carbohydrate to a protein is termed glycation (1). However, in common usage, glycation almost always refers to nonenzymatic glucosylation, and it is in this sense that the term 'glycation' and 'glycated' are used in this thesis. Glycation occurs when the carbonyl group of an aldose or ketose condenses with a free amino group of protein to form a reversible Schiff base or aldimine linkage, followed by an Amadori rearrangement yielding the more stable ketoamine (Fig.1). This process was first described in 1912 by L.C. Maillard, who observed that solutions of amino acids heated in the presence of reducing sugars developed a yellow-brown color and stable brown pigments formed in them (2). Maillard hypothesized that this reaction could occur *in vivo* and be of importance in diabetes. Maillard-type reactions were observed during preparation and storage of foods. Proteins and reducing sugars present in foods reacted in dehydrated and concentrated food preparations (for example, milk and egg powder, dried fruits) forming Amadori compounds (early Maillard reaction). Depending on temperature, time, humidity and pH, the Amadori compounds can remain unchanged for a long time or decompose rapidly to produce intense browning. In this advanced Maillard reaction, the sugar moiety of the Amadori compound can undergo 1,2- or 2,3- enolization reaction, followed by dehydration or scission reactions with formation of secondary products like: dicarbonyls, ketols, aldehydes, reductones, etc. (3,4) (Fig.2).

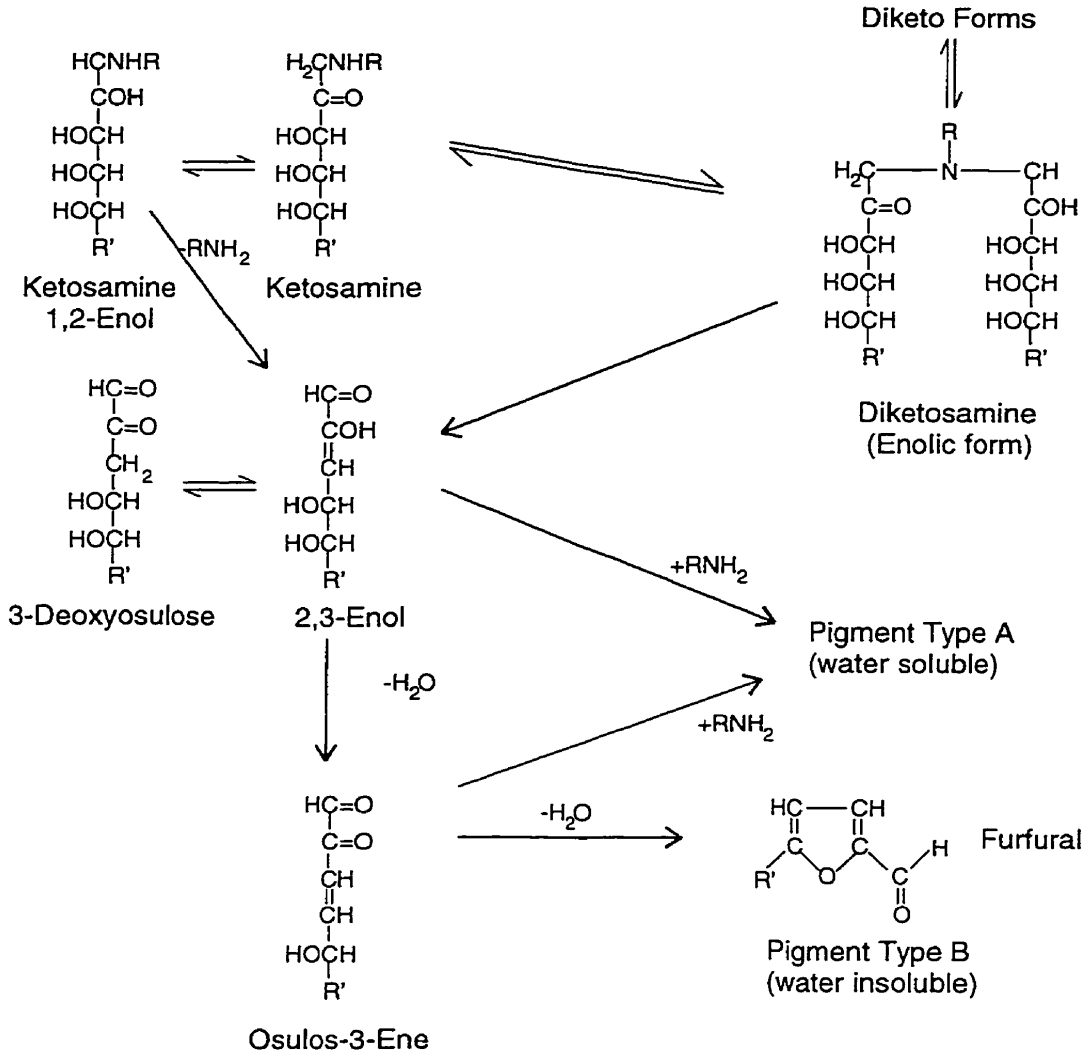


Fig. 1.1 Proposed pathways for pigment formation during Maillard reactions.

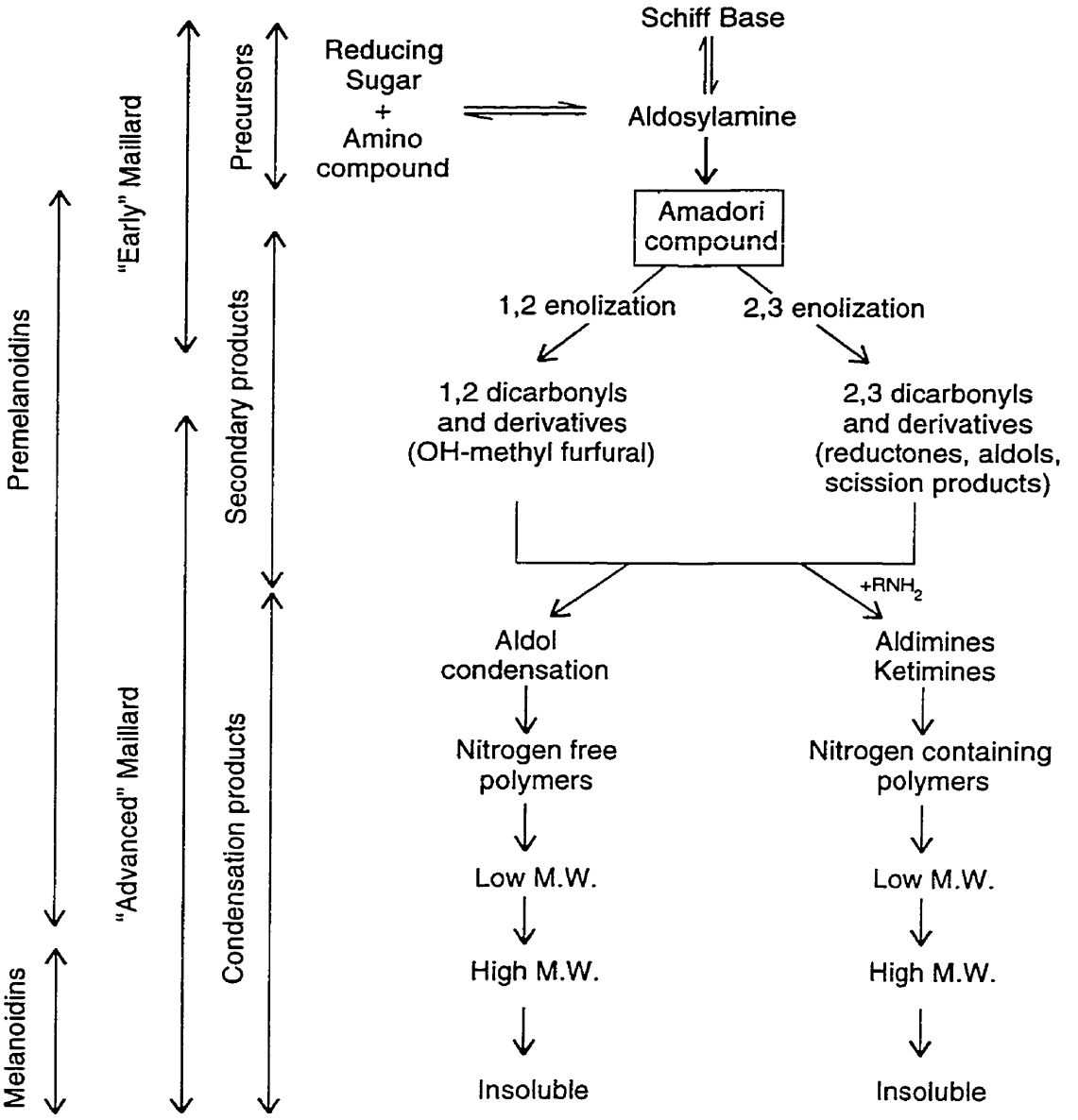


Fig. 2.1 A simplified scheme of the Maillard reaction products

The secondary products can polymerize to produce nitrogen-free brown pigments (malanoidins), or they can react with free amino groups to form aldimines and ketimines, which polymerize to produce nitrogen-containing melanoidins (5). Nonenzymatic browning of foods causes loss of nutritive value of proteins, decrease in the digestibility of proteins and in the nutritional availability of amino acids and carbohydrates. The indigestible premelanoidins (low molecular weight, soluble brown pigments) reduce proteolysis (6), inhibit intestinal disaccharidase activity and suppress absorption of amino acids in experimental animals (7). These phenomena lead to an accumulation of indigestible materials in the cecum, liver and kidneys, and subsequently, to the hypertrophy of these organs (8), which is most pronounced in the cecum. Mineral metabolism can be altered by premelanoidins present in the diet; for example, calcium is excreted in the urine as a Maillard polymer (9). Similarly, in parenteral nutrition, urinary zinc, copper, and iron were higher in patients infused with sterilized solutions of glucose plus casein hydrolysates than after infusion with a mixture of the two solutions sterilized separately (10). Nutritional losses, physiological effects, toxicity and possible mutagenicity (11) of browned food products need to be continuously evaluated, since Maillard reactions are often used to produce flavor and color characteristics in processed foods. In the last twenty-five years it has become evident that the Maillard reaction also occurs in living organisms.

Glycation of hemoglobin

The first evidence for non-enzymatic protein glycation *in vivo* was obtained with hemoglobin. Various studies of the most abundant minor hemoglobin A_{IC} (4 percent) showed that the NH_2-terminus of each of the chains contained 1-deoxy-1-(N-

valyl)fructose (12-14). Formation of hemoglobin A_{Ic} involves two steps. Initially, in the reversible reaction, the aldimine (Hb pre- A_{Ic}) is formed by a nucleophilic attack of unprotonated NH_2-terminal amino group of the β chain of hemoglobin on open-chain carbonyl group of glucose (15,16). In studies of this reaction with hemoglobin, fifteen monosaccharides were analysed with aldoses showing higher reactivities than ketoses. The reactivity of each sugar was dependent on the extent to which it exists in the open-chain structure. Glucose was the least reactive of the aldohexoses (15). In the second step, a labile aldimine under-goes a very slow and nearly irreversible Amadori rearrangement forming hemoglobin A_{Ic} (16). The Amadori product may assume different ring structures. In this case the ε-amino group of lysine reacts with glucose (17). Chemical studies done by Fisher and Winterhalter (18) suggest that glycated hemoglobin exists almost exclusively in the ring form. This has been confirmed by ^{13}C NMR studies of glycated pancreatic RNase A used as a model compound alongside reference compounds such as D-fructose, fructose-glycine, N^{α}-formyl-N^{ε}-fructose-lysine and glycated poly-L-lysine (17). As with fructose, the β-pyranose anomer was the most intense peak in these spectrum. The α-pyranose anomer, as well as α- and β-fructofuranose anomers were identified at much lower intensities. Cyclization of the ketoamine apparently contributed to its stability. Apart from A_{Ic}, other minor hemoglobins were modified as allows: β-NH_2 group of A_{Ia2} by fructose-1,6-P_2 (0.5 %), (19), α-NH_2 group of A_{Id3} probably by glucose (1.9%),(20).

Hemoglobin A_{Ic} has been studied most extensively, because it is elevated 2- to 3-fold in patients with diabetes mellitus (21,22). Experiments on mice showed that

hemoglobin A_{IC} was formed throughout the life of the RBC at a constant rate of 0.1 percent per day (23). In diabetic mice, the formation rate was 2.7-fold higher. In diabetic patients, the level of Hb A_{IC} depended on the average concentration of glucose in the plasma during the preceding 2-3 months (24). Measurement of HbA_{IC}, is routinely made by HPLC, which provides a clinical index for control of hyperglycemia in diabetes, as well as the screening test for unsuspected diabetes (25). Glycation of hemoglobin has spurred interest in the potential modification of other proteins.

Human serum proteins were found to be glycated (26,27). Circulating serum albumin (half-life about 19 days) was glycated up to 10-12 percent in normal subjects and up to 30 percent in diabetics (27,28). The predominant site of glycation (50 %) of human albumin *in vivo* is the ε-NH_2 group of lysine-525, which is the second lysine of a lysine-lysine sequence. A different lysine, namely, lysine-199 was glycated during *in vitro* incubations of human serum albumin with glucose. Glycation at this site would decrease drug binding to albumin, since, for example, aspirin competes for lysine-199 (29). Horse spleen and rat liver ferritins were glycated *in vitro* (30), and the modification was suggested as a possible explanation for the occurrence of isoferritins (31)

Other proteins, for example, lens crystallins (32) and peripheral nerve protein (33) were found to be non-enzymatically glycated. Recently, histone Hl was shown to have its lysine involved in glycation (34).

Kinetics and rates of glycation *in vivo*

There has been only one direct study on the kinetics of glycation of a protein *in vitro*. Bunn et al (35) measured the rate of formation of human HbA_{IC} following infusion of ^{59}Fe- transferrin. The rate of formation of HbA_{IC} *in vivo*, i.e., glycation of the β-

terminal valine residue, was 0.018%/mM Glc/day, which was consistent with the mean level of Hb A_{IC} in normal human blood. In subsequent studies on glycation of Hb *in vitro* (16), the observed rate of formation of HbA_{IC} was 0.009%/mM Glc/day, or approximately one-half the *in vivo* rate. In retrospect, the faster reaction *in vivo* probably resulted from the presence of other effectors in the erythrocyte milieu (36). Bunn et al (37) also reported that Hb glycated *in vivo* contains ~2.5 times as many glucose adducts to lysine as to valine residues, indicating that the rate of glycation of Hb at lysine residues was ~0.045% $GlcHbA_{IC}$/mM Glc/day. Baynes et al (38) studied the rate of glycation of HSA *in vitro,* and concluded that the rate of glycation of HSA was ~0.21% GlcHSA/mM Glc/day, consistent with the extent of glycation of HSA and its biological half-life *in vivo.* After adjusting for the difference in lysine content of the proteins (22 per αβ-dimer in Hb and 57 in HSA), the rates of glycation of lysine residues in Hb and HSA were -0.0008% and 0.0037%/mM Glc/day, respectively. Thus, the rate of glycation of lysines in HSA is, on average, 4-5 times as fast as glycation of those in Hb.

Advanced Glycosylation Endproducts (AGE)

AGEs were originally characterized by their yellow – brown fluorescent color and their ability to form crosslinks to and between amino groups (39). The term 'AGEs' is now used for a broad range of advanced products of the Maillard reaction (6,7) including compounds as (3,4)N'-(carboxymethyl)lysine (CML) and pyrraline, which neither show color and fluorescence, nor occur as crosslinks in proteins (42) (Fig3.1). The formation of AGEs *in vitro* and *in vivo* is dependent on the turnover rate of the chemically modified target, time and sugar concentration. The products of the reaction that have been shown to cause many of the pathological effects of non-enzymatic glycation are the Advanced

Glycosylation Endproducts (AGE). AGEs are chemically stable products of the Maillard reaction which under physiological conditions can result in protein crosslinks.

Cellular consequences of AGEs

AGE formation was originally thought to specifically tag senescent proteins, thereby providing a specific signal for recognition, degradation and removal of senescent macromolecules (43,44). Low molecular weight AGE-rich peptides were identified as degradation products and are presumably released into the circulation to be cleared by the kidneys (45,46). Recent studies, however, demonstrate that interactions of AGE modified proteins with AGE-receptor complexes serve not only to degrade AGE-proteins, but also to activate signal transduction pathways, that induce the synthesis and release of cytokines and growth factors responsible for initiating tissue repair and protein turnover (47). AGE also contribute to the development of vascular disease and diabetic complications (48,43). A large number of studies have confirmed the close correlation between AGE formation and the physiological changes observed in vascular disease, diabetes, atherosclerosis and aging. Since intracellular sugars are much more reactive than glucose (49), due to an increase in glucose in the open chain form (fructose, glucose-6- phosphate), intracellular AGE formation occurs extremely fast. *In vitro* experiments by Giardino et al (50) have demonstrated that 1 week incubation of endothelial cells in the presence of high glucose results in an 13.8-fold increase in the intracellular AGE- content measured by anti AGE antibody. In parallel, the mitogenic activity of high glucose cultivated endothelial cells markedly decreased. The observed loss in mitogenic activity was due to post-translational modifications of basic fibroblast growth factor (bFGF) by

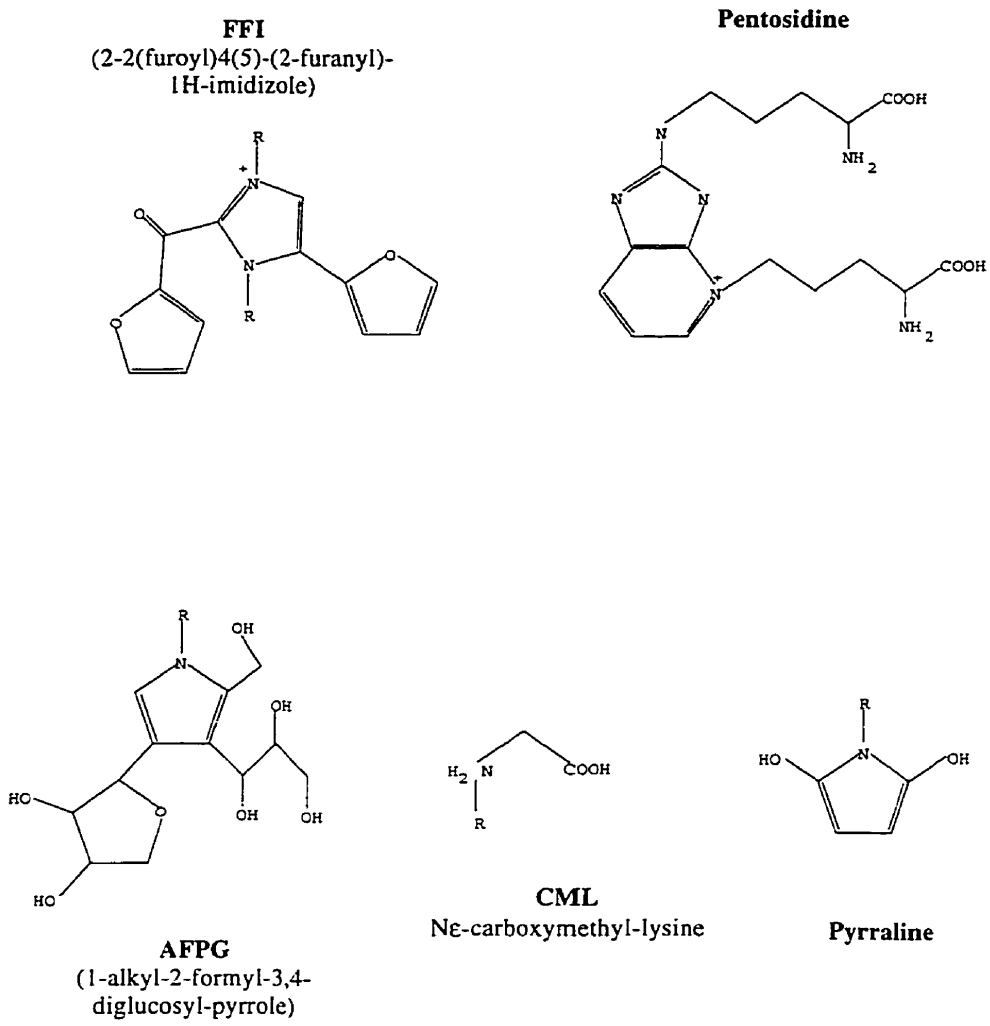


Fig. 3.1 Advanced Glycosylation Endproducts (AGE) identified in *in vitro* and *in vivo* systems.

AGEs representing the major AGE-modified protein in endothelial cells (4). No specific AGE molecule was measured and the increase was determined by antibodies against bFGF glucosylated *in vitro*.

Effects of advanced glycation endproducts *in vivo*

AGEs formation proceeds slowly under normal ambient sugar concentrations, but is enhanced in the presence of hyperglycemia and/or under conditions, where the protein degradation and turnover is prolonged. The structure of the different crosslinked AGEs, that are generated *in vivo*, has not yet been completely determined. Because of their heterogeneity and the complexity of the chemical reactions involved, only some AGE structures have been structurally characterized *in vivo*. CML and pentosidine have been found to accumulate in tissue collagen of the human skin with age and at accelerated rate in diabetes mellitus (51,52) by utilizing GC/MS with single ion monitoring in human lens proteins, plasma, skin collagen and urine (54). In addition, measurements have been made with antibodies directed against caproyl-pyrraline, which detected AGEs in sclerosed glomeruli of kidneys from old nondiabetic animals as well as in diabetic kidneys (55). Increased pyrraline contents of plasma proteins have also been described in diabetes mellitus (56). However, there are still doubts whether pyrraline forms under physiological conditions since cross reactivity of the antibodies used, prevents direct determination (57-60). Dicarbonyl intermediates such as methylglyoxal have been found to be elevated in plasma and urine of patients with diabetes mellitus (61,62) and a 3- to 6-fold increase has been reported in serum of patients with non-insulin dependent diabetes mellitus (NIDDM) and insulin dependent diabetes mellitus (IDDM), respectively (63). Beside these structurally characterized AGEs, a large number of studies have reported the

detection of uncharacterized AGEs in serum and tissue proteins, identified by solely ELISA and radio receptor assay (RRA) techniques (64,65) or immunohistochemistry using antibodies developed to proteins browned by glucose (66-68). The AGEs recognized by these antibodies were defined by (i) yellow-brown color, (ii) fluorescence, (iii) crosslinking and/or (iv) their interaction with AGE- specific receptors, but were not structurally defined. Although CML might represent the majority of the structures recognized by these antibodies (42), it cannot be excluded that other AGE structures are also detected (42). Using these antibodies increased serum levels of AGEs were detected in very young prepubertal and pubertal IDDM diabetic patients shortly after manifestation of diabetes (69). This indicates that the pathological process leading to diabetic late complications starts at a very early timepoint. Again using antibodies, AGEs were further detected in cardiac and renal tissues of patients with diabetes mellitus (70,71). In diabetic kidneys, AGEs were preferentially localized in the renal cortex (72), mesangial areas (73), vascular lesions (71) and glomerular basement membranes (71-74). The accumulation of AGEs in the glomerular extracellular matrix is supposed to contribute to renal diabetic nephropathy (47,75). Consistently, serum AGE levels correlate with the progressive loss of kidney function and are up to 8-fold elevated in individuals with end-stage renal disease requiring dialysis (46). Diabetic patients with uremia have also an increased risk for cardiovascular complications (43). Inefficient clearance of degraded low molecular weight AGE-rich peptides and recirculation of these 'toxic' molecules might therefore not only account for uremia (70,71), but also perpetuate extrarenal vascular damage in these patients. AGEs were also detected in diabetic red blood cells and in liver histones of diabetic rats (77). The later one might account for

increased heterogeneity associated with diabetes (77). The recently identified guanine advanced glycation endproduct, *N* -(1-carboxyethyl)guanine is also supposed to contribute to mutations and DNA transpositions (78).

AGE present in Atherosclerotic tissue

The availability of highly specific antibodies has permitted the investigation of protein glycation products in atherosclerotic tissues. Thus, Nakamura et al (70) using AGE antibodies, found significant amounts of AGE protein in diabetic atherosclerotic plaques. This identification was made using a polyclonal anti-glycated RNase antibody after proteinase K digestion of formalin-fixed and paraffin-embedded sections of coronary arteries and cardiac tissues from three autopsy cases. Fibrous extracellular protein deposits in the atheromatous plaques were also detectable by the antibody. Further experiments using a monoclonal antibody allowed the localization of AGE protein in human atherosclerotic lesions in frozen sections of aortas of 25 autopsy cases by Horiuchi et al (79). In all cases (fatty steaks to advanced plaques) a low to moderate extracellular AGE deposition was demonstrated. The intracellular AGE, on the other hand, was more dense and clearer. AGE accumulation was observed in both macrophage and vascular smooth muscle cell derived foam cells. It was not clear whether or not AGE modification per se is the cause of the disease or simply its effect and whether or not these AGE are deposited by lipoproteins or formed locally. A more selective staining for lipoprotein AGE antibody is required to show the presence of glycated lipoproteins in atherosclerotic plaques. Recently, Stitt et al (80) have claimed correlation between arterial tissue AGEs and circulating AGE-ApoB, which would suggest a causal link between AGE modification of lipoproteins and atherosclerosis. Further confirmatory

evidence for AGEs being central mediators of late diabetic complications was provided by animal models, *in* which an 8 week long administration of AGEs to euglycemic rats resulted in glomerular and arteriolar basement thickening, mesangial expansion and glomerulosclerosis with proteinuria and albuminuria and vascular dysfunction (81-83).

Significant AGE deposits were also detected in the retinal vasculature of AGE-infused rats as well as in rats with long-term diabetes (84). Short-term administration of *in vitro* prepared AGE-albumin produced vascular defects, such as vascular permeability and leakage, unresponsiveness to vasodilatory agents, subendothelial mononuclear recruitment (65,85), activation of the transcription factor NFκ–B and subsequent VCAM-1 gene expression (86-88). These observations underline the concept of AGEs as modulators of the vascular tone in diabetes and vascular disease. Besides diabetes mellitus, increased AGE levels were described in diseases associated with amyloid formation as haemodialysis-associated p2-microglobulin containing amyloidosis (89) and Alzheimer's disease (90-92). Furthermore, AGEs were detected in healthy persons with a long history of smoking (93), in patients with vascular disorders in the absence of diabetes (93) and in aortic atherosclerotic lesions in patients lacking a history of diabetes (68). In the latter instance, extracellular deposition and intracellular accumulation of AGEs in the intimal lesions correlated with age and progression of atherogenesis (68) which indicated that the presence of redox active AGEs might accelerate lipoprotein oxidation and thereby increase the process of atherogenesis (94). Consistently, long term infusion of physiological amounts of AGE-modified serum albumin into nondiabetic rabbits resulted in AGE accumulation in aortic tissues associated with intimal changes and focal expression of the adhesion molecules VCAM-1 and ICAM-1 (84). Thus, it

appears that continuous AGE deposition might potentially promote the development of atherosclerotic lesions (95). This view is emphasized by the finding that AGEs are detected in atherosclerotic lesions of euglycemic LDL-receptor deficient rabbits in areas rich in lipids and lipoproteins (96) and in lung collagen of old euglycemic rats (97)

NON-ENZYMATIC LIPOPROTEIN GLYCATION

The first demonstration of glycation of lipoproteins exposed to elevated glucose concentrations, both *in vitro* and *in vivo*, was reported in 1981 (98). After incubating human lipoproteins (LDL and HDL) with (^{14}C) glucose *in vitro*, Schleicher et al (98) found that glucose incorporation into lipoproteins AI, AII, B, C and E was directly proportional to the length of incubation and to the glucose concentration. They also found a two-fold increase in glycation of Apo-B in LDL from diabetic human subjects and were the first to suggest that increased glycation of lipoproteins *in vivo* might have significant metabolic consequences.

Many investigators have since confirmed the occurrence of glycated lipoproteins *in vivo* utilizing specific antibodies and boronate affinity chromatography (99-100). It has been established that the extent of glycation of LDL in insulin dependent diabetes mellitus (IDDM) patients correlates well with other short- and long term indicators of glycemic control (mean plasma glucose, plasma protein glycation, and glycated hemoglobin) (101). In these patients, the relative increase in Apo B glycation compared with control patients (1.6 fold) was similar to that for glycated hemoglobin (1.5-fold) and for total plasma proteins (2.2 fold) (101). There is much variation in the levels of *in vivo*

glycation of lipoproteins in diabetics and controls. This is attributed to the relative heterogeneity of the populations studied in terms of hyper glycemic control (102).

Subsequently Curtis et al (100) have shown that lipoproteins isolated from plasma of diabetic subjects in poor metabolic control contain up to 33 fold more of glucitolysine (reaction product of glucose and lysine) residues/mg of isolated lipoprotein protein, and on an absolute basis, contain between 36 and 383 nmol of glucitolysine in their total lipoprotein fraction, compared to controls, who had a mean value of 2.9 nmol (100).

Although glycated hemoglobin has been utilized as a diagnostic marker for long-term glycemic control this is not the case for glycated lipoproteins (102). Throughout current literature there is a lack of consistency on the levels of either lipoprotein glycation or AGE levels in diabetes and hyperglycemia. This could be due to the fact that a standard method for isolation and quantitation of glycated lipoproteins has not been developed. The heterogeneity of lipoprotein glycation products is responsible for the difficulty of measuring absolute lipoprotein glycation. This factor has compromised many of the epidemiological studies attempting to correlate lipoprotein glycation and cardiovascular disease (104). Although all lipoproteins are glycated *in vivo* the most extensively studied lipoprotein is LDL. In work concerning glycation, investigation is facilitated by the fact that LDL possesses only one apolipoprotein, apoB100. Efforts continue to develop new and easier ways to measure LDL glycation. Most involve boronate affinity chromatography, a technique depending on the adherence of glycated residues to the affinity matrix (105). Hydrolyzed Apo B, intact LDL, or even serum may be applied. But these methods are not standardized and also harbor complications. There have also been ELISA based methods for the quantitation of glycated versus non-

glycated LDL. These assays are dependent on glycation at a few specific sites on LDL, and may not represent glycation at other sites (106).

Consequences of lipoprotein glycation

A multitude of secondary complications of diabetes have been attributed to non-enzymatic glycation. Non-enzymatic glycation of lipoproteins can affect their function and hence their atherogenicity. Non-enzymatic glycation can alter both lipoprotein structure and their susceptibility to oxidation. The term metabolic consequences is used to illustrate changes in lipoprotein function that do not involve oxidative modification. Therefore, oxidative changes resulting from lipoprotein glycation are considered separately.

Cellular and Metabolic consequences

LDL Glycation

One of the most compelling lines of evidence, which allows us to consider glycated lipoprotein atherogenic, is the altered biological activity of glycated lipoproteins. Both *in vitro* and *in vivo* evidence suggested this modified activity. The first study to show an altered biological activity of a glycated lipoprotein measured LDL uptake by normal human fibroblasts (98). Recognition of *in vitro* glycated LDL by these cells, which only have classical LDL receptors, was impaired. This impairment was proportional to the degree of modification of the lysine residues. It was shown that by glycating as few as 6-8% of the lysine residues the LDL uptake was completely inhibited. Later Klein et al (107) showed that recognition of LDL from diabetic patients in poor glycemic control by human fibroblasts was also impaired, supporting the role of glycation in altering recognition of LDL by classical LDL receptor. Subsequent results showed that incubation of macrophages with glycated LDL resulted in transformation of

these macrophages into cholesterol loaded foam cells (108). Uptake of glycated LDL also resulted in increased CE synthesis in human macrophages that correlated with the intracellular CE accumulation. Human macrophages in culture also showed increased in CE accumulation and synthesis when exposed to LDL from diabetic subjects (108). It was shown that the scavenger receptors were not involved in glycated LDL uptake. Competition studies using acetylated LDL showed that the scavenger receptor pathway was not involved and the presence of a separate high affinity receptor was proposed (99).

In vivo studies by Steinbrecher et al (109-110) demonstrated the diminished LDL receptor activity with glycated LDL in man. When radiolabeled glycated LDL was used, it had an increased half-life compared to control LDL.

Glycated LDL has been also shown to exhibit altered behavior towards two other cell types important in the development of atherosclerosis, endothelial and vascular smooth muscle cells. There have been few studies with glycated LDL and these two cell types and the reason for this is not known. In both cell types, incubation with glycated LDL resulted in increased CE accumulation and increased uptake of glycated LDL (111,112).

VLDL Glycation

Of interest in diabetes is the metabolic alteration caused by VLDL glycation since triacylglycerols rich VLDL levels are increased in most diabetic subjects. Using a rat model Mamo et al (113) showed that glycated VLDL was poor substrate for lipoprotein lipase. Glycation of VLDL also interfered with the lipolysis of VLDL triacylglycerols. This explained the delayed clearance of triacylglycerols of glycated VLDL *in vivo*. VLDL glycation also extended the mean residence time of VLDL particle, which the

authors (113) attributed in part to the hypertriglyceridemia observed in subjects with diabetes mellitus.

In a study utilizing human macrophage cultures, glycated VLDL, isolated from diabetic subjects, caused increased CE deposition and synthesis (114). Thus glycated VLDL particles are not only removed from plasma at a slower rate but also promote foam cell formation. It has also been shown that VLDL isolated from normolipemic patients with either IDDM or NIDDM interact abnormally with cultured human monocyte-macrophages (115,116), stimulating increased CE synthesis and accumulation. However, the effect was not as marked as with LDL. Also, subtle alterations in lipid and apolipoprotein composition were observed in the VLDL from diabetic patients, and these may have accounted for some of the findings. Glycation of individual apolipoproteins of VLDL was not determined, and it remains uncertain whether or not increased glycation of all apolipoproteins contributed equally to the altered metabolism of the particles. Investigation of this problem is, hampered by the difficulties of measuring glycation of the individual apolipoproteins on the VLDL particle.

HDL Glycation

The glycation of the HDL apolipoproteins results in decreased plasma half-life of the human HDL in a guinea pig model. Most of the glucose incorporated in HDL was localized to Apo-AI, but all other HDL associated apolipoproteins were also glycated (117). An increase of glucose incorporation in HDL was associated with a decrease in HDL half-life. When 60% of lysine were derivatized, clearance of glycated HDL was 60% faster than that of control HDL. When as few as 2% of lysines were glycated, there was still an 8% increase in the rate of clearance. Duell et al (118) demonstrated that *in*

vitro glycation of HDL inhibits its high affinity binding to cultured fibroblasts and to the candidate HDL receptor protein. They went on to show that due to this lack of binding, glycated HDL caused a 25-40% reduction in intracellular cholesterol efflux and that the steady state efflux of LDL-derived cholesterol was also markedly reduced. These studies and others (119) suggest that, because the removal of cholesterol from extra hepatic cells by HDL the critical step in reverse cholesterol transport, the non-enzymatic glycation of HDL may be associated with a reduction in reverse cholesterol transport in diabetes.

Therefore, non-enzymatic glycation of lipoproteins results in increased plasma LDL and VLDL, reduction in HDL, and an increase in cholesterol ester deposition in macrophages resulting in foam cells. These events support an atherogenic profile often seen in diabetic hyperglycemia.

Lipoprotein Oxidation

One of the current hypothesis for initiation and progression of atherosclerosis is based on oxidized LDL as the major atherogenic agent. Several lines of evidence separately implicate oxidatively modified LDL: first, oxidized LDL has been isolated by gentle extraction from atherosclerotic plaques (120); second, antibodies to oxidized LDL indicate its presence in plasma and plaque material (121); third, in both human and animal antibodies have been identified that can react with oxidized LDL (OxLDL) molecules (121); and fourth, administration of antioxidants prevents oxidative modification of LDL and slows the progression of atherosclerosis in several animal models (122). In addition, there have been numerous studies that have shown that oxidized LDL is not recognized by its normal receptors, but is taken up by special receptors on macrophages resulting in CE deposition and foam cell formation. OxLDL has also been shown to be a chemoattractant for

monocytes and to be cytotoxic to endothelial cells and mitogenic for macrophages and SMC, as well as to inhibit NO induced vasodilation (123). Some 20 additional biological effects have been described that show the atherogenicity of oxidized LDL. Oxidative modification of LDL apparently leads to possibly a very large array of consequences other than the generation of foam cells thought to be important in atherogenesis (122). The major site of attack in oxidation of lipoproteins is the lipid moiety due to the abundance of polyunsaturated fatty acid. It is noteworthy that oxidized lipids have been implicated in the development of diseases ranging from diabetes to arthritis. Thus, lipids derived from oxidatively damaged lipoproteins are of central importance in atherogenesis.

LDL lipid oxidation

The classical studies of Brown and Goldstein (124) demonstrated that in order for LDL to be recognized by a macrophage scavenger receptor it had to be chemically modified. The simplest modification of LDL was acetylation of the LDL particle (124). Subsequently other methods of LDL modification, such as maleylation, acetoacetylation, carbamylation, succinilation and treatment with glutaraldehyde also resulted in its increased uptake by macrophages (125). All these reagents have in common a high reactivity with free amino groups (e.g. ε- amino group of lysine). However none of these modifications occur *in vivo*, and thus it remained obscure what the biological modifiers might be. Fogelman et al (126) proposed in 1980 that malonaldehyde, a short chain bifunctional aldehyde, generated from lipid peroxidation reactions can modify LDL resulting in CE deposition in macrophages. Again the necessity for amino group modification was shown to be important in LDL modification. Subsequently many other *in vitro* studies suggested that LDL lipid oxidation is the key event in atherogenic modification of LDL (127,128). Many other

changes are associated with the oxidative degradation of the LDL lipids as, for example, an extensive fragmentation of the Apo B to smaller peptides. It has been suggested (128,129) that these fragments together with the covalent binding of aldehydes generated by lipid peroxidation can lead to a complete structural rearrangement of the protein creating new epitopes which do not bind to the classical LDL receptor but to the scavenger receptor.

Although oxidized LDL molecules have been identified both in atherosclerotic tissue and in plasma (130) it has yet to be determined what pathway is involved in LDL oxidation. Several systems that have been utilized in order to mimic the *in vivo* process. These include incubation of LDL with metal ions such as Cu^{2+} and Fe^{2+}, autoxidation of thiols resulting in superoxide generation, enzymatically generated super oxide, lipoxygenase, nitric oxide, myeloperoxidase and glucose (130). The utilization of all these various methods stems from the fact that the oxidized LDL as a molecule is not a well defined due to the complexity of the oxidation reactions and their products. This hinders the ability to bridge the gap between *in vitro* OxLDL and *in vivo* OxLDL. One common factor to all these various oxidation methods is the production of both saturated and unsaturated aldehydes from the lipid moiety of LDL.

Low molecular weight oxidation products

During LDL oxidation the primary reaction is the hydroperoxidation of the unsaturated fatty acids bound to phospholipids, cholesteryl esters and triacylglycerols, which through homolytic scission (β-cleavage) of C-C bonds on either side of the hydroperoxy group (131). This reaction proceeds via the lipid alkoxyl radical and it is accelerated strongly by traces of reduced forms of transition metal ions such as Fe^{2+} and Cu^{2+} (132). From the multitude of low molecular weight aldehydes generated during LDL lipid

oxidation two molecules have been studied extensively, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (133). Due to the high reactivity of these two molecules towards crosslinking and covalent bonding of free amino groups, it has been suggested that many of the structural changes seen in OxLDL, such as increase in negative charge and aggregation can be attributed to these two molecules (133). Some of the toxic characteristics of OxLDL can be mimicked by direct chemical modification of LDL with HNE. Recently Bolgar et al (134) have demonstrated the addition of HNE to Apo B following oxidation of LDL, which provides the first evidence for the previously hypothesized lipid protein conjugation. By using a HPLC/ES/MS/MS technique, they identified the modified residue as a cysteine present at the surface of the Apo B molecule, which the authors suggest could significantly affect the interaction with macrophage-bound receptors. It remains to be shown whether HNE or MDA can also modify aminophospholipids present in LDL.

It has also been demonstrated that HNE treated LDL shows growth inhibitory properties toward fibroblasts comparable to those of OxLDL, and that the inhibitory properties reside in the lipid phase of OxLDL. Kaneko et al (135) have tested in cultured fibroblasts and endothelial cells, the cytotoxiticy and growth inhibition of several products of lipid peroxidation. The most toxic compounds were 2,4-alkadienols (nonadienal, decadienal) and HNE, which produced 50% inhibition of endothelial cell proliferation at 10-25 mM; linoleic acid hydroperoxides had similar toxicity (25 mM), whereas 2-alkenals (hexenal to nonenal) alkanals were less toxic (136). As mentioned above, MDA was one of the first lipid oxidation products that was shown to induce atherogenic properties in LDL (126). Throughout literature it has been repeatedly suggested that oxidized LDL uptake by macrophages strongly depends on the extent of its oxidation, primarily MDA (137,138).

Although MDA reactivity and products have been shown with other model proteins and peptides, a direct evidence for MDA reactivity with LDL is still lacking. It may be noted, however, that MDA and HNE represent a small fraction of the lipid oxidation products and of the aldehydes that are generated during LDL oxidation (139). Many studies, including the vast majority of cell culture experiments, have utilized oxidized LDL without isolating the active components of LDL lipid peroxides that could be responsible for the observed activity. The problem stems from lack of specific detection assays for either hydroperoxides or aldehydes. Many studies rely on the thiobarbituric acid reactive substance (TBARS) color reactions to measure oxidation products. It has been shown by several groups that TBARS lack specificity and also suffer from cross reactivity with compounds that are not lipid peroxidation products (140). Recently it has been demonstrated that by utilizing HPLC and mass spectrometric techniques a majority of LDL low molecular weight lipid oxidation products can be identified and quantitated (141).

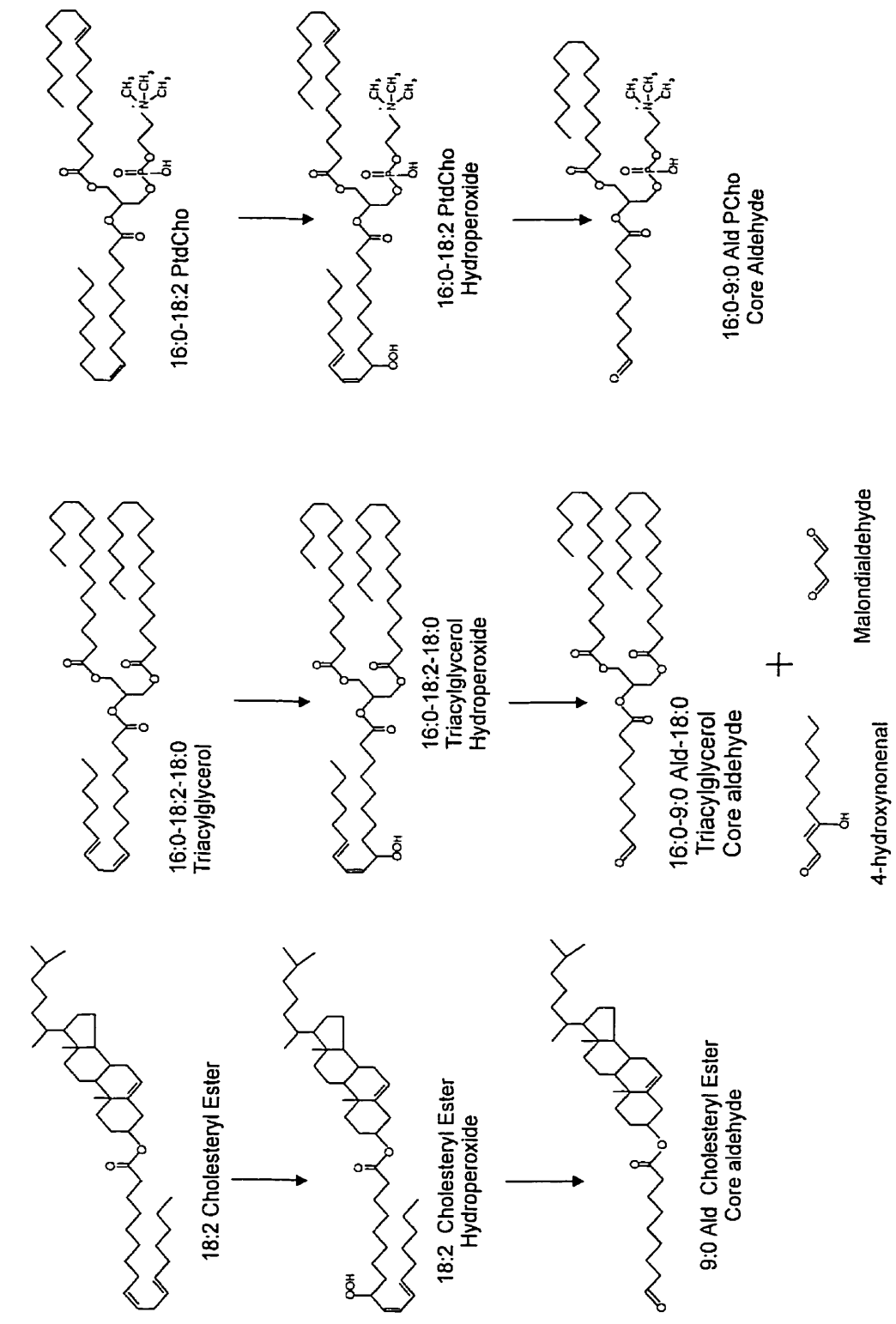
High molecular weight oxidation products

The intensive investigation of LDL lipid oxidation products has until recently been concerned only with the low molecular weight compounds (i.e. MDA and HNE). As shown in Fig 4.1 the production of short chain aldehydes results in generation of saturated aldehydes bound to the glycerol back bone of the parent molecule (core aldehydes) whether it be a phospholipid, cholesteryl ester or a triacylglycerols molecule. Kamido et al (142) in 1992 showed that OxLDL contains these higher molecular weight aldehydes that are still attached to the parent lipid ester molecule. The chain length of these aldehydes, depending on the original esterified fatty acid, ranges from 4 to 9 carbons. Unlike volatile aldehydes, the core aldehydes are not water soluble and are retained in the LDL molecule allowing for

their accumulation. Of interest are the phospholipid core aldehydes which have been shown to have potent biological activity as mimics of platelet activating factor (143), and induce increased endothelial macrophage interactions *in vitro* (144). It has also been shown that these core aldehydes are present in atherosclerotic tissue. Itabe et al (145) has shown that antibodies raised against complexes of oxidized phospholipids and albumin can recognize foam cells in atherosclerotic tissue. Although the specific structure of these adducts were not identified it lends support to the significant role that high molecular weight aldehydes can play in atherogenesis. These oxidized phospholipids specially OxPtdCho can also play a significant role in other diseases. Antiphospholipid antibodies which are usually present in patients with Anti-phospholipid antibody syndrome (APS) have been shown to have cross reactivity towards oxidized LDL as a result of the presence of oxidatively modified PtdCho molecules (146). The reactivity of the aldehyde group can result in generation of novel epitopes recognized by the antibodies present in APS condition.

Other aldehydes such as cholesteryl ester aldehydes are also present in oxidized LDL. They have been identified as dinitrophenyl hydrazine derivatives by HPLC/MS (147). Although CE are the most abundant molecule in LDL very little is known about CE aldehydes. It would be expected that their reaction with amino groups can also result in modification of proteins.

Fig. 4.1 Pathway for the generation of low and high molecular weight aldehydes from phospholipids, cholesteryl esters and triacylglycerols.


18:2 Cholesteryl Ester
18:2 Cholesteryl Ester Hydroperoxide
9:0 Ald Cholesteryl Ester Core aldehyde
16:0-18:2-18:0 Triacylglycerol
16:0-18:2-18:0 Triacylglycerol Hydroperoxide
16:0-9:0 Ald-18:0 Triacylglycerol Core aldehyde
4-hydroxynonenal
Malondialdehyde
16:0-18:2 PtdCho
16:0-18:2 PtdCho Hydroperoxide
16:0-9:0 Ald PCho Core Aldehyde

LDL Lipid glycoxidation

Oxidative modification of LDL has been established as atherogenic, it is not however clear how the glycation of lipoproteins, specifically LDL, is related to oxidative modifications. It is difficult to separate the effects of glycation of lipoproteins from those of free radical oxidation. The two processes are intimately connected. That is why the term glycoxidation is used to illustrate the glucose induced oxidative reactions. Enhanced susceptibility of LDL to *in vitro* oxidative damage following prior glycation has been described recently and is in accordance with previous studies showing that glucose enhances oxidative susceptibility of LDL (148). Studies by Gugliucci et al (149) demonstrate that LDL and VLDL from diabetic patients are more susceptible to oxidation. In contrast LDL from type 1 diabetic patients with good glycemic control exhibited no increase in oxidizability, nor were there abnormalities in particle glycation, size or composition (150). These studies are consistent with the possibility that increased *in vivo* glycation of LDL is required for the increase in susceptibility to oxidation. The pathogenic properties of OxLDL are clearly enhanced when LDL is glycated. Some of these studies have used on cultured macrophages and have reported increased CE deposition and CE synthesis when incubated with oxidized glycated LDL compared to oxidized LDL (151). Although originally glucose was thought to have pro-oxidant activity both in free and bound form, more recent work has suggested that glycoxidation reactions resulting from bound glucose are the major rout for glucose mediated oxidation of biological macromolecules (152). Hunt et al (153), using albumin as a model protein, showed that protein-glucose adducts, as opposed to glucose itself are, the reason for the inextricable link between glucose attachment to protein and lipid oxidation. Hicks et al

(154) demonstrated that glycated collagen can catalyze the peroxidation of membranes containing linoleic and arachidonic acid. Al-Abed et al (155) demonstrated that during LDL glycation, under antioxidative conditions (EDTA), can result in the production of aldehydes such as 4-hydroxyhexenal and HNE. Several studies have indirectly addressed the complex issue of how glycation affects LDL oxidizability. In 1993 Bucala et al (156) using polyclonal anti-AGE antibodies suggested that the aminophospholipids in LDL can also participate in LDL glycation. It was hypothesized that the covalent bonding between lipid and glucose can play a role in *in vivo* LDL oxidation (157), but none of the products of the reaction were isolated or identified

There are also indications that the early glycation products can also play a major role in accelerated atherosclerosis in diabetes. Mullarkey et al (158) demonstrated that both Schiff base and Amadori glycated products of model proteins resulted in fifty-fold increase in superoxide generation and at physiological pH. This resulted in a two-fold increase in peroxidation of membranes of linoleic/arachidonic acid vesicles. Not only was glycated LDL more susceptible to oxidation but when oxidized it has more potent atherogenic properties as suggested by Galle et al (159). It was shown that exposure of aortic rings to glycated and oxidized LDL resulted in a significant attenuated endothelium-dependent relaxation which was a result of increased O_2^- formation resulting in inactivation of nitric oxide. Even plasma of diabetic individuals has been shown to be more susceptible to lipid oxidation in which lack of antioxidants was not a factor.

Summary and working hypothesis

The review of literature shows that since its description some ninety years ago many studies have been carried out on the glycation reactions both *in vitro* and *in vivo*. The

pathogenic consequences, whether being through functional alteration of enzymes and receptors or through oxidative pathways, have also been demonstrated. Previous work had established some of the products of the protein glycation reaction *in vitro* and *in vivo*, but the identification and characterization of aminophospholipid glycation reactions have been over looked specifically in the case of lipoproteins. These products have been claimed to exist as products of the glycation reaction but their biological activity has not been demonstrated and the structural identification of lipid glycation compounds need to be investigated. Due to the complexity of the reaction products powerful analytic tools such as HPLC and mass spectrometry are needed to clarify the identity and allow for their characterization. Many of the protein glycation products are not only prooxidative but also harbor a multitude of atherogenic properties of LDL. Thus it is hypothesized that:

1) PtdEtn is a candidate for glycation reactions; 2) Glycation of PtdEtn occurs *in vivo* with increased levels present in diabetics; 3) Since 5-6% of total LDL phospholipids is PtdEtn, this aminophospholipid is the candidate for glycation reactions; 4) Specific presence of glycated PtdEtn in LDL, in absence of any protein glycation, promotes LDL oxidative modification; 5)The interaction of glucosylated PtdEtn with LDL also leads to the formation of a complex, which is readily phagocytosed by macrophages resulting in creased CE and triacylglycerol accumulation in these cells.

Outline of thesis

In this thesis, an attempt was made to address several of the gaps in knowledge as follows: Chapter 2 describes the first identification and characterization of aminophospholipid glycation products utilizing a novel HPLC/MS technique. Both reduced and non-reduced glucosylated PtdEtn and PtdSer were identified. Chapter 3

demonstrates for the first time the *in vivo* presence of glycated PtdEtn by using LC/ES/MS. Glycated PtdEtns were identified in both normal and diabetic red blood cells and plasma. Chapter 4 is concerned with the PtdEtn glycation in LDL. It demonstrates that PtdEtn is the major LDL glycation product and results in increased LDL oxidative susceptibility. The major phospholipid products of oxidation were identified as the PtdCho hydroperoxides and PtdCho aldehydes. Chapter 5 addresses the reactions of PtdCho aldehydes, which are generated during LDL oxidation, with amino groups present in aminophospholipids and proteins. Chapter 6 presents evidence for the increased uptake of LDL and neutral lipid deposition by macrophages as result of glucosylated PtdEtn. Chapter 7 concludes my work and contains a general discussion of results, an assessment of their significance, and a series of suggestions for possible future directions of research in this area. Each experimental chapter contains a brief introduction and discussion pertinent only to the subject studied. Cited references are listed at the end of each chapter.

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Chapter 2: Preparation and Characterization of Glucosylated Aminoglycerophospholipids*

* The majority of the results in this chapter have been published in the following reference: Ravandi, A., Kuksis, A., Myher, J.J., Marai, L. (1995) Preparation and characterization of glucosylated aminoglycerophospholipids Lipids 30, 885-91

ABSTRACT

Natural aminophospholipids were isolated from egg yolk and from the red blood cells of man. Glucosylated ethanolamine and serine phosphatides were prepared by exposing synthetic and natural aminophospholipids to glucose for 3-18 hours at pH 7.4. The glucosylation products were resolved from parent phospholipids by normal phase HPLC and were identified by on-line mass spectrometry with an electrospray interface. The soft ionization method allowed us to detect the glucosylation products as molecular ions of the Schiff bases. The Schiff bases could be stabilized by sodium cyanoborohydride reduction. The molecular species of the ethanolamine and serine phosphatides reacted in proportion to their molar concentration in the mixtures. The yields of the glucosylation products varied with time of reaction and the concentration of glucose in the medium. At 50 mM glucose and 0.6 mg/ml phosphatidylethanolamine, 20% of the aminophospholipid was glycated in 18 hours.

INTRODUCTION

Advanced glycosylation is believed to be a major pathway for the post-translational modification of tissue proteins, which begins with the non-enzymatic addition of sugars (such as glucose) to the primary amino groups of proteins (1,2). Although the non-enzymatic glycation of the primary amino groups of proteins has been extensively investigated, little attention has been given to phospholipid glycation reactions. The presence of reactive primary amino groups in phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) has prompted the hypothesis (3) that glucose might react with lipids to initiate advanced glycosylation. However, glycated aminophospholipids have not been specifically isolated from any of the sources.

In this study we have prepared the glucosylation products of PtdEtn and PtdSer, which are Schiff bases formed by non-enzymatic attachment of glucose to the amino moiety of the aminophospholipid, and have characterized them by high performance liquid chromatography and on-line mass spectrometry with electrospray ionization (LC/ESI/MS).

MATERIALS AND METHODS

Materials

D-glucose was obtained from BDH Chemicals (Toronto, Ontario). Synthetic sn-1,2-dipalmitoylglycero-3-phosphoethanolamine and bovine brain sn-1,2-dipalmitoylglycero-3-phosphoserine were purchased from Sigma Chemical Co. (St. Louis, MO). Egg yolk PtdEtn was isolated in the laboratory from chloroform-methanol (2:1, vol/vol) extracts of egg yolk by thin-layer chromatography (TLC) on Silica gel H (Merck) using chloroform/methanol/

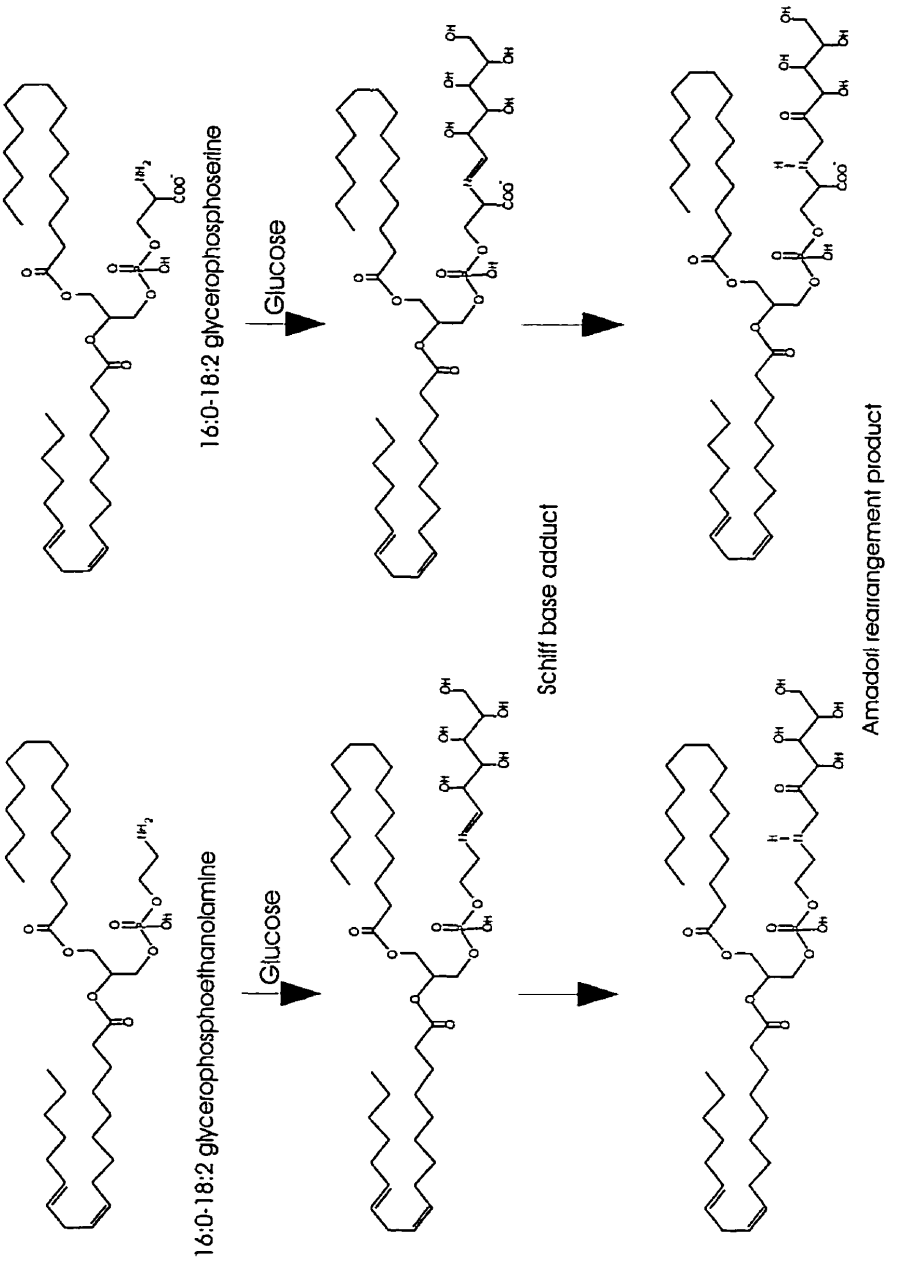
acetic acid/water (75:35:12:6, by vol) as developing solvent (4). Human red blood cell phospholipids were extracted as described by Rose and Oaklander (5). Red cell PtdEtn and PtdSer were isolated by TLC with chloroform/methanol/ammonia (30%) (65:35:7, by vol) as the solvent (6). The phospholipids were recovered from the silica gel by extraction with the developing solvent.

Glucosylation of aminophospholipids

Two mg of either PtdEtn or PtdSer was dissolved in 1 mL methanol. To this solution were added 2 mL of the glucosylation solution, which consisted of different concentrations of glucose (5-100 mM, in 0.1M phosphate buffer, pH 7.4). The test tubes were intermittently sonicated for 30 min at room temperature to produce lipid emulsions. These emulsions were incubated on a mechanical shaker in the dark at 37°C for periods of 3 to 18 hours. After incubation the lipid soluble material was separated from unreacted glucose by extraction with 2 mL chloroform/methanol (2:1, vol/vol). The tubes were centrifuged for 10 min and the organic phase was collected. The extraction was repeated two more times and the extracts combined, blown down under nitrogen and redissolved in chloroform/methanol (2:1, vol/vol). (Scheme 1). The glucosylations were performed several times at each glucose level and each time interval.

Isolation of glucosylated aminophospholipids

The glucosylated and non-glucosylated lipids (1 mg) were resolved by TLC (20 x 20 cm plates, 250 micron thick layer of Silica gel H) using chloroform/methanol/ammonia (30%) (65:35:7, by vol). The resolved lipids were located by spraying the TLC plate with 2,7-dichlorofluorescein and viewing it under UV light. Both glucosylated and non-glucosylated lipids were recovered by scraping the gel from the fluorescent areas and


16:0-18:2 glycerophosphoethanolamine
Glucose
Schiff base adduct
Amadori rearrangement product
16:0-18:2 glycerophosphoserine
Glucose

Scheme 1.2

extracting it with the developing solvent. After solvent evaporation, the residues were dissolved in chloroform or methanol.

$NaCNBH_3$ reduction

The glycated PtdEtn species were reduced by adding freshly prepared $NaCNBH_4$ in methanol (final concentration 70 mM). The sample was incubated at 4°C for 1 hour. The product was washed three times with water to remove excess reagent.

GLC analysis of fatty acid methyl esters

Fatty acid methyl esters were prepared from aliquots of each sample resolved on the TLC plates by scraping the gel from the appropriate areas into 6% H_2SO_4 in methanol and heating at 80°C for 2 hours. After the reaction the methyl esters were extracted twice with hexane. The solvents were blown down under nitrogen and the samples redissolved in hexane. The fatty acids were analyzed on a polar capillary column (SP 2380, 15 m x 0.32 mm i. d., Supelco. Canada) installed in a Hewlett-Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a hydrogen flame ionization detector (7). Hydrogen was the carrier gas at 3 psi.

HPLC of phospholipids.

Normal phase HPLC separations of phospholipids were performed on Spherisorb 3 micron columns (100 mm x 4.6 mm i. d., Alltech Associates, Deerfield, IL) installed into a Hewlett-Packard Model 1050 Liquid Chromatograph connected to an evaporative light scattering detector (ELSD) (Varex, Model ELSD II, Rockville, Maryland). The column was eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/water/30%

ammonium hydroxide, 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min (8). The flow rate was set at 1 ml/min.

LC/MS of phospholipids

Normal phase LC/MS was performed by admitting the entire HPLC column effluent into a Hewlett Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer assisted ESI interface (HP 59987A). Negative ESI spectra were taken in the mass range 400-1100. The capillary exit voltage was set at 150 volts with the electron multiplier at 1795 volts. Selected ion spectra were retrieved from the total ion spectra by computer. The masses given in the tables and figures are the actual masses of the ($[M-H]^-$) ions. The nominal masses are one unit lower.

The molecular species of the various glycerophospholipids were identified on the basis of the molecular mass provided by mass spectrometry, the knowledge of the fatty acid composition of the aminophospholipid classes, and the relative HPLC retention time (more saturated species migrating ahead of the unsaturated species) of the phospholipids.

RESULTS

TLC of Glucosylated Aminophospholipids

Figure 1.2 shows that on normal phase TLC the glucosylated phospholipids migrate more slowly than the corresponding non-glucosylated derivatives and that there is an effective resolution of the glucosylated aminophospholipids from other common phospholipids. The Rf values of PtdEtn and PtdSer were 0.69 and 0.21, and of glucosylated PtdEtn and glucosylated PtdSer 0.46 and 0.09, respectively. Trans-methylation of the TLC bands

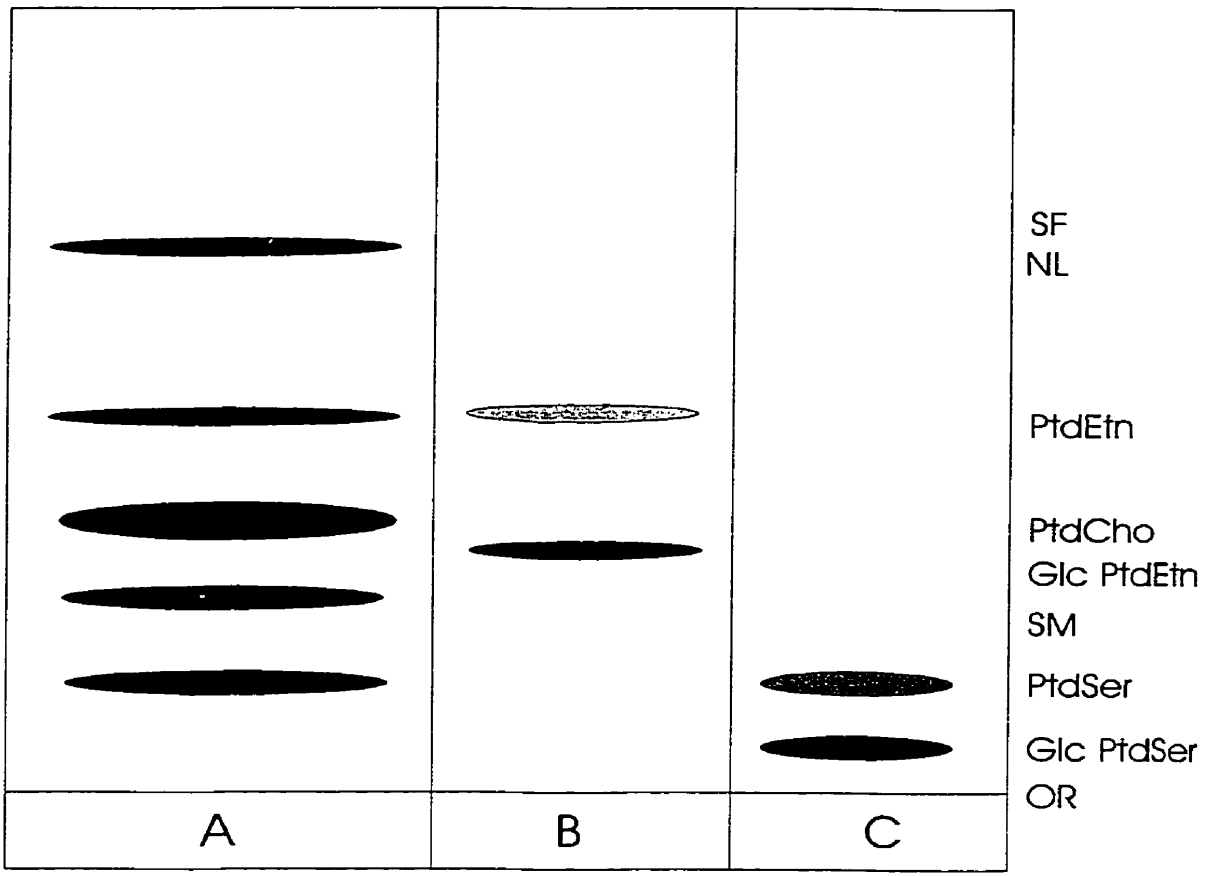


Fig. 1.2 TLC of common phospholipids before and after glucosylation with 50 mM glucose. A, reference phospholipids; B, glucosylated PtdEtn; C, glucosylated PtdSer; SF, solvent front; NL, neutral lipid; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; SM, sphingomyelin; PtdSer, phosptnhatidylserine; Glc PtdEtn, glucosylated PtdEtn; Glc PtdSer, glucosylated PtdSer; OR, origin. Solvent: chloroform/methanol/ammonia(30%) (65:35:7, by vol). Detection: 2,7-dichlorofluorescein showed identical fatty acid composition for the corresponding precursor and product molecules.

HPLC separation of phospholipids with ELSD detection

Figure 2.2 shows the normal phase HPLC separation of glucosylated PtdEtn relative to PtdEtn and other red blood cell phospholipids. The glucosylated PtdEtn is eluted later than non-glucosylated PtdEtn. Furthermore, the glucosylated PtdEtn is seen to overlap in part with phosphatidylcholine. Similarly, glucosylated PtdSer was eluted later than non-glucosylated PtdSer, both being eluted after the glucosylated PtdEtn (see below). The peaks were detected by ELSD and their identity was confirmed by on-line mass spectrometry.

Time course of PtdEtn Glucosylation

Figure 3.2 shows that the yield of the glucosylated product depends on the concentration of the glucose in the incubation medium. Detectable glucosylation was seen at 5 mM, which corresponds to the glucose level in healthy subjects (9). The 50 mM glucose yielded 15-20% glucosylation in 18 hours. Increasing the glucose concentration to 100 μM doubled the yield of the Schiff base, and further increases in glucose led to additional glucosylation of the PtdEtn. The small S. D. values show that the glucosylation is reproducible. Similar results were obtained with PtdSer.

LC/ES/MS of reduced Glucosylated PtdEtn and PtdSer

Figure 4.2 shows the total negative ion current profile and full spectra as obtained by LC/ESI/MS for the dipalmitoyl species of PtdEtn after glucosylation and $NaCNBH_3$ reduction and the dipalmitoyl species of PtdSer after glucosylation. In each instance the symmetrical ion current profiles are accompanied by single $[M-H]^-$ ions of the correct mass. Following reduction the glucosylated compounds were stable to acid and showed an increase in the m/z value of 2 amu, indicating a saturation of the original Schiff base

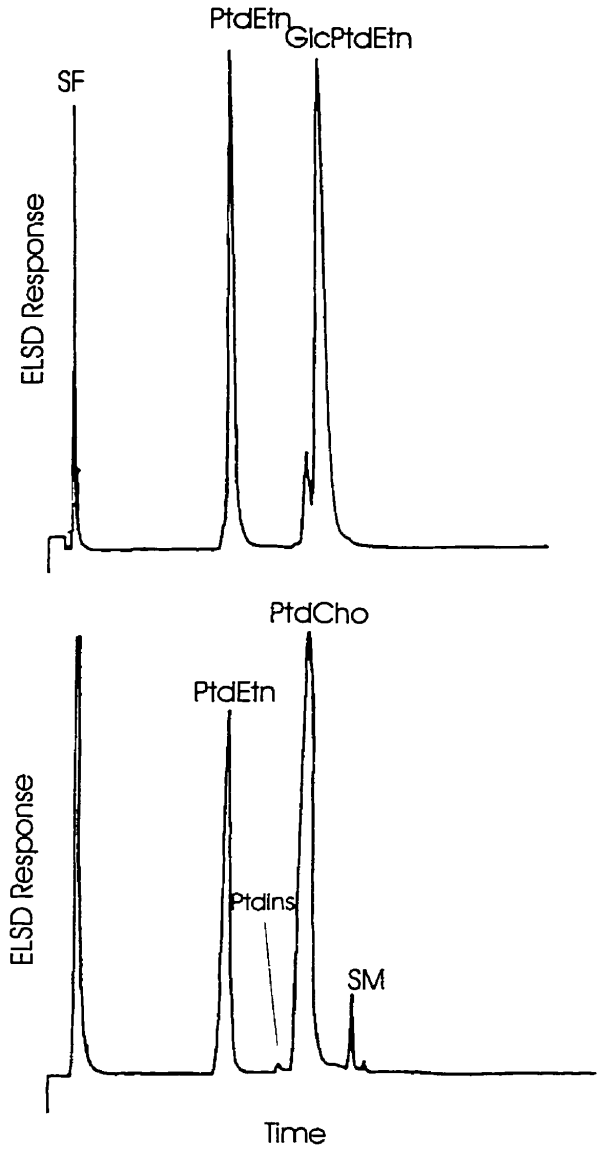


Fig.2.2 Normal phase HPLC of glucosylated and non-glucosylated phospholipids. A, partially glucosylated egg yolk PtdEtn; B, non-glucosylated red blood cell phospholipids. Solvent: Linear gradient of 100% Solvent A to 100% Solvent B in 14 min, then Solvent B for 10 min. Solvent A: chloroform/methanol/30%ammonium hydroxide (80:19.5:0.5, by vol); Solvent B, chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:0.5, by vol). Detection: ELSD. Phospholipid abbreviations as in Fig. 1. PtdSer and PtdCho overlap in this solvent system.

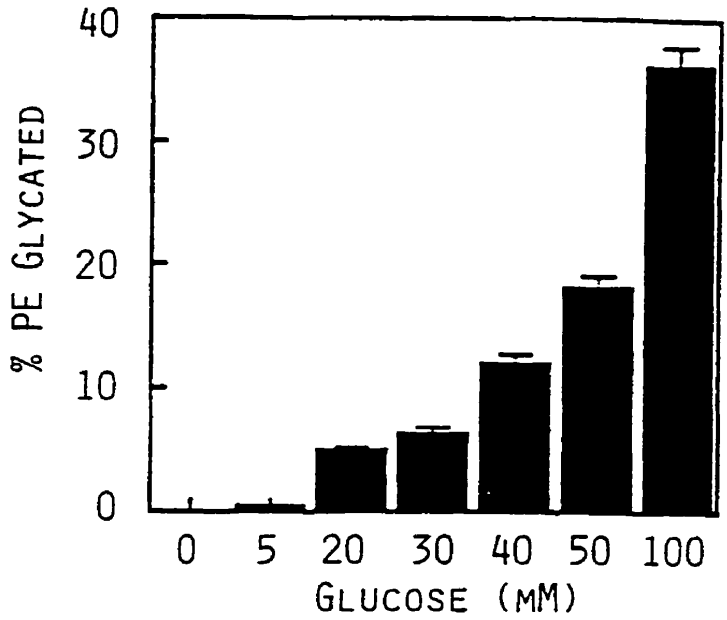


Fig. 3.2 Formation of glucosylated PtdEtn during an overnight (18 hours) incubation (37°C, pH 7.4) with various concentrations of glucose. The glucosylated and non-glucosylated PtdEtn were quantitated by normal phase HPLC with ELSD.

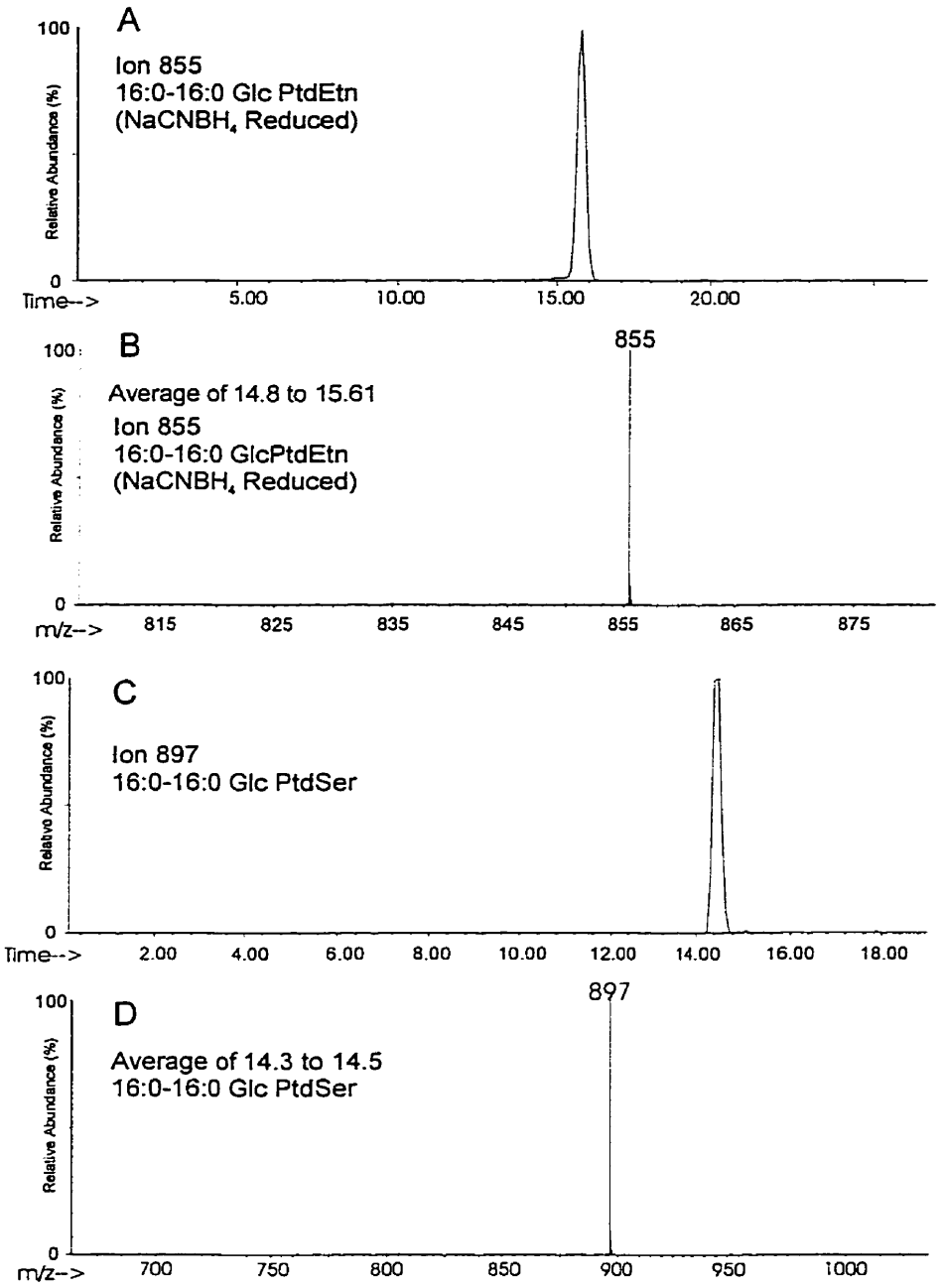


Fig. 4.2 Normal phase LC/MS with ESI of glucosylated and NaCNBH4-reduced dipalmitoyl aminophospholipids. Selected ion plot (A) and full spectrum (B) of glucosylated and reduced PtdEtn; selected ion plot (C) and full spectrum (D) of glucosylated PtdSer. HPLC and MS conditions are given in Fig. 2 and under Experimental Procedures.

structure. There was a slight increase in the retention time of the reduced in comparison to the non-reduced glucosylation products.

Glucosylation of egg yolk PtdEtn

Figure 5.2 shows the total ion current profile obtained by normal phase LC/MS for a mixture of non-glucosylated and glucosylated (18 hours) egg yolk PtdEtn along with the ES spectra averaged over the peaks of the two types of PtdEtn molecules. The egg yolk PtdEtn was incubated with 25 mM glucose. The major ions for the non-glucosylated PtdEtn are m/z 715 (16:0-18:2), m/z 717 (16:0-18:1), m/z 739 (16:0-20:4), m/z 743 (18:0-18:2 and 18:1-18:1), m/z 767 (18:0-20:4) and m/z 793 (18:0-22:5), corresponding to the major diacyl species in the original egg yolk PtdEtn (10). There are also minor ions corresponding to alkenylacyl species, e. g. m/z 775 (18:0-22:6), m/z 777 (18:0-22:5) and m/z 779 (18:0-22:4). The major molecular ions for the glycated PtdEtn are m/z 877 (16:0-18:2), m/z 879 (16:0-18:1), m/z 901 (16:0-20:4), m/z 905 (18:0-18:2 and 18:1-18:1), m/z 907 (18:0-18:1), m/z 929 (18:0-20:4). It appears that the oligoenoic species of PtdEtn are glucosylated as readily as the polyunsaturated and the alkenylacyl species.

Glucosylation of RBC aminophospholipids

Figure 6.2 shows the normal phase LC/MS profile of human red blood cell phospholipids following incubation with 50 mM glucose (18 hours), along with the mass spectra averaged over the glucosylated and non-glucosylated PtdEtn and PtdSer subclasses. The total ion current profile of non-glucosylated PtdSer shows two peaks, because the more unsaturated species migrate slightly faster than the more saturated species. This difference is not apparent following glucosylation when they overlap. About 50% of each phospholipid class was glucosylated. The PtdEtn showed glucosylation of both diacyl and the alkenylacyl

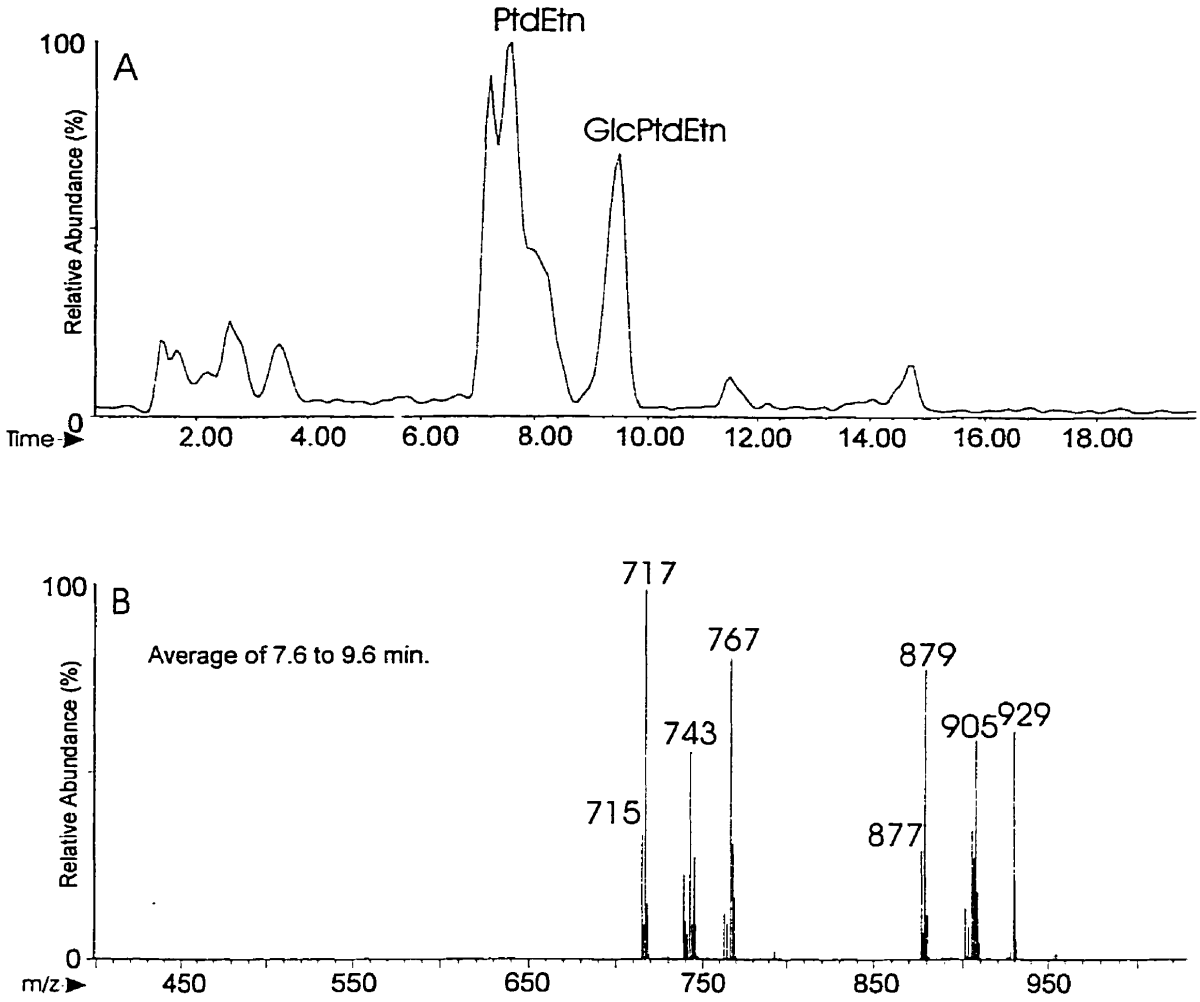
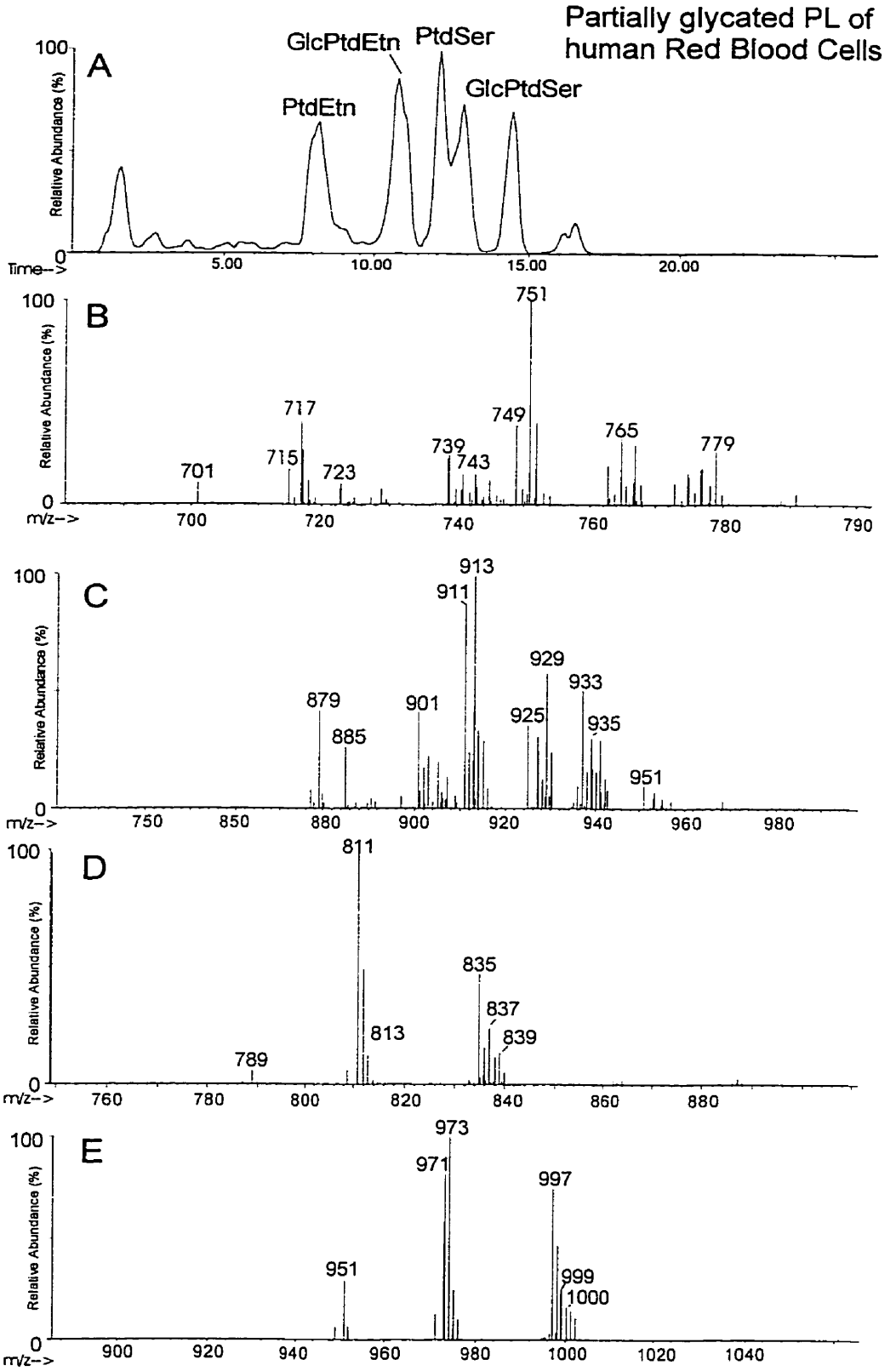


Fig.5.2 Normal phase LC/MS with electrospray of partially glucosylated egg yolk PtdEtn. A, total ion current profile; B, mass spectra averaged over the peak of non-glucosylated and glucosylated PtdEtn; HPLC and MS conditions are given in Fig. 2 and under Experimental Procedures.

Fig.6.2 Normal phase LC/MS with electrospray of partially glucosylated aminophospholipids of human red blood cells. A, total ion current profile; B, mass spectra of non-glucosylated PtdEtn; C, mass spectra of glucosylated PtdEtn; D, mass spectra of non-glucosylated PtdSer; E, mass spectra of glucosylated PtdSer. LC/MS conditions are given in Fig. 4 and under Experimental Procedures.


Partially glycated PL of human Red Blood Cells
A
GlcPtdEtn
PtdSer
PtdEtn
GlcPtdSer
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
B
751
717
749
765
779
715
739
743
701
723
m/z-->
700
720
740
760
780
790
C
913
911
929
933
879
901
925
935
885
951
750
850
880
900
920
940
960
980
D
811
835
837
813
839
789
760
780
800
820
840
860
880
E
973
971
997
951
999
1000
900
920
940
960
980
1000
1020
1040

species. The molecular species of the PtdEtn and PtdSer were glucosylated roughly in proportion to their mole percentages in the phospholipid classes in the human red blood cell. The major component in both non-glucosylated PtdEtn (m/z 751) and in glucosylated PtdEtn (m/z 913) was the alkenylacyl (18:0-20:4) species. The major component in both non-glucosylated PtdSer (m/z 811) and in glucosylated PtdSer (m/z 973) was the diacyl (18:0-20:4) species. Table 1.2 lists the $[M-H]^-$ values, identities and the relative proportions of the major molecular species in the glucosylated and non-glucosylated portions of the aminophospholipids. The proportions of the major molecular species within the glucosylated and non-glucosylated forms of each aminophospholipid class were calculated from the $[M-H]^-$ ion (corrected for M+1 and M+2, where necessary) intensity and are given as percent of total. These proportions agreed closely with the known composition of the major species of egg yolk and red blood cell PtdEtn and PtdSer (11). The proportions of glucosylated and non-glucosylated forms of each aminophospholipid were determined by normal phase HPLC with ELSD. The ESI intensity differed significantly among the different phospholipid classes and between glucosylated and non-glucosylated PtdEtn and PtdSer, and required appropriate correction during quantitation in either positive or negative ion mode.

DISCUSSION

Although glucose is the least reactive of the common monosaccharides in Schiff base formation (12), it has been suggested that it might react with aminophospholipids and

Table1.2
Molecular Species of Aminophospholipids of Human Red Blood Cells Following Exposure to 50 mM Glucose.
PtdEtn

Molecular Species	DiacylPtdEtn		Diacyl Glc PtdEtn		Alk-acyl PtdEtn		Alk-Acyl GlcPtdEtn	
	$[M-H]^-$	%	$[M-H]^-$	%	$[M-H]^-$	%	$[M-H]^-$	%
16:0-18:1	717	14.7	879	15.3				
16:0-18:2	715	5.2	877	4.7				
18:0-18:1	745	2.8	907	1.3	729	2.3	891	1.1
16:0-20:4	739	15.3	901	13.9	723	7.1	885	6.8
18:1-18:1	743	5.8	905	6.4				
18:1-18:2	741	9.7	903	11.7	725	11.1	887	5.3
16:0-20:5	737	0.4	897	0.2	721	0.1	883	0.4
18:0-20:4	767	16.9	929	19.8	751	35.8	913	31.6
16:0-22:4								
18:1-20:3								
18:0-20:3	769	4.3	931	5.8	753	0.1	915	2.1
18:1-20:4	765	13.2	927	10.2	749	15.7	911	21.7
18:0-20:5								
16:0-22:5								
16:0-22:6	763	7.6	925	9.4	747	1.4	909	1.6
18:2-20:4								
18:2-20:5	793	0.8	955	0.7	777	9.9	939	13.7
18:1-22:4								
18:0-22:6	791	2.1	953	0.1	775	11.9	937	12.2
18:1-22:6	789	0.8	951	1.3	773	3.4	935	3.1

PtdSer

Molecular Species	PtdSer		Glc PtdSer	
	$[M-H]^-$	%	$[M-H]^-$	%
18:0-18:1	789	6.6	951	8.3
18:0-20:4	811	50.6	973	45.6
16:0-22:4				
18:1-20:3				
18:0-20:3	813	7.3	975	10.3
18:1-20:4	809	2.9	971	3.6
16:0-22:5				
18:0-22:5	837	9.1	999	12.6
18:0-22:6	835	22.4	997	17.2
18:1-22:6	833	1.1	995	0.8

Diacyl PtdEtn, 20.2%; Diacyl GlcPtdEtn, 24.5%; Alkenylacyl PtdEtn, 24.0; Alkenylacyl GlcPtdEtn, 31.1%; PtdSer, 48.6%; GlcPtdSer, 51.3%.

play a role in production of age pigments. The products of the reaction, however, have not been isolated. Previous attempts had not proven successful in the isolation of glycated PtdEtn from phospholipid-containing membranes (9). The earlier failure to isolate glycated PtdEtn may have been due to the easy dissociation of the Schiff base in the presence of mild acid, which is frequently employed in lipid extraction and TLC of phospholipids. We have not determined the exact time course of acid hydrolysis of glucosylated aminophospholipids. However, we have repeatedly observed the decomposition of the glucosylation products during extraction and chromatography (HPLC and TLC) with solvents containing small amounts of acetic, trifluoroacetic or phosphoric acid. In contrast, these solvent systems allowed effective extraction and chromatography of the cyanoborohydride reduction products of the glucosylated aminophospholipids. In the present study we noted that alkaline solvent systems were best suited for the recovery of the glucosylated aminophospholipids from *in vitro* incubations.

The effect of pH was investigated only over a limited range, keeping close to physiological conditions. This was done in order to approximate the non-enzymatic *in vivo* formation of Schiff bases between the aldehyde form of glucose and the aminophospholipids, and to avoid subsequent transformations. More alkaline conditions (pH > 7.4) were not used because of potential hydrolysis of the lipid ester bonds during prolonged incubation. The alkaline HPLC solvent system and the soft ESI allowed us to demonstrate the glucosylation of both PtdEtn and PtdSer in the presence of glucose concentrations found in severe diabetes and hyperglycemia. Such glucose concentrations have been shown to cause membrane damage and cell death of cultured pericytes, endothelial cells, kidney cells, retinal rods and red blood cells (13-16). Incubation of intact red blood cells

with increasing concentrations of glucose under isotonic conditions and pH 7.4 led to glucosylation of largely PtdEtn, which we hope to described in greater detail elsewhere along with the *in vivo* glucosylation of red blood cell. We have demonstrated that glucosylation of PtdEtn and PtdSer results in increased accumulation of lipid ester hydroperoxides and core aldehydes in the aminophospholipids. It is not known whether this is due to increased susceptibility of the glucosylated aminophospholipids to peroxidation or to an interference of the glucosylated base structure with the removal of the hydroperoxy fatty acids from the phospholipid molecules by phospholipases A_2. Phospholipase A_2 is believed to be involved in cleavage of oxidized fatty acids as part of oxidation damage repair mechanism in cell membranes (17,18). The availability of the glucosylated standards along with well defined working conditions for the isolation of these structures should allow an assessment of the natural distribution of the glucosylated aminophospholipids in normal and pathological tissues. Comparable ESI spectra of non-glucosylated aminophospholipids have been recently reported (19,20).

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Chapter 3: Isolation and identification of glycated aminophospholipids from red cells and plasma of diabetic blood*

* The majority of the results in this chapter have been published in the following reference: Ravandi, A., Kuksis, A,. Marai, L., Myher J.J. Steiner, G., Lewis, G., Kamido, H.(1996) *FEBS Lett.* **381**, 77-81.

ABSTRACT

Glycosylation is a major pathway for post-translational modification of tissue protein and begins with non- enzymatic addition of carbohydrate to the primary amino groups. Excessive glycation of tissue protein has been implicated in the pathogenesis of diabetes and aging. While glycation of aminophospholipids has also been postulated, glycated amino- phospholipids have not been isolated. Using normal phase HPLC with on-line electrospray mass spectrometry we found glycated ethanolamine phospholipids to make up 10-16% of the total phosphatidylethanolamine (PtdEtn) of the red blood cells and plasma of the diabetic subjects. The corresponding values for glycated PtdEtn of control subjects were 1 – 2%.

INTRODUCTION

We have previously reported on the identification of high molecular weight secondary products of peroxidation of standard lipids and lipoproteins during *in vitro* incubation with tert-butyl hydroperoxide (1,2) or copper ions (3,4). Using these products as reference standards we have identified lipid ester hydroperoxides and core aldehydes in plasma and atheromas of patients with atherosclerosis and diabetes (5). Bucala et al. (6) have postulated that aminophospholipids might react with the increased glucose in diabetes to form Schiff bases, which promote fatty acid oxidation. The glycation products of amino group-containing phospholipids, however, have never been isolated and their role in lipid peroxidation has not been directly determined. We have recently synthesized glucosylated phosphatidylethanolamine (Glc PtdEtn) and phosphatidylserine (Glc PtdSer) and have determined their chromatographic properties (7). In the present study we have used the glucosylated aminophospholipids as reference compounds to identify and quantitate glucosylated PtdEtn in lipid extracts from red blood cells and plasma of diabetics and of control subjects.

MATERIALS AND METHODS

Materials

Glucosylation products of the dipalmitoyl species of PtdEtn and PtdSer as well as the PtdEtn of egg yolk and PtdSer of human red blood cells were available from previous work (7). Non-glucosylated egg yolk PtdEtn and bovine brain PtdSer, as well as egg yolk phosphatidylcholine (PtdCho) and bovine brain phosphatidylinositol (PI) and sphingomyelin (SM) were obtained from Sigma Chemical Co., St. Louis, MO. All chemicals were of reagent grade quality, while the solvents were of chromatographic

purity and were obtained from local suppliers. The purity of the reference compounds was ascertained by thin-layer chromatography (TLC) (3,7).

Isolation of phospholipids from blood

Blood was obtained from six diabetic patients and six non-diabetic donors. The diabetics were selected for elevated blood glucose levels indicated by their content of glycosylated hemoglobin (9 – 15%). EDTA blood was centrifuged (2300xg for 10 min) in a swinging bucket rotor to separate the plasma from the red cells. The cells were washed three times with five volumes of phosphate buffered saline (150 mM NaC1, 50 mM sodium phosphate, pH 8.0) and centrifuged (2300 x g for 10 min). The red blood cell phospholipids were extracted according to Rose and Oaklander (8). The plasma phospholipids were extracted with chloroform-methanol 2: 1 modified from Folch et al. (9). Glucosylated PtdEtn could be stored at – 20'C in neutral chloroform – methanol for several days without decomposition. The Schiff base dissociated in dilute acetic acid.

$NaCNBH_3$ reduction

Aliquots of phospholipids containing glucosylated PtdEtn in chloroform were reduced by adding freshly prepared $NaCNBH_3$ in methanol (final concentration 70 mM) as previously described (7).

Normal phase HPLC LC/ESI/MS of phospholipids

Normal phase HPLC separations of phospholipids were performed on Spherisorb 3 micron columns (100 mmx4.6 mm ID, Analtech, Deerfield, IL) installed into a Hewlett-Packard (Palo Alto, CA) Model 1090 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B Quadrupole mass spectrometer equipped with a nebulizer assisted electrospray interface. The column was eluted with a linear gradient of 100% Solvent A

(chloroform/fnethanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/ methanol/water/30% ammonium hydroxide, 60: 34: 5: 0.5, by vol) in 14 min, then at 100% B for 10 min (10). Both negative and positive ionization spectra were taken in the mass range 400 – 1100. Selected ion spectra were retrieved from the total ion spectra by computer. The molecular species of the various glycerophospholipids were identified on the basis of the molecular mass provided by the mass spectrometer, the knowledge of the fatty acid composition of the aminophospholipid class, and the relative elution order (the less polar long chain species emerging ahead of the more polar short chain species) of the phospholipids from the normal phase column.

Statistical analysis

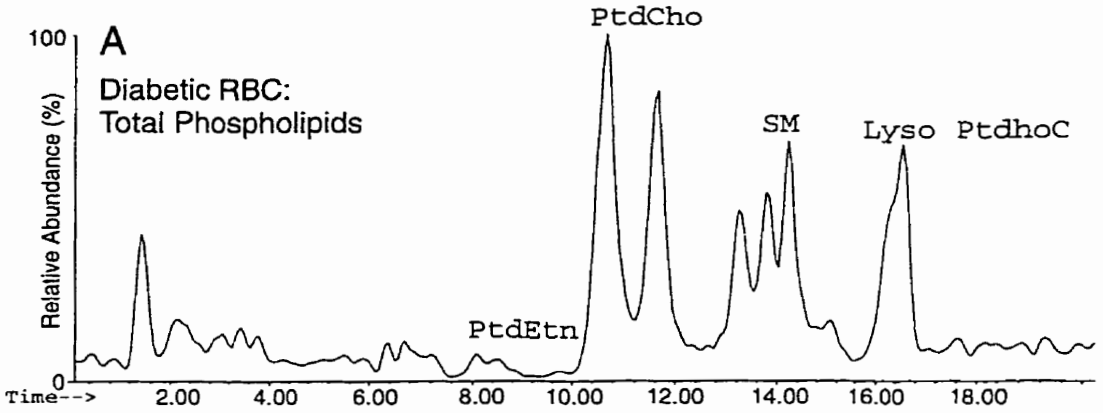
Statistical significance was performed with ANOVA.

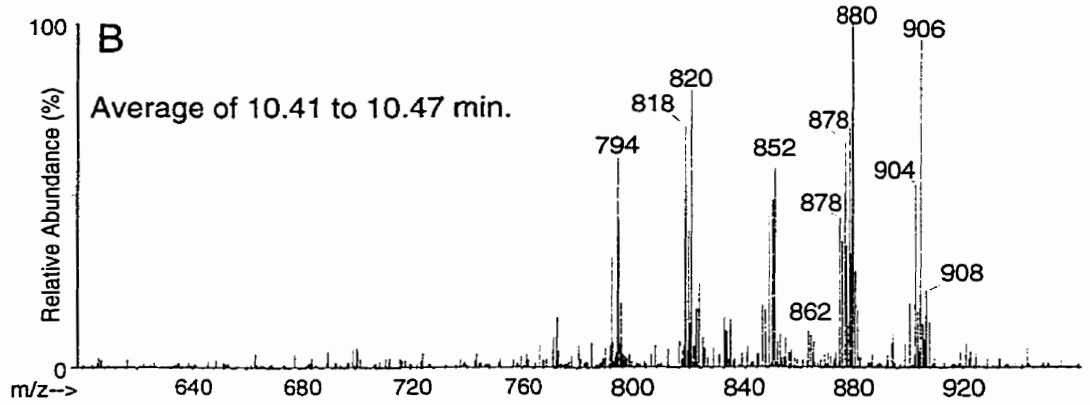
RESULTS

Isolation of Glycated PtdEtn in diabetic RBC

Fig. 1.3A shows the total positive ion current profile of red blood cell phospholipids as obtained by LC/MS for a 50 year old patient with diabetes. Only PtdCho, SM and lysophosphatidylcholine (Lyso PtdCho) are seen as they are readily ionized under these conditions, but PtdEtn can also be discerned. The PtdCho and SM are resolved into two or more subfractions with the longer chain species being eluted earlier than the shorter chain species. LC/ESI/MS yielded molecular masses characteristic of the major molecular species in each phospholipid class, except for the species associated with the front of the first PtdCho peak, which were of higher molecular mass. Fig. 1.3B gives the mass spectra averaged over the front part of the earliest emerging PtdCho peak (10.418 – 10.479 min) in Fig. 1.3A. The major masses correspond to the [M+1]+ ions of the mono-

Fig. 1.3 LC/ES/MS of the phospholipids of red blood cells of a male diabetic subject with 9% glycation of hemoglobin. (A) Total positive ion profile; (B) mass spectra averaged over the front part of the earliest emerging PtdCho peak (10.418-10.479 min). PE, phosphatidylethanolamine; PtdCho, phosphatidylcholine; SPH, sphingomyelin; Lyso PtdCho, lysophosphatidylcholine. The masses in C correspond to the [M+1]+ ions of the 18:0 18:1 (m/z 908), 18:1 18:1 (m/z 906); 18:1 18:2 (m/z 904); 16:0 20:4 (m/z 902); 16:0 18:0 (m/z 882); 16:0 18:1 (m/z 880); 16:0 18:2 (m/ z 878); and 16:0 16:0 (m/z 854) of glucosylated PE. HPLC conditions: Column, Spherisorb 3 micron (100 mmx4.6 mm ID, Analtech, Deerfield, IL); Solvent: Linear gradient of 100% Solvent A to 100% Solvent B in 14 min, then Solvent B for. 10 min. Solvent A: chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5, by vol). Solvent B: chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:0.5, by vol). MS instrumentation and operating conditions were as given in section 2.


A
Diabetic RBC:
Total Phospholipids
PtdCho
SM
Lyso PtdhoC
PtdEtn
Relative Abundance (%)
100
0
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00


B
Average of 10.41 to 10.47 min.
880
906
820
818
878
794
852
904
878
908
862
Relative Abundance (%)
100
0
m/z-->
640
680
720
760
800
840
880
920

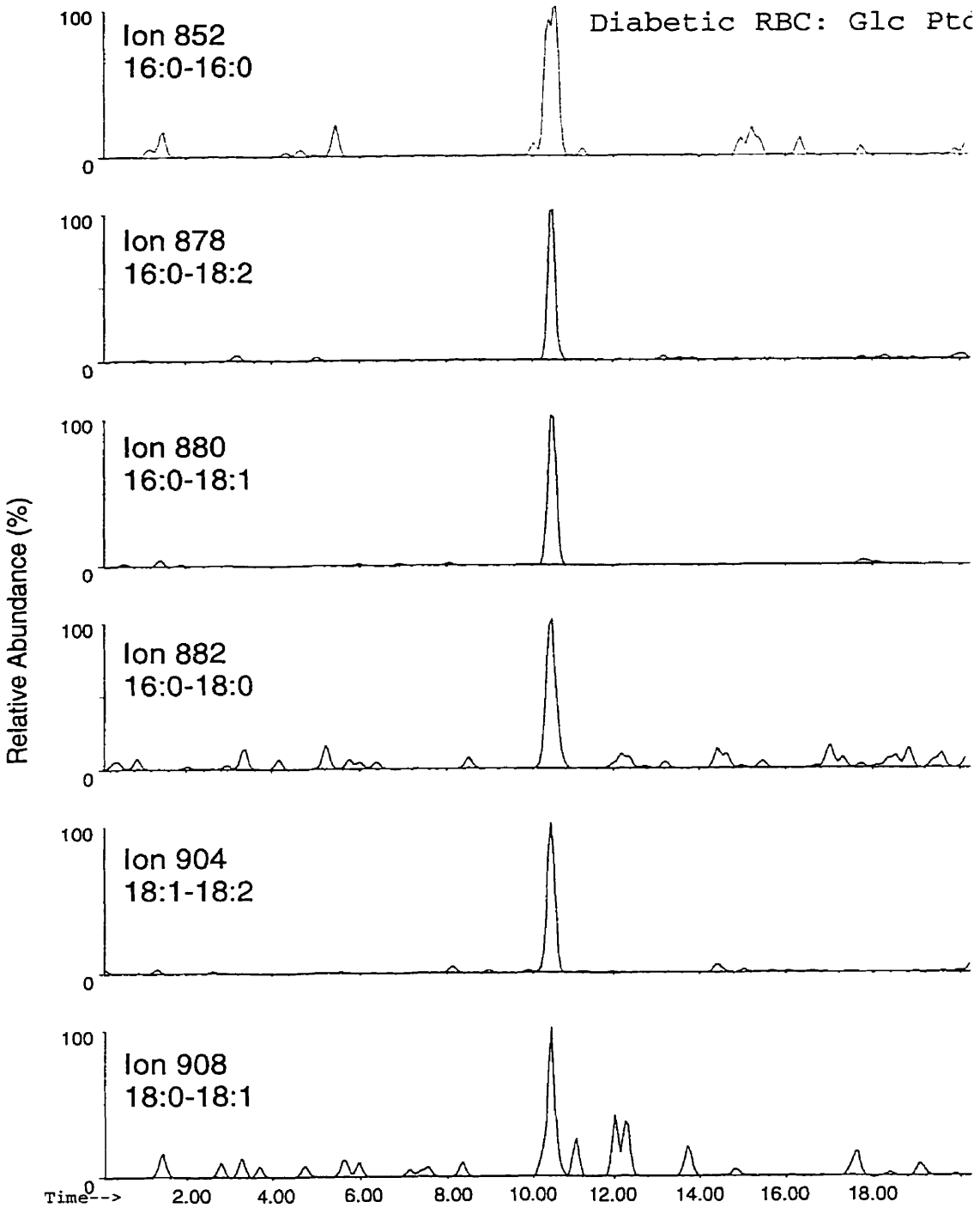


Fig. 2.3 Single ion plots of the masses corresponding to the $[M+I]^+$ ions of the major molecular species of glycated PE from red blood cells of a diabetic subject. The ions are identified as shown in the figure. LC/ESI/MS conditions are as given in Fig. 1.3.

and di-unsaturated species of glucosylated PtdEtn, e.g. m/z 852 (16: 0 – 16: 1), m/z 878 (16: 0 – 18: 2), m/z 880 (16: 0 – 18: 1), m/z 882 (18: 0 – 16: 0), m/z 902 (18: 2 – 18: 2), m/z 904 (18: 1 – 18: 2), m/z 906 (18: 1 – 18: 1) and m/z 908 (18: 0 – 18: I). We have shown previously (7) that glucosylated PtdEtn emerge with the front of the first PtdCho peak, while glucosylated PtdSer overlap with the Lyso PtdCho peak. The masses at m/z 794 and 820 were assigned to the 18:0"-20:4 and 18:0"-22:5 and other isobaric alkenylacyl PtdCho species, respectively, to which they also correspond in HPLC retention time.

Molecular species of Glycated PtdEtn

Fig. 2.3 gives single ion plots for the major species of glycated along with the proposed identities. These ions appeared just ahead of those corresponding to the major poly-unsaturated species of PtdCho, but there were some overlaps. The opportunity for overlapping of co-incidence ions from PtdCho and glucosylated PtdEtn was eliminated by sodium cyanoborohydride reduction of the glucosylated PtdEtn. The reduction increased the mass of the [M+1]+ ions by two mass units and led to a marked increase (14 min) in HPLC retention time, which resulted in the elution of the glucosylated PtdEtn species in the sphingomyelin range (7). Similar results were obtained with the red blood cells of the other diabetic subjects.

Isolation of Glycated PtdEtn in diabetic Plasma

Fig. 3.3A shows the total positive ion current profile of the plasma phospholipids as obtained by normal phase LC/ESI/ MS for another patient with diabetes. The phospholipid classes yield a pattern slightly different from that seen for the red cells.

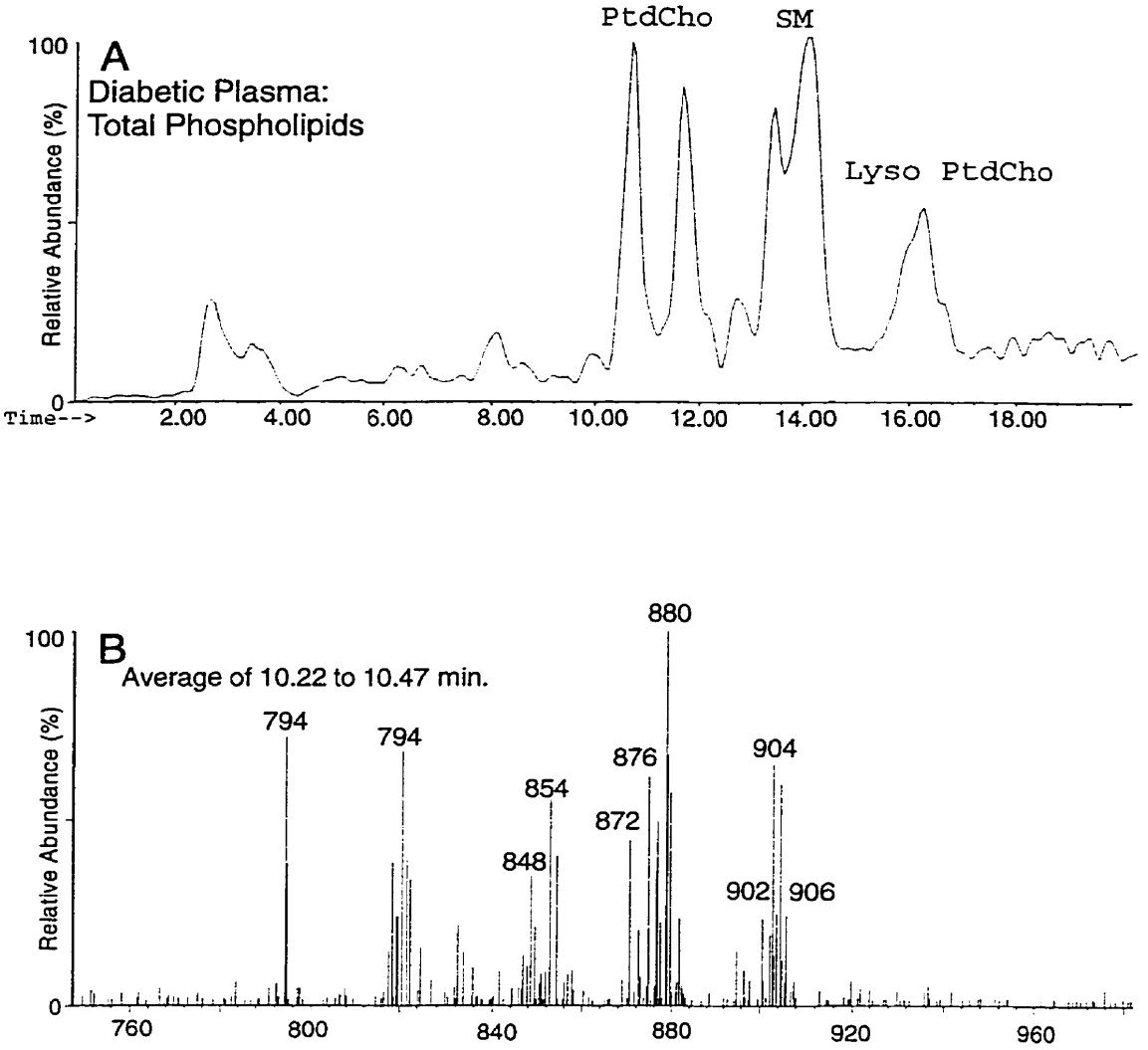


Fig. 3.3 LC/ES/MS of plasma phospholipids of a female diabetic with 12% glycation of hemoglobin. (A) Total positive ion current profile; (B) mass spectra averaged over the front part of the earliest emerging PtdCho peak (10.22 - 10.47 min). Phospholipid classes are identified as in Fig. 1. The masses in (B) correspond to the glucosylated PtdEtn as indicated in Fig. 4. LC/ESI/MS conditions were as given in Fig. 1.3.

However, PtdCho is again split into two peaks, with the earlier emerging one overlapping with glucosylated PtdEtn. There is relatively more SM, again being split into subfractions and Lyso PtdCho in the plasma than in the red cells. Again the various phospholipid classes gave molecular masses characteristic of the major molecular species, except for the front of the first PtdCho peak, which contained a series of higher molecular weight components corresponding to glucosylated PtdEtn. Fig. 3.3C shows that the higher masses are concentrated at the front of the earliest emerging PtdCho peak (10.226 – 10.471 min) in Fig. 3.3A. The major masses in the averaged spectrum correspond to the [M+1]+ ions of glucosylated mono- and di-unsaturated species of PtdEtn as noted above for the red cells, e.g. m/z 852, m/z 876, m/z 878, m/z 880, m/z 904, and m/z 908). The ions at m/z 794 and 822 correspond to the 18: 0"-20:4 and 18:0"-22:4 and isobaric alkenylacyl species of PtdCho, which are eluted earlier than the other PtdCho species because of the lower polarity. The proportions of the glucosylated PtdEtn species differ only slightly from those of the red cells.

Molecular species of Glycated PtdEtn in Plasma

Fig. 4.3 gives single ion plots for the glucosylated PtdEtn masses along with the proposed identities of the species. Again the glucosylated species exhibited the increases in the mass and in retention time as a result of the cyanoborohydride reduction, as already noted for the glucosylated PtdEtn of the red cells. Neither positive nor negative ion mode indicated the presence of any glucosylated PtdSer in the red cells or in plasma, although PtdSer was demonstrated to be readily glucosylated *in vitro* (7). Table 1.3 compares the species of the diacyl, alkenylacyl, and glycated diacyl PtdEtn from the red cells of diabetic and control subjects. Only the diacyl species have become glycated and

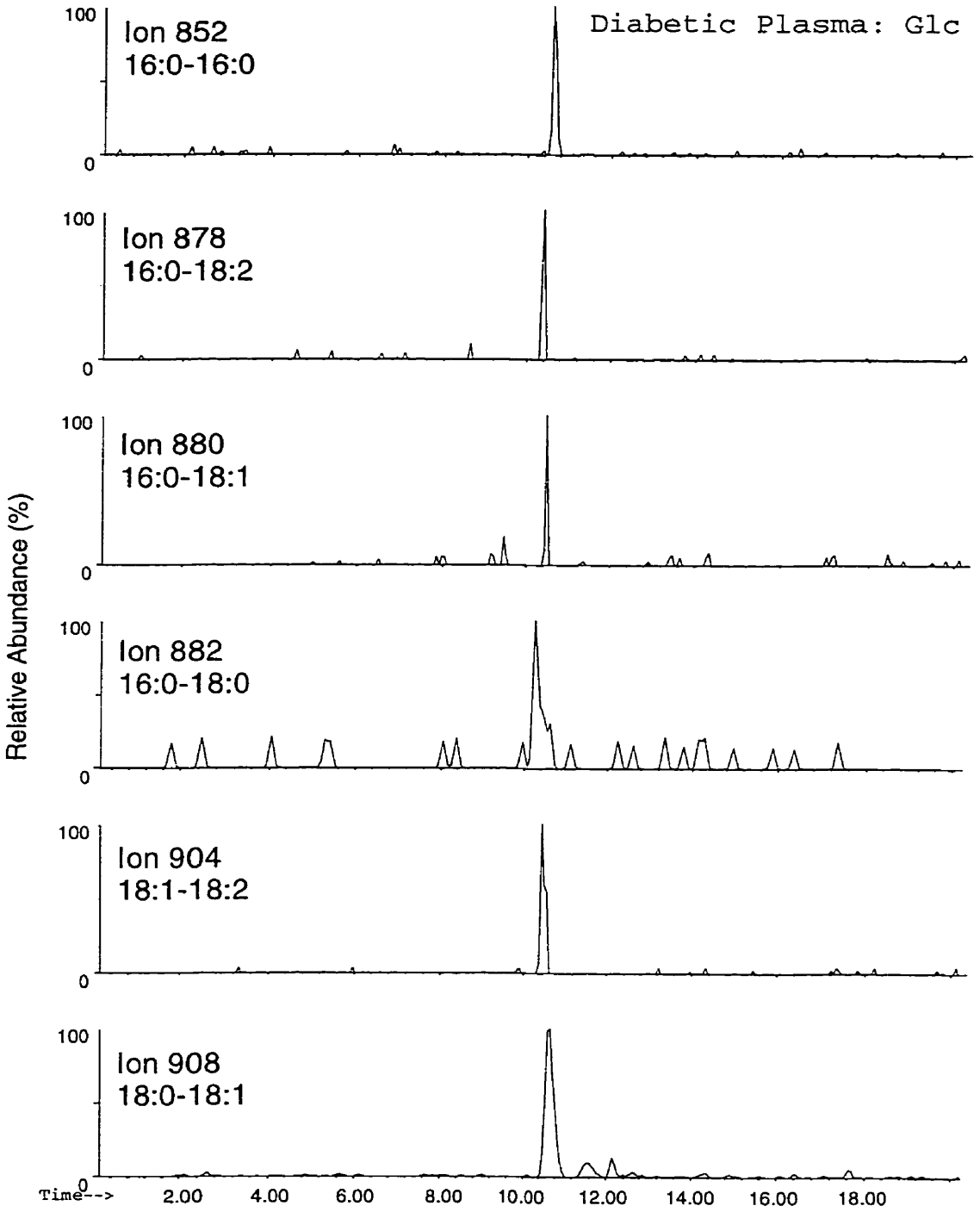


Fig. 4.3 Single ion plots of the masses corresponding to $[M+1]^+$ of the molecular species of glucosylated PE as obtained in Fig. 3B for the plasma of a female diabetic subject. The ions are identified as shown in the Fig. 4. LC/ESI/MS conditions are as given in Fig. 1.3.

not the alkylacyl or alkenylacyl ones, although the plasmalogenic species make up 50% of total red blood cell PtdEtn. Further- more, the glycation of the diacyl PtdEtn appears not to be random, which contrasts with random glycation of both diacyl and plasmalogenic species *in vitro* (7). It may be noted that several of the glucosylated species represent very minor red cell PtdEtn components of control and diabetic subjects. The proportions of the major molecular species within the underivatized PtdEtn were comparable in the controls and diabetics, including a high proportion of the plasmalogens (11,12). Table 2.3 shows that the glucosylated PtdEtn made up some-what higher proportion of plasma than red cell PtdEtn, although there was much less total PtdEtn in plasma. On the basis of the major PtdEtn species it was calculated that 16% and 10% of the diacyl PtdEtn of plasma and red cells, respectively, became glucosylated. The overall glycation of PtdEtn (10 – 16%) was similar to the overall glycation of hemoglobin (9 – 15%) in the diabetic patients. The control red cells and plasma showed less than 1% glucosylation.

DISCUSSION

Although glucose is the least reactive of the monosaccharides in Schiff base formation (14), it has been suggested (6) that it might react with aminophospholipids and play a role in production of lipid advanced glycation end-products (AGE). In support of this hypothesis Bucala et al. (6) have reported evidence for the formation of phospholipid-linked AGE *in vitro*, mimicking the absorption, fluorescence and immunochemical properties of AGE that result from advanced glycosylation of proteins.

Table 1.3
Glycated diacyl (PtdEtn), and alkenylacyl (PlsEtn) phosphoethanolamines in red blood cells of control and diabetic subjects (mole %)

	Controls			Diabetics		
Molecular species	Gly PtdEtn	PtdEtn	PlsEtn	Gly PtdEtn	PtdEtn	PlsEtn
16:0-16:0	7.1 ±1.1	1.2 ± 0.2		6.8 ± 0.8	2.7 ±0.5	
16:0-18:0		1.0± 0.3	0.3± 0.1	2.5±0.6	1.0± 0.2	
16:0-18:1		26.9± 5.3	5.3 ± 1.6	20.2± 3.7	23.6 ± 4.2	2.9± 0.8
16:0-18:2	70.3± 6.9	11.8 ± 3.2		21.7± 4.1	8.5 ± 2.6	0.5 ±0.1
16:1-18:3	1.6± 0.5	0.1 ± 0.1		14.2± 2.3	0.8 ± 0.2	
18:0-18:0		0.1± 0.1			0.9± 0.2	0.3± 0.1
18:0-18:1		5.1 ± 1.4	3.7 ± 1.1	1.9 ± 0.8	5.0± 1.8	1.7± 0.7
18:0-18:2	11.2 ± 2.5	8.2± 1.8	3.4± 0.9	2.7± 0.8	7.0± 1.9	2.1 ±0.9
18:1-18:2		8.3± 2.3	0.9± 0.2	20.6± 4.3	9.4± 2.1	0.5 ± 0.1
16:0-20:4		1 1.7 ± 2.6	6.1 ± 1.0	3.1 ± 0.6	9.8 ± 1.8	4.6 ± 1.2
16:0-20:5	6.7± 1.5	0.7± 0.1		3.5 ± 0.7	2.1± 0.7	1.3± 0.2
16:1-20:5			2.6± 0.4	1.5± 0.3		3.0 ± 0.5
18:0-20:3			2.5± 0.6			0.5 ± 0.1
18:0-20:4		8.1 ± 1.8	23.5± 4.2		7.1 ± 1.6	18.8± 3.6
18:1-20:4		8.8± 2.1	11.0± 2.6		7.0 ± 1.8	8.7 ± 1.1
16:0-22:6		3.4 ± 0.8	1.1± 0.3		4.0 ± 1.2	6.4±1.6
18: 1-22: 6			5.9± 1.2		0.6± 0.1	3.5± 0.2
18:0-22:3		0.1± 0.1	1.1± 0.2		0.1 ± 0.1	0.3± 0.1
18.0-22:4		0.1 ± 0.1	0.7± 0.1			1.3± 0.4
18:0-22:5		0.6± 0.1	11.5± 2.1		0.7± 0.2	10.2±2.0
18:0-22:6		1.0± 0.3	11.3± 2.3		2.4± 0.9	19.3± 3.7
18:1-22:6		1.1± 0.2	5.1 ± 1.8		3.9± 0.8	9.0± 1.4
Other	3.0 ± 0.8	1.9± 0.4	4.1 ± 1.7	1.5 ± 0.5	3.3± 0.8	5.1 ± 1.7
Total	1.1 ± 0.2	54.0± 3.5	44.9 ± 4.1	9.8 ± 1.1	40.1± 2.2	50.1 ± 2.9

Table 2.3.
Content of glycated PtdEtn in plasma and red blood cells of diabetics and control subjects as estimated on basis of total, diacyl and the palmitoyl-linoleoyl species of GroPEtn.

Subjects	% Total diradyl Gro PEtn	% Diacyl GroPEtn	% 16:0 – 18:2 GroPEtn
Diabetics			
RBC	10.2± 2*	18.4± 3*	
Plasma	16.1± 3*	27.7± 3*	17.6± 2*
Non-diabetics			
RBC	1.2 ± 0.5	5.6± 2	
Plasma	2.3 ± 1	1.5± 0.5	6.1± 4

The percentages were calculated by expressing the sum of intensities in the various glucosylated species as a percentage of the total of each species or group or species. Means ±S.D. (n=4). Compared between diabetics and controls *P<0.01

Furthermore, AGE-ELISA analysis of LDL specimens isolated from diabetic individuals revealed (6) increased levels of both apoprotein and lipid-linked AGE, when compared to specimens obtained from normal, non-diabetic controls. Glycated aminophospholipids were not isolated from any of the sources.

The effect of diabetes on blood lipid composition is controversial (15). Some studies had shown decreased (16) and others increased (17) PtdEtn levels in the red blood cells of diabetics. In one instance (17) an unidentified phospholipid had been detected by thin-layer chromatography (TLC). We estimated the Rf value of this unknown as 0.35 corresponding to an Rf value of 0.37 obtained for the standard glycated PtdEtn in the same solvent system in our laboratory. Still other studies (18) have shown significantly increased membrane lipid peroxidation and a formation of an adduct of phospholipids and malondialdehyde in erythrocytes from diabetic compared to erythrocytes from non-diabetic subjects. The degree of membrane lipid peroxidation was significantly correlated with the level of glycosylated hemoglobin.

Because of the apparent preferential glucosylation of the diacyl PtdEtn species, it would have been anticipated that the residual PtdEtn in the red blood cells of diabetics would be increased in plasmalogens. This was not obvious in the present experiments. Previously Freyburger et al. (17) had noted minimal amounts of dimethylacetals among the methylation products of the red blood cell PtdEtn. However, these workers also failed to detect the normal amounts of dimethylacetals in the PtdEtn of control subjects (17).

In view of random glycation of diacyl and alkenylacyl PtdEtn *in vitro* (7), the preferential glycation of the diacyl species of PtdEtn of the red blood cells and plasma

observed *in vivo* in the present study requires an explanation. The phospholipid classes of the lipid bilayer of the red blood cell are known to be extensively segregated (19,20), and work with liposomes has demonstrated (21) that the sidedness of the free amino group influences the peroxidation of the aminophospholipids and their interaction with the secondary peroxidation products. Alternately, the diacyl and alkenylacyl species of PtdEtn might be subject to differential interaction with proteins, protecting the alkenylacyl and exposing the diacyl species of PtdEtn to glucosylation, regardless of their location in the bilayer. It is also possible that glucosylated alkenylacyl species of PtdEtn might have become preferentially deglucosylated by some of the defense mechanisms operating in the red blood cell membrane or plasma.

The failure to isolate glycated PtdEtn from the red blood cells of diabetics previously may have been due to the relatively easy dissociation of the Schiff base in the presence of dilute acid frequently employed in phospholipid recovery from TLC (e.g. organic solvents with 10% acetic acid). Using reference standards we had noted (7) that alkaline solvent systems were better suited than acid solvent systems for recovery of glycated PtdEtn from *in vitro* and *in vivo* samples. The mild electrospray ionization facilitated the detection of glycated aminophospholipids, which was not possible with fast atom bombardment mass spectrometry (7).

The glucosylation of a major proportion of the PtdEtn would be anticipated to affect the structure and function of the red cell membrane due to alterations in charge and the bulk of the glucosylated polar head group. It would also be expected to affect the susceptibility of the glucosylated phospholipid to phospholipases. We have reported elsewhere (13) that glucosylated PtdEtn is more susceptible to peroxidation than non-

glucosylated PtdEtn, which is more resistant to peroxidation than PtdCho (21). Recently, Zommara et al. (22) have reported the inhibitory effect of ethanolamine plasmalogen on lipid peroxidation to be due to binding of iron and copper to liposomal lipids. It is therefore possible that hyperglycemia might play a role in promoting membrane lipid disorganization leading to peroxidation and atherosclerosis in diabetics.

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Chapter 4: Glucosylated aminophospholipids are the major LDL glycation products and increase LDL susceptibility to oxidation: evidence for their presence in atherosclerotic lesions

ABSTRACT

Glycation of both protein and lipid components are believed to be involved in low density lipoprotein (LDL) oxidation, but the relative importance of lipid and protein glycation in the oxidation process has not been established and the lipid glycation products have not been isolated. Using glucosylated phosphatidylethanolamine prepared synthetically, we have identified both diacyl and alkenylacyl species among the glycated ethanolamine phospholipids in LDL incubated with glucose. The accumulation of the stable Amadori transformation products of the initially formed Schiff bases was time and glucose concentration dependent. LDL specifically enriched in glucosylated phosphatidylethanolamine (25 nmol/mg protein) showed increased susceptibility to lipid oxidation when dialyzed against a 5 μM Cu^{++} solution. The presence of this glucosylated lipid resulted in 5 fold increase in production of phospholipid-bound hydroperoxides and 4 fold increase in phospholipid-bound aldehydes. The inclusion of glucosylated phosphatidylethanolamine in the surface lipid monolayer of the LDL resulted in rapid loss of polyunsaturated cholesteryl esters from the interior of the particle during oxidation. Glycated ethanolamine phospholipids were also isolated and identified from atherosclerotic plaques collected from both diabetic and non-diabetic subjects. The present findings provide direct evidence for the previously proposed causative effect of lipid glycation on LDL oxidation.

INTRODUCTION

Recent work on the pathogenesis of atherosclerosis implicates "oxidized" low density lipoprotein (LDL) as a key factor in the initiation of atherosclerotic lesions (1). It has been suggested (2) that non-enzymatic glycation of apoprotein B and possibly aminophospholipids could promote the generation of "oxidized" LDL. However, the specific contributions of lipid and protein glycation to LDL oxidation have not been determined.

Non-enzymatic glycosylation is defined as a post-translational modification of a protein by the covalent attachment of a sugar residue, resulting in an amino-carbonyl bonding or a Schiff base linkage. The stable Amadori rearrangement products of these Schiff bases have become known as advanced glycation end products (AGE) (3). Glycation has been shown to alter the biological activity of LDL resulting in reduced rate of degradation by fibroblasts (4) and an increased cholesteryl ester deposition in human aortic cells compared to normal LDL (5). Using antibodies to oxidized LDL, Bucala et al (6) have claimed that lipid advanced glycation constitutes an important pathway for lipid oxidation *in vivo*. Gugliucci et al (7) suggested that LDL glycation constitutes a predisposing event to its subsequent oxidative modification, and that LDL/very low density lipoprotein (VLDL) fractions from diabetic patients are more susceptible to oxidation. Furthermore, glycation of LDL has been reported (8) to accelerate LDL oxidation by copper ions, while glycation and lipid oxidation increase the uptake of LDL particles by macrophages (9). In none of these instances were glycated and/or oxidized lipids isolated or identified, or a distinction made between effects of protein and lipid glycation. We have recently shown (10,11) that plasma aminophospholipids, which

comprise 5-6% of total LDL phospholipid, can be readily glucosylated and the glucosylation products isolated. The present study demonstrates that glucosylated glycerophosphoethanolamines are the major LDL lipid glycation products. Furthermore, glucosylated PtdEtn added to non-glycated LDL promotes the oxidation of PtdCho and cholesteryl esters.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylethanolamine (PtdEtn), bovine brain PtdEtn, 1-Palmitoyl-2-linoleoyl GroPEtn, $NaCNBH_3$, $NaBH_4$ were obtained from Sigma-Aldrich (Oakville, Canada). A standard mixture of phospholipids made up of equal weight proportions of PtdCho, PtdEtn, PtdSer, PtdIns, $(Ptd)_2Gro$, LysoPtdCho and SM was also purchased from Sigma. D-Glucose-1-[^{14}C] was obtained from New England Nuclear (Montreal, Canada). All solvents were of HPLC grade or higher quality and were obtained from local suppliers.

Synthesis and Isolation of Glucosylated PtdEtn

Glucosylated PtdEtn was prepared and purified as described in detail previously (10). Briefly, PtdEtn (2 mg) dissolved in 1 ml of methanol was transferred to a 15 ml test tube and the solvent evaporated under nitrogen. Four ml of 0.1 M phosphate buffer containing 0-400 mM glucose and 0.1 mM EDTA were added and sonicated at low power for 5 min. at room temperature and the mixture incubated under nitrogen at 37 ℃ for various periods of time. Lipids were extracted into chloroform/methanol (2:1, v/v) as described by Folch et al (12) and the solvents evaporated under nitrogen. Some samples were reduced by adding a methanolic solution of either $NaCNBH_3$ or $NaBH_4$ to a final concentration of 70 mM and incubated at 4°C for 1 hr. The reduction products were washed with water, re-extracted with chloroform/methanol (2:1, v/v) and dried under

nitrogen. Samples were redissolved in chloroform/methanol (2:1, v/v) and kept at -20°C until analysis. Glucosylated PtdEtn (2 mg) was purified by preparative TLC (20 x 20 cm glass plates) coated with silica gel H (250 μ thick layer). The chromatoplates were developed using chloroform/methanol/30% ammonia (65:35:7, by vol.) as described (10). Phospholipids were identified by co-chromatography with appropriate standards and visualizing any lipid bands under ultraviolet light after spraying the plate with 0.05% 2,7-dichlorofluorescein in methanol. Both glucosylated and non-glucosylated lipids were recovered by scraping the gel from appropriate areas of the plate and extracting twice with the developing solvent.

Liposomal oxidation

Unilamellar liposomes of egg yolk phospholipids or of the standard phospholipid mix (0.5 mg/ml) were prepared by the ethanol injection method (13) in 0.1 M phosphate buffer. Unilamellar liposomes containing 10% glucosylated egg yolk PtdEtn were prepared similarly. Liposomes in the phosphate buffer, including those supplemented with glucosylated PtdEtn, were peroxidized by incubation in 10 mM tert-butyl hydroperoxide as the oxidant. At different times aliquots were withdrawn and the lipids were extracted with chloroform-methanol (2:1, v/v), and were washed repeatedly with buffer to remove residual tert-butyl hydroperoxide. Samples were analyzed by LC/ESI/MS immediately after extraction.

Lipoprotein Isolation

LDL (1.019-1.069 g/ml) was obtained by density gradient ultracentrifugation (14) from plasma of fasted normolipidemic individuals. LDL (2 mg protein/ml) was subsequently dialyzed against 0.1 M phosphate buffer (pH 7.4) containing 0.1mM EDTA

for 24 hrs (three buffer changes). LDL samples were sterilized by passing through a 0.22 micron filter (Millipore, Milford, MA), kept at 4 °C, and used within 1 week. Lipoprotein concentration was determined by the method of Lowry et al (15) and expressed as mg/ml. Oxidation of LDL (5 mg protein/5 ml) was performed by dialysis against 5 μM $CuSO_4 \cdot 5H_2O$ in 0.1M phosphate buffer, pH 7.4, for 24 hr at 37°C in the dark. Lipids were extracted into chloroform/methanol (2:1,v/v) as described above.

Enrichment of LDL with PtdEtn

Glucosylated and non-glucosylated PtdEtn was incorporated into LDL essentially as described by Engelmann et al. (16) for enriching human plasma lipoproteins with phospholipids. Glucosylated PtdEtn (1 mg) in chloroform-methanol (2:1, v/v) was transferred to a 15 ml test tube, the solvent evaporated under nitrogen, and the lipids dispersed by vortexing in 1.5 ml buffer containing 50 mM Tris /HCl, 1 mM dithiothreitol and 0.03 mM EDTA (pH 7.4). The solutions were sonicated in a bath sonicator for 5 min at 1 min intervals while being kept on ice under a stream of nitrogen. The liposome mixture was centrifuged at 3500 g and the supernatant collected and passed through a 0.45 μ filter. The liposomal mixture (1 ml) was added to fresh plasma (4 ml) containing 3 mM NaN_3 under gentle mixing. The mixture was incubated under nitrogen at 37 °C for 24 hours. Lipoproteins were isolated as described above.

LDL glucosylation

LDL (2 mg protein/ml) in 1 mM EDTA, containing 0.1 mg/ml chloramphenicol and 3mM NaN_3 was incubated with 5-400 mM glucose at 37°C for 1 week under nitrogen. Other incubation mixtures contained 3μCi/mL D-Glucose-1-^{14}C. At the end of incubation time, LDL samples were extracted with chloroform/methanol (2:1, v/v). LDL

samples containing radiolabeled glucose were reduced with $NaBH_4$ for 1 hr at 4°C and were dialyzed against 0.1 M phosphate buffer containing 0.1 mM EDTA for 24 hrs to remove free radioactive glucose. After dialysis the lipids were extracted and radioactivity measured in both protein and lipid fractions.

Extraction of atherosclerotic plaques

Atherosclerotic plaques obtained from patients during carotid endarterectomy and from postmortem carotid arteries were available from previous studies (18). The plaques were immediately placed into phosphate buffered saline, pH 7.4, containing 0.1% EDTA. Plaque material (100 mg) was separated from media and adventitia, minced into small (0.5-1.0 mm^2) pieces, and total lipid extracts prepared with chloroform/methanol (2:1, v/v) (19). The tissue lipid extracts were stored in chloroform at -20 ℃ under nitrogen.

Analysis of fatty acid methyl esters (FAME)

LDL lipid classes were isolated by preparative TLC on silica gel H using heptane/isopropyl ether/acetic acid (60:40:4, by vol) as solvent (20). In this system the phospholipids were retained at origin, while free fatty acids, triacylglycerols and cholesteryl esters were resolved in order of decreasing polarity. Appropriate areas of the plate were cleared of silica gel and FAME were prepared by treating the gel with 6% H_2SO_4 in methanol for 2 hrs at 80 °C. Heptadecanoic acid was included as an internal standard at approximately 10% of total fatty acid concentration. After reaction the FAME were extracted twice with hexane. The solvent was blown down under nitrogen and the samples redissolved in hexane. The fatty acids were analyzed on a polar capillary column (SP 2380, 15 m x 0.32 mm id., Supelco, Mississauga, ON, Canada) installed in a

Hewlett Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a flame ionization detector. Hydrogen was the carrier gas at 3 psi (21).

Analysis of Phospholipid Classes

Normal phase high performance liquid chromatography (HPLC) of phospholipids was performed on a 3μ Spherisorb column (100 mm x 4.6 mm i.d.) or a longer 5μ Spherisorb column (250 mm x 4.6 mm i. d., Alltech Associates, Deerfield, IL). The columns were installed into a Hewlett-Packard Model 1090 Liquid chromatograph and eluted with a linear gradient of 100 % Solvent A (chloroform /methanol/ 30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/ water/30% ammonium hydroxide 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min. (10,22) The flow was set at 1 ml/min. The peaks were monitored by on-line ES/MS.

Analysis of Molecular Species of Phospholipids

Normal phase HPLC with on-line electrospray mass spectrometry (LC/ES/MS) was performed by splitting the HPLC flow by 1/50 resulting in 20μL/mL being admitted to a Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface (HP 59987A) (22). Tuning and calibration of the system was achieved in the mass range of 400-1500 by using the standard phospholipid mix dissolved in the HPLC solvent A and flow-injected at 50 μL/min into the mass spectrometer. Capillary voltage was set at 4 kV, the endplate voltage at 3.5 kV and the cylinder voltage at 5 kV in the positive mode of ionization. In the negative mode, the voltages were 3.5 kV, 3 kV and 3.5 kV, respectively. Both positive and negative ion spectra were taken in the mass range 100-1100 amu. The capillary exit (Cap Ex) voltage

was set at 120 volts in the positive and 160 volts in the negative ion mode. For fragmentation studies the Cap Ex voltage was raised to 300 volts. Nitrogen gas was used as both nebulizing gas (40 psi) and drying gas (60 psi, 270° C). Phospholipids were quantified on basis of standard curves established for each phospholipid class. The equimolar ion intensities of different species of each phospholipid class varied by less than 5% (23) in each of the ion modes. The LC/ES/MS response to different phospholipid classes varied greatly and required the regular use of standards.

Measurement of radioactivity

Radioactivity in glucosylated diradyl GroPEtn and proteins was determined by scintillation counting following lipid extraction and TLC. The silica gel containing the glucosylated ethanolamine phospholipids was scraped into scintillation vials with CytoScint (ICN Pharmaceuticals). The radioactivity in the protein precipitate was determined after dissolving the precipitate in CytoScint.

RESULTS

Isolation of glucosylated PtdEtn from glucosylated LDL.

Glucosylated PtdEtn may occur in LDL as the Schiff base adduct or its Amadori rearrangement product (Scheme 1.4). The glucose adducts of PtdEtn were originally recognized as part of the front of the PtdCho peak detected by conventional normal phase LC/ES/MS as already described for total plasma and red blood cell extracts of diabetic subjects (11). Using longer HPLC columns, a complete resolution was obtained for the PtdCho, PtdIns and glucosylated PtdEtn components overlapping on the shorter column. Furthermore, employing the negative ion mode, the major PtdCho peak was seen only as minor PtdCho+Cl adduct providing a clear view (Fig. 1.4A) of the Glc diradylGroPEtn

peak following incubation of LDL with 50 mM glucose. Fig 1.4B shows the single ion plots of the major species of both glucosylated PtdEtn and PlsEtn, which elute with the same retention time as standard glucosylated PtdEtn. Fig. 1.4C shows the full mass spectrum averaged over the elution time of the Glc diradylGroPEtn. It includes many molecular species which are quantified in Table 1.4. Table I shows that the glucosylation of the ethanolamine phospholipids of LDL was largely indiscriminate as both PtdEtn and PlsEtn components were glucosylated. However, on the average the diacyl species represent a somewhat higher proportion of the total glucosylated ethanolamine

LC/ES/MS Characterization of PtdEtn

Fig. 2.4 shows the fragment ions obtained for the palmitoyllinoleoyl GroPEtn glucosylate in the negative and positive ionization mode as obtained for the HPLC peak eluting at 15.8 min. In the negative ion mode (Fig. 2.4A), fragmentation of the molecular ion (*m/z* 876) gave palmitic (*m/z* 255) and linoleic (*m/z* 279) acids. The ion at m/z 712 is due to loss of glucose moiety, while the ion at *m/z* 756 results from cleavage of the attached glucose between carbons 2 and 3. In the positive ion mode (Fig. 2.4B), the ion at *m/z* 303 represents glucosylated PEtn and the ion at *m/z* 576 the diacylGro fragment. The ion at *m/z* 180 was due to cleavage of the adduct with a retention of nitrogen with the glucose moiety. In both modes of ionization, the fragments produced were consistent with either Schiff base or Amadori rearrangement product of glucosylated PtdEtn, but the relatively higher stability of the complex suggested the Amadori product, as already

Fig 1.4 LC/ES/MS analysis of total lipid extract of LDL incubated with 50 mM glucose for 7 days. (A)Total negative ion current profile of LDL phospholipids. (B) single ion plots for the major glucosylated PtdEtn and PlsEtn species. (C) Averaged spectra over the glycated PE peak (15.05 to 15.75 min.). LDL total lipid extract was dissolved in chloroform/methanol (2:1 vol/vol) and 20 µl of the sample containing 10 µg lipid was analysed. LC/ES/MS conditions. Normal phase 5µ Spherisorb column (250 mm x 4.6 mm i. d.) eluted with a linear gradient of 100 % Solvent A (chloroform /methanol/ 30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/ water/30% ammonium hydroxide 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min. at 1 ml/min. The effluent was split by 1/50 resulting in 20µL/mL being admitted into the mass spectrometer and scanning in the negative ion mode from 400-1100 amu.

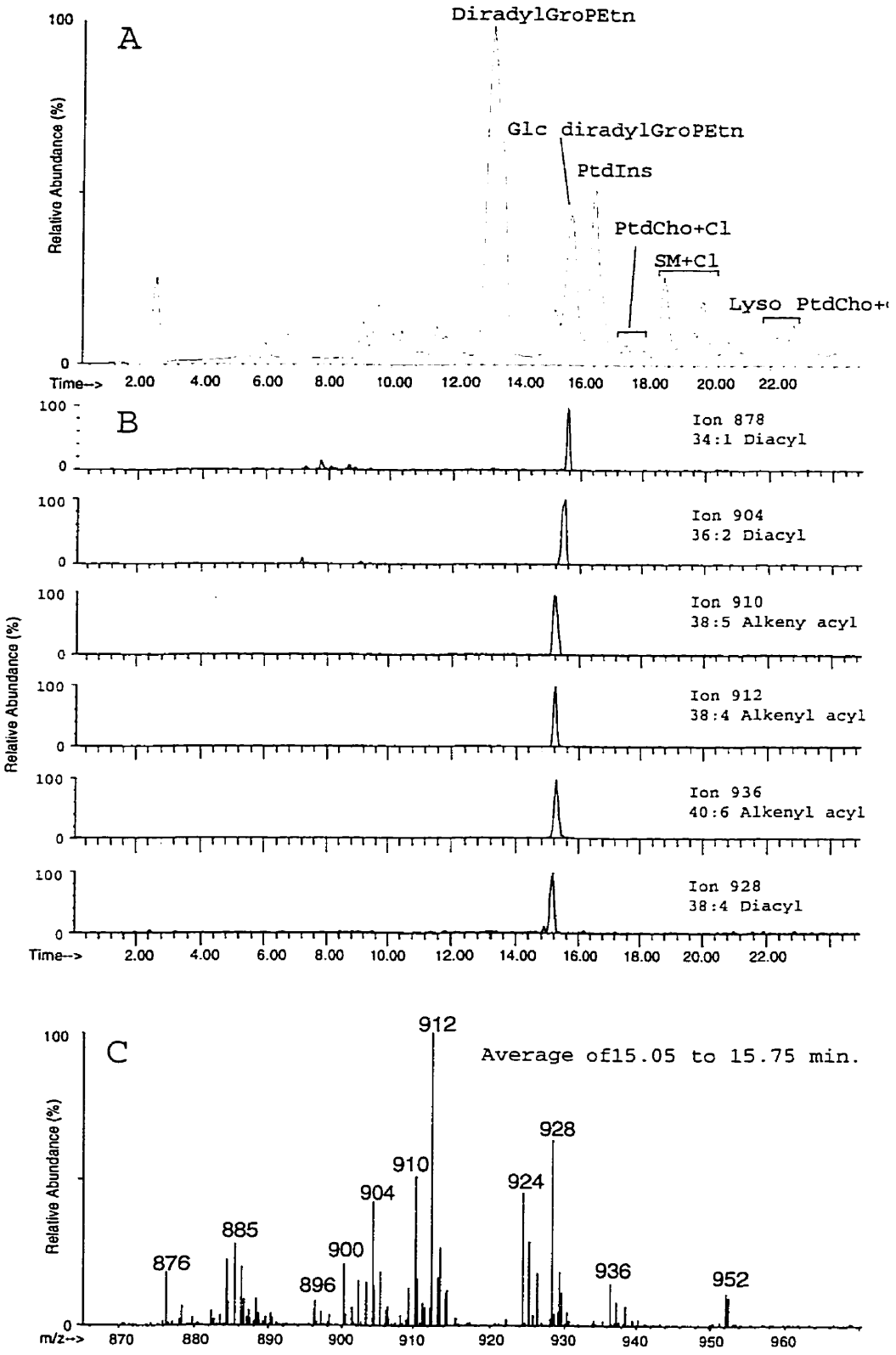

A
DiradylGroPEtn
Glc diradylGroPEtn
PtdIns
PtdCho+Cl
SM+Cl
Lyso PtdCho+
Relative Abundance (%)
Time-->
B
Ion 878
34:1 Diacyl
Ion 904
36:2 Diacyl
Ion 910
38:5 Alkeny acyl
Ion 912
38:4 Alkenyl acyl
Ion 936
40:6 Alkenyl acyl
Ion 928
38:4 Diacyl
C
Average of15.05 to 15.75 min.
912
928
910
924
904
885
900
876
896
936
952
m/z-->

Table 1.4.

Molecular species of glucosylated and non-glucosylated diradyl GroPEtn in LDL.LDL was incubated with 20 mM glucose for 7 days pH 7.4, at 37°C (n=4). Lipids were extracted and different phospholipid classes were separated by normal phased silica column HPLC and resolved peaks analysed by in-line electrospray mass spectrometry.

		Non-glucosylated				Glucosylated			
Species	C#	m/z	mole%		SD	m/z	mole%		SD
Alkenylacyl									
16:0-20:4	36:4	722	4.25	±	1.39	884	2.15	±	1.63
18:0-18:3	36:3	724	2.09	±	0.97	886	4.22	±	2.25
16:0-20:3									
16:0-22:6	38:6	746	5.51	±	1.86	908	0.69	±	0.24
18:2-20:4									
18:0-20:5	38:5	748	10.75	±	2.14	910	9.16	±	3.58
16:0-22:5									
18:0-20:4	38:4	750	19.30	±	4.53	912	12.00	±	4.27
18:0-20:3	38:3	752	3.63	±	0.23	914	2.49	±	0.65
18:0-22:6	40:6	774	3.45	±	1.87	936	1.90	±	1.22
Total			**48.98**	**±**	**3.37**		**32.61**	**±**	**3.86**
Diacyl									
16:0-18:2	34:2	714	3.84	±	1.24	876	3.58	±	1.44
16:0-18:1	34:1	716	1.20	±	0.25	878	1.60	±	0.34
16:0-20:4	36:4	738	3.45	±	1.32	900	6.90	±	2.28
18:0-18:3	36:3	740	2.29	±	1.68	902	2.56	±	0.57
18:0-18:2	36:2	742	8.11	±	3.17	904	12.52	±	3.36
18:0-18:1	36:1	744	1.99	±	0.79	906	3.01	±	1.43
16:0-22:6	38:6	762	5.51	±	2.43	924	8.17	±	3.78
18:2-20:4									
18:0-20:5	38:5	764	6.10	±	1.81	926	4.92	±	2.83
16:0-22:5									
18:0-20:4	38:4	766	12.36	±	3.92	928	18.70	±	4.54
18:0-20:3	38:3	768	1.89	±	0.65	930	1.40	±	0.61
18:0-22:6	40:6	790	1.41	±	1.64	952	0.17	±	0.15
18:0-22:5	40:5	792	2.88	±	0.85	950	3.85	±	1.23
Total			**51.02**	**±**	**4.34**		**67.39**	**±**	**5.81**

concluded by Lederer et al (24). The two major species were alkenylacylGroPEtn (18:0-20:4) and diacylGroPEtn (18:0-20:4).

Rate of glucosylation of LDL diradyl GroPEtn

The concentration dependence of LDL lipid glucosylation was investigated by determining the time course of Glc PtdEtn and Glc PlsEtn accumulation in LDL by LC/ES/MS. Fig 3.4 shows the concentration dependence of LDL diradylGroPEtn glucosylation. Even at 5 mM glucose 4-nmol Glc GroPEtn / mg LDL protein was present. This represents 8.6% of total LDL diradylGroPEtn (52 nmole/mg LDL protein) in glucosylated form at physiological glucose concentrations. The level of Glc diradylGroPEtn in LDL incubated at 400 mM glucose for 7 days 35 nmole/mg LDL protein represents 67% of total LDL diradylGroPEtn being glucosylated. Even at these high glucose concentrations there was incomplete glycation of LDL diradylGroPEtn. Using radiolabeled glucose, the differences between the rate of lipid and protein glycation in LDL were investigated. Fig. 4.4 shows that the initial rate of incorporation of the radioactivity was significantly higher in the lipid than in the protein fraction. A TLC examination of the lipid phase indicated that about 92% of the radioactivity was recovered in the band corresponding to the $NaBH_4$ reduction product of Glc diradylGroPEtn and further analysis by LC/ES/MS gave the correct molecular weights for the reduced Glc diradylGroPEtn products (data not shown). This demonstrates that Glc diradylPEtn is the initial and major LDL glucosylation product

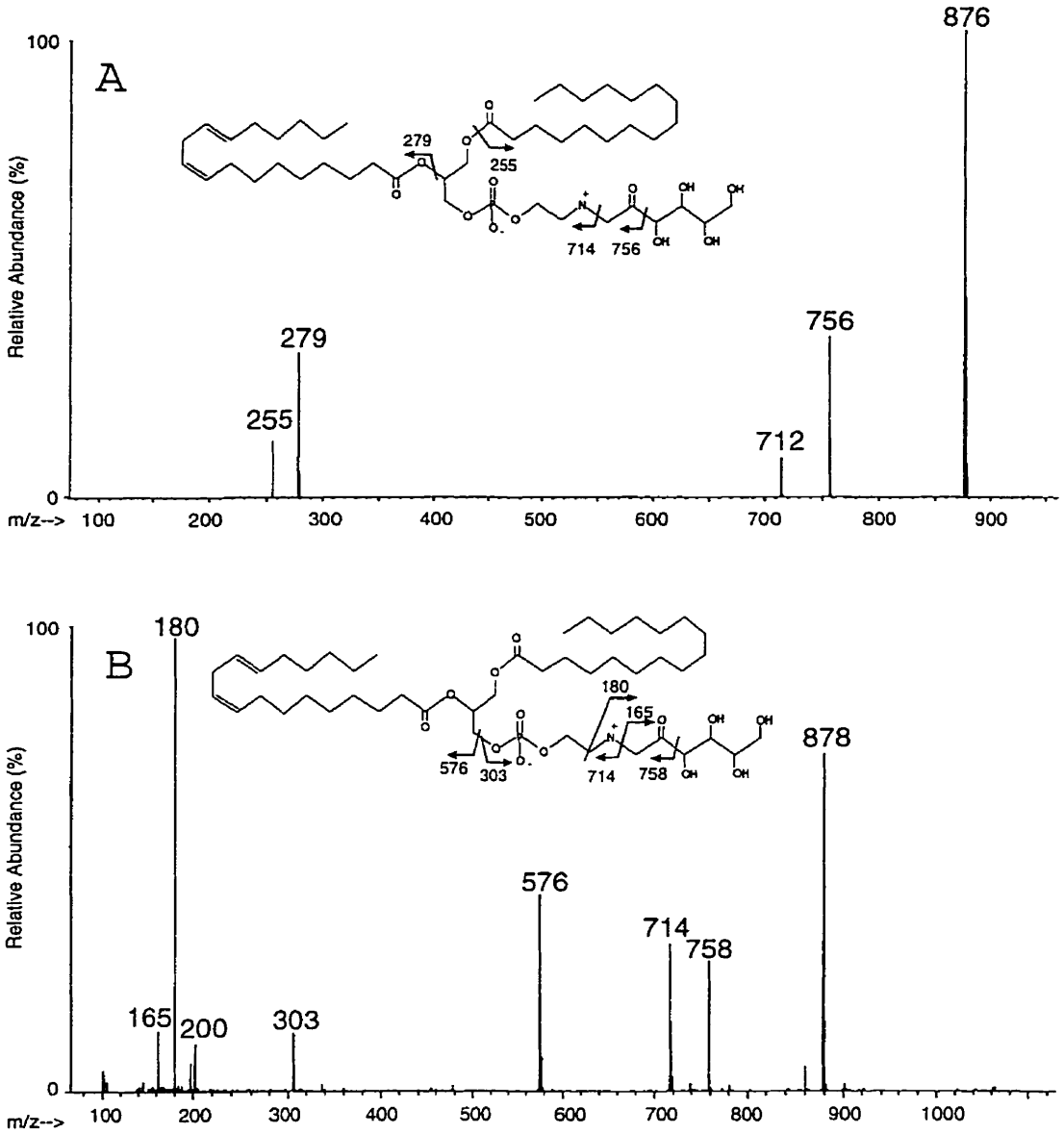


Fig. 2.4. Fragmentation pattern of 16:0-18:2 GroPEtn generated by increasing the capillary exit voltage from 160 to 300 volts. (A) Fragmentation pattern in the negative mode of ionization; (B) fragmentation in the positive mode of ionization. LC/ES/MS as described in Fig1.4.

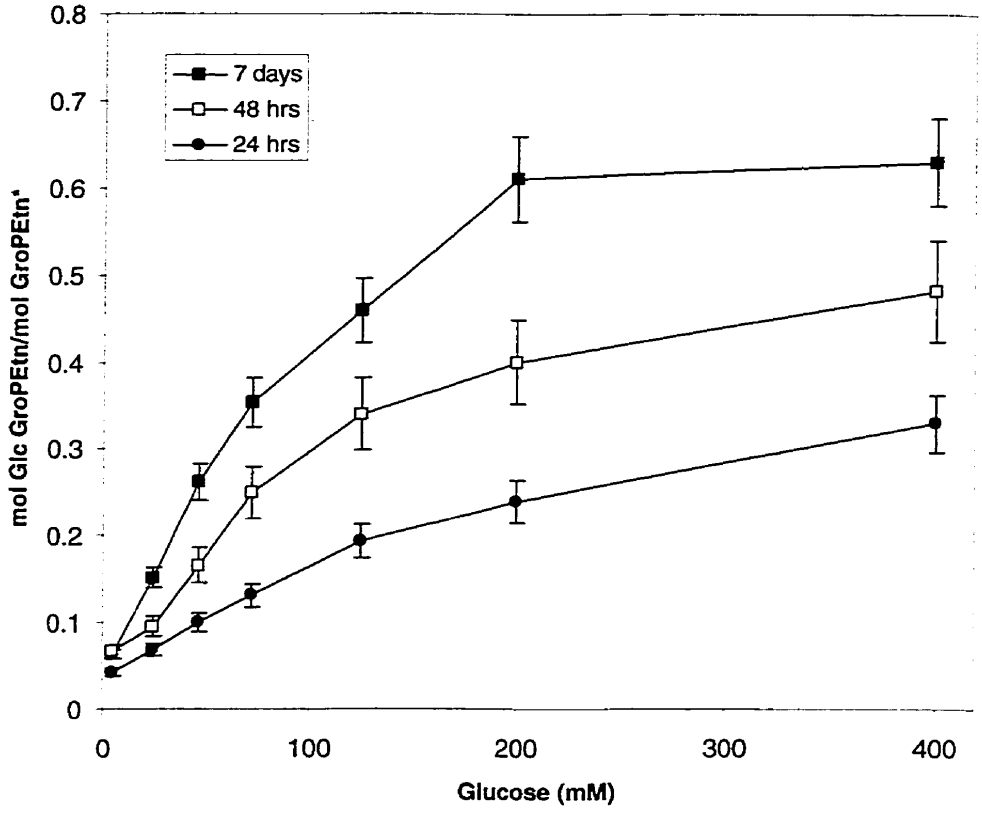


Fig. 3.4. Glucosylation of LDL ethanolamine phospholipids at different incubation times with increasing glucose concentrations. The various time and concentration points represent the sum of PlsEtn and PtdEtn. *diradylGroPEtn represents both the glucosylated and the non glucosylated diradylGroPEtn. LC/ES/MS conditions as described in Fig1.4.

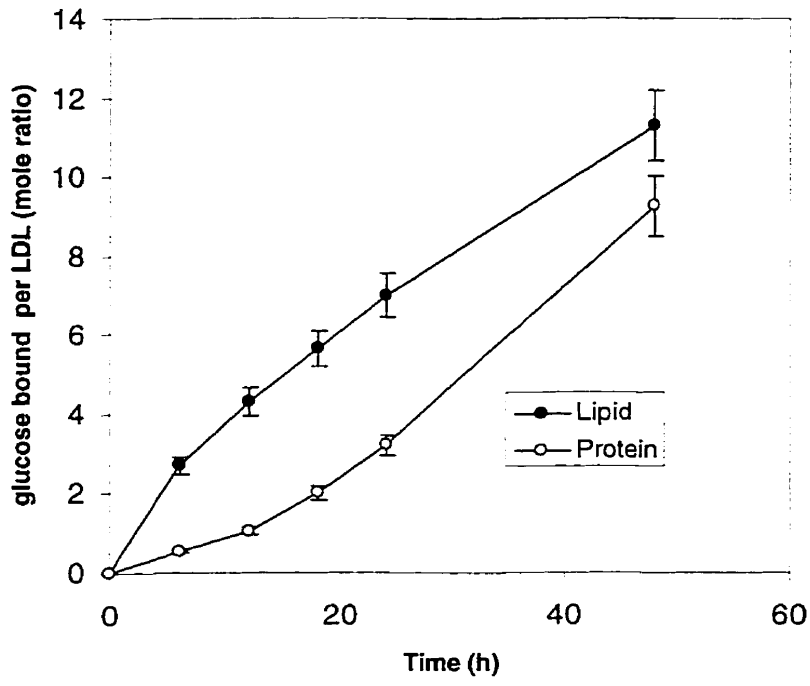


Fig. 4.4 Glycation of LDL lipid (●) and protein (○) as measured by radiolabeled glucose incorporation. LDL was incubated with 20 mM glucose containing 3mCi/mL D-glucose-1-[^{14}C]. The incubation mixture was reduced with $NaBH_4$ and dialyzed extensively. Radioactivity was measured by scintillation counting for both lipid and protein fractions and is given as moles of glucose per LDL molecule. Molecular weight of ApoB was assumed to be 500kDa.

Relative rates of oxidation of PtdEtn and Glc PtdEtn

The effect of glycation on PtdEtn oxidation was determined by comparing the rates of *tert*-butyl hydroperoxidation of glucosylated and non-glucosylated PtdEtn incorporated at 10% mass into unilamellar egg yolk PtdCho liposomes (Fig. 5.4). Fig. 5.4A shows the time course of hydroperoxidation of 18:0-18:2 GroPEtn and glucosylated 16:0-18:2 GroPEtn when incorporated into PtdCho liposomes, as determined by LC/ES/MS. Mono-hydroperoxides were the major oxidized molecules monitored in both PtdEtn molecules. It is seen that the Glc PtdEtn was peroxidized 2-3 times ($P<0.01$) more readily than non-glucosylated PtdEtn. Furthermore, Glc PtdEtn had a pro-oxidant activity towards non-glucosylated PtdEtn and PtdCho. Fig. 5.4B shows that 16:0-20:4 and 16:0-18:2 GroPCho liposomes in presence of Glc PtdEtn were peroxidized at 2-3 times higher rate than the same PtdCho liposomes supplemented with non-glucosylated PtdEtn.

Pro-oxidant activity of glucosylated PtdEtn in LDL

In order to investigate further the apparent pro-oxidant effect of glucosylated ethanolamine phospholipids, we measured the peroxidation of LDL by copper ions in the presence and absence of added glucosylated 16:0-18:2 Gro PEtn (10-30 nmole/mg LDL protein). This amount corresponds to levels obtained for LDL incubation with 50 mM glucose for 48 h. Control LDL contained 10-20 nmole /mg LDL protein of added non-glucosylated 16:0-about four fold.

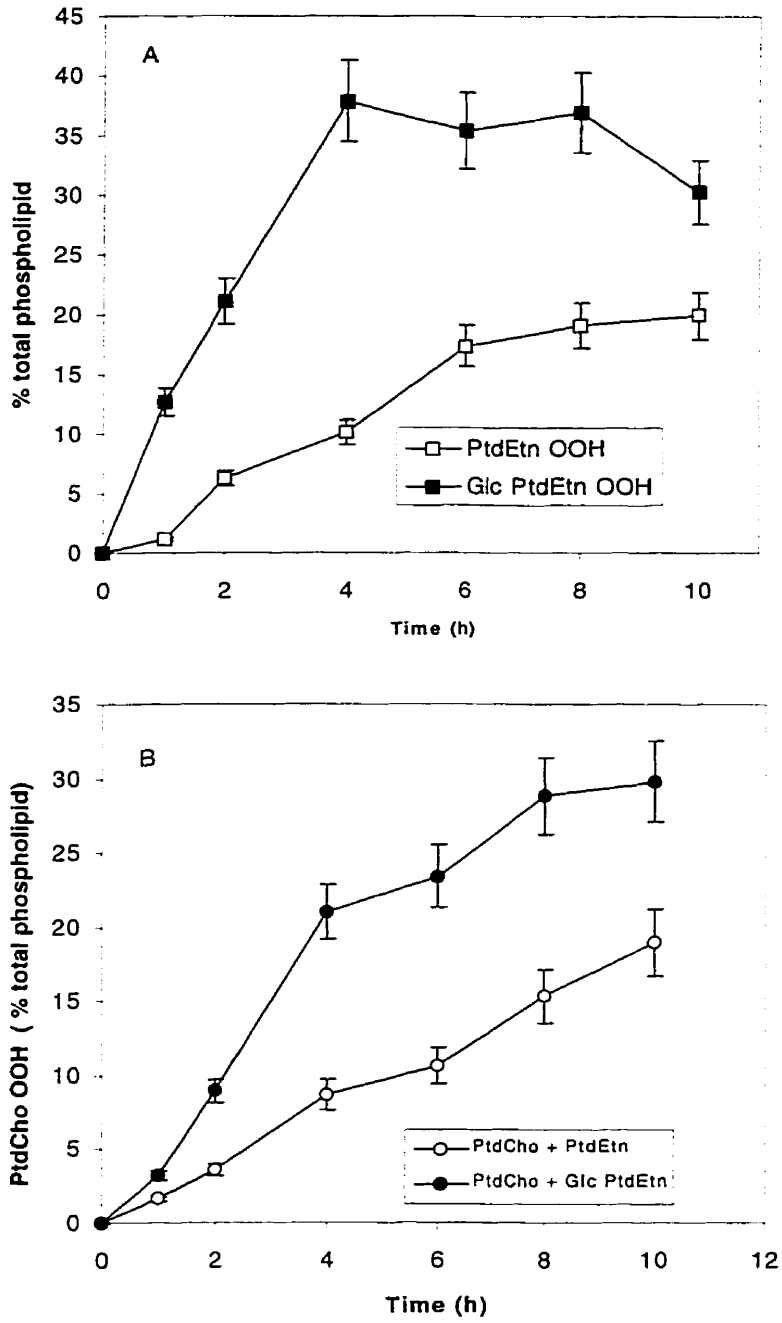
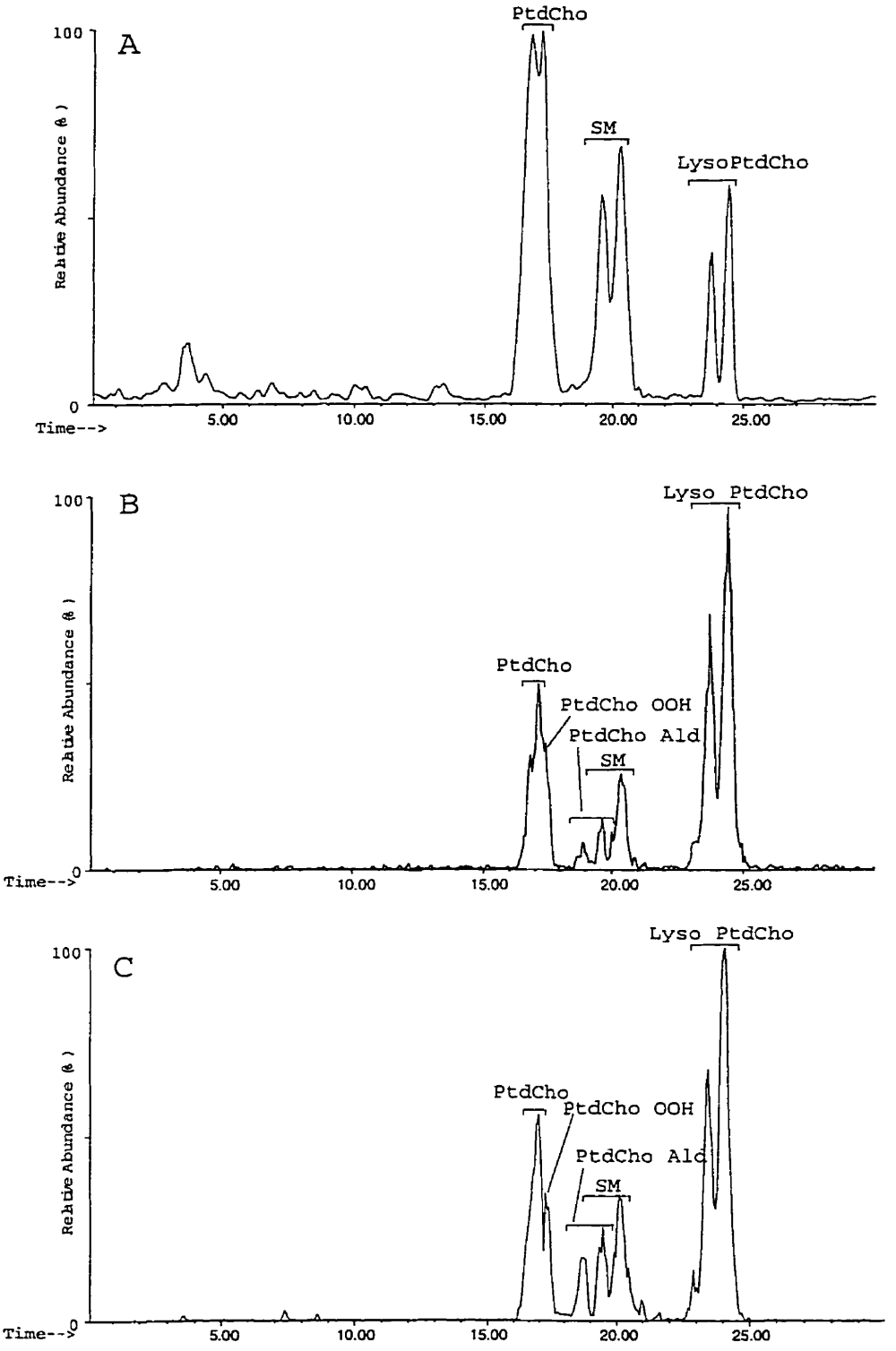


Fig. 5.4 Effect of Glc PtdEtn on phospholipid oxidation by tert-butyl hydroperoxide. (A) Liposomes containing egg yolk Glc PtdEtn and egg yolk PtdEtn; (B) Liposomes containing PtdCho in presence and absence of Glc PtdEtn. The peroxidation was measured by monitoring the generated mono-hydroperoxide ions by LC/ES/MS. LC/ES/MS as described in Fig 1.4.

Fig 6.4 Copper catalyzed oxidation of LDL phospholipids in presence of PtdEtn or Glc PtdEtn. (A) total positive ion current profile of non-oxidized LDL; (B) total positive ion current profile of oxidized LDL supplemented with PtdEtn (12 hrs, 37 °C); (C) total positive ion current profile of oxidized LDL supplemented with Glc PtdEtn (12hrs, 37°C). Peak identification as given in figure. LC/ES/MS conditions as described in Fig1.4.


A
PtdCho
SM
LysoPtdCho
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00
B
Lyso PtdCho
PtdCho
PtdCho OOH
PtdCho Ald
SM
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00
C
Lyso PtdCho
PtdCho
PtdCho OOH
PtdCho Ald
SM
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00

18:2 GroPEtn. The extent of lipid peroxidation was determined by measuring the hydroperoxides and core aldehydes of the most abundant PtdCho species of the two LDL preparations. Fig. 6.4 compares the phospholipid profiles of untreated LDL (A) and copper oxidized LDL + PtdEtn (12 hrs, 37°C) (B) and copper oxidized LDL + Glc PtdEtn (12 hrs, 37°C) as obtained by positive ion LC/ES/MS. Fig. 6.4A shows the resolution of the PtdCho, SM and lyso PtdCho, which are the major phospholipid components of native LDL. The PtdCho, SM and LysoPtdCho peaks are split due to a resolution of the short and long carbon chain species. Following copper ion oxidation (Fig. 6.4B), there is a dramatic change in the composition of the LDL phospholipids largely as a result of conversion of the unsaturated PtdCho into the hydroperoxides and core aldehydes, which are eluted later than the saturated PtdCho species. In addition there has been a relative increase in the proportion of LysoPtdCho possibly due to hydrolysis of the peroxidized PtdCho by the phospholipases present in LDL. Following supplementation of the LDL with Glc PtdEtn (Fig. 6.4C), there is a further increase in the lipid peroxidation. The absolute amount of LDL SM remained unchanged in both PtdEtn supplemented (27.9 % total phospholipid) and Glc PtdEtn supplemented LDL preparations (26.7 %), although this is not immediately obvious from either Fig. 6.4B or Fig. 6.4C.

Fig. 7.4A shows the full mass spectrum averaged over the range of elution times of the hydroperoxy PtdCho (17.070-18.603 min), while Fig. 7.4B shows the single ion plots corresponding to the monohydroperoxy and dihydroperoxy derivatives of the major PtdCho species. The arachidonate-containing species (16:0-20:4GroPCho) with two hydroperoxy groups (2xOOH) are resolved into three subfractions presumably due to the presence of positional and *cis, trans* isomers of the hydroperoxides.

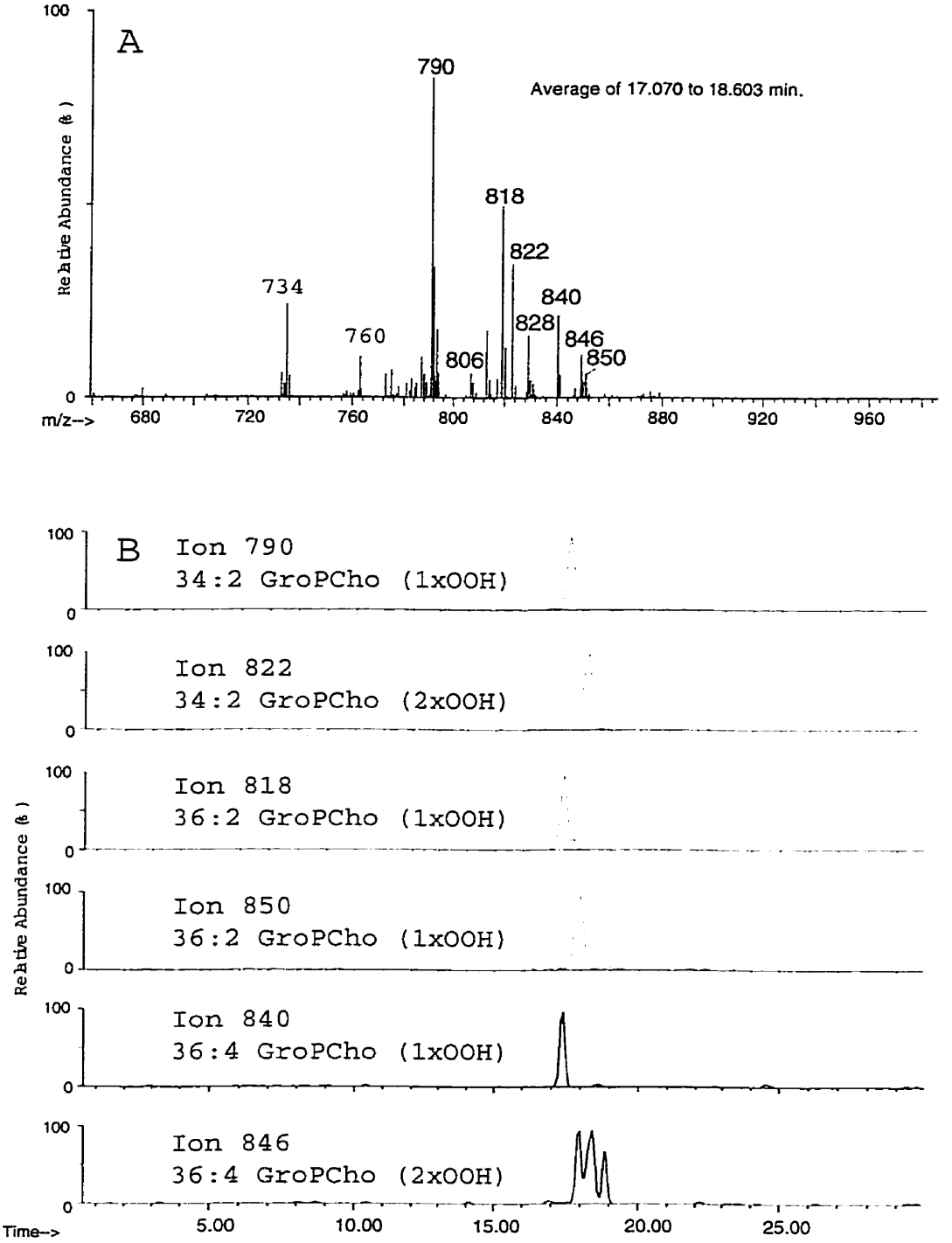


Fig. 7.4 Identification of LDL PtdCho hydroperoxy ions generated due to copper oxidation (A) spectra averaged over the PtdCho hydroperoxide peak; (B) single ion plots of the major PtdCho hydroperoxides present in oxidized LDL containing Glc PtdEtn. LC/ES/MS conditions as described in Materials and Methods.

Fig. 8.4 shows the time course of PtdCho hydroperoxide formation during dialysis against copper ions of LDL supplemented with Glc PtdEtn or PtdEtn. The major PtdCho hydroperoxides: 34:2 1xOOH (m/z 790) ; 36:2 1xOOH (m/z 818); 34:2 2xOOH (m/z 822); and 36:4 1xOOH (m/z 840) are all increased significantly in Glc PtdEtn supplemented LDL, with the 34:2 1xOOH being the most abundant. LC/ES/MS analysis of the $NaBH_4$ reduced peroxidation products indicated that the hydroperoxy PtdCho had been converted into the corresponding hydroxy PtdCho. The addition of Glc PtdEtn to LDL resulted in about 5 fold increase in the formation of phospholipid-bound hydroperoxides as calculated from averaged differences from all time points.

Fig. 9.4A shows the full mass spectrum averaged over the elution range (18.76-19.19 min), of the C_9 core aldehydes of PtdCho, which are eluted ahead of the C_5 core aldehydes. Fig 9.4B shows the full spectrum averaged over the entire range (19.19-21.00 min) of the C_5 aldehydes which trail into the SM species (m/z 675, palmitoyl SphPCho). Fig. 9.4C shows the single ion plots for all four major molecular species of the PtdCho core aldehydes. The 9-oxo-nonanonates and 5-oxo-valerates of the palmitoyl and stearoyl GroPCho would be anticipated to represent the major components on the basis of the fatty acid composition of the molecular species of LDL PtdCho.

Fig. 10.4 shows the time course of PtdCho core aldehyde formation during copper oxidation of LDL supplemented with Glc PtdEtn or PtdEtn. The major core aldehydes are the 16:0-9:0 Ald and the 18:0-9:0 Ald species as expected from presence of linoleic acid as the major unsaturated fatty acid (61%) in the sn-2 position of LDL PtdCho. $NaBH_4$ treatment resulted in a reduction of the aldehydes to the corresponding alcohols as reflected in the increase of 2 mass units in the molecular ion of each PtdCho core aldehyde (data not

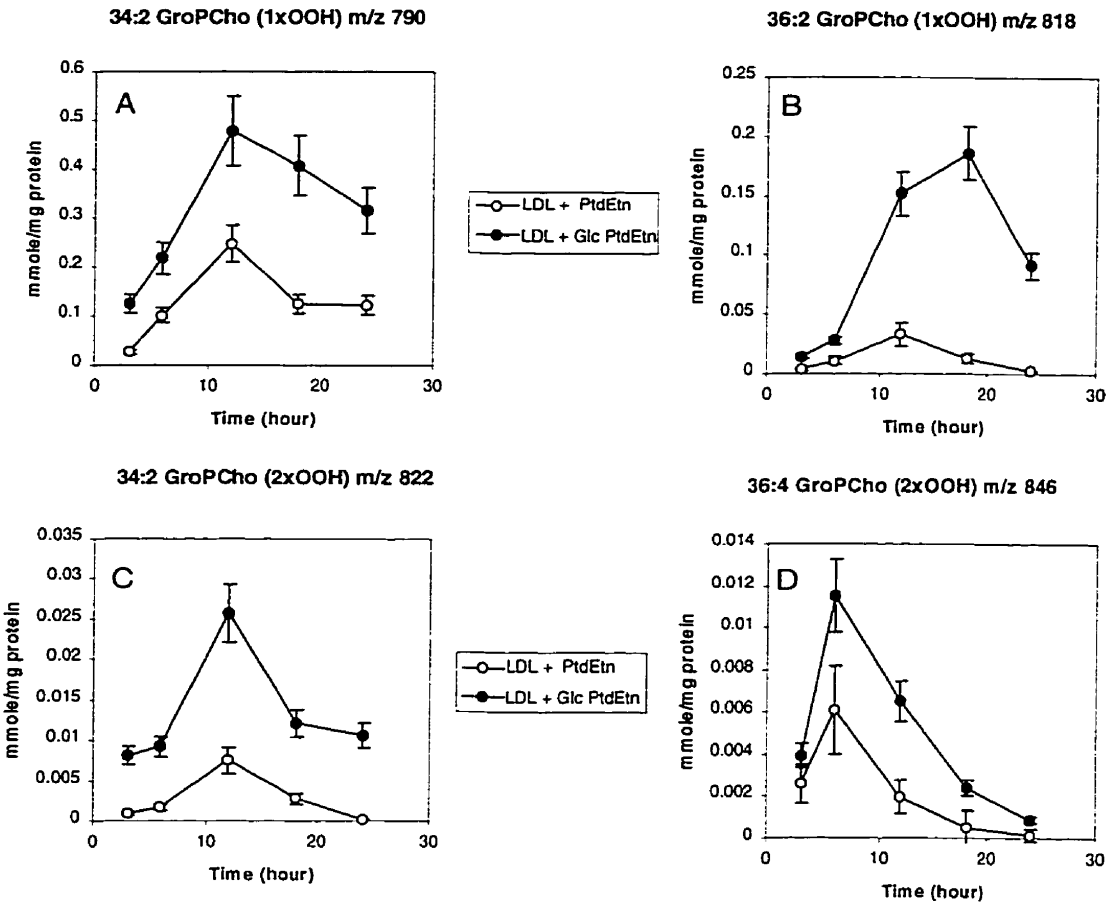
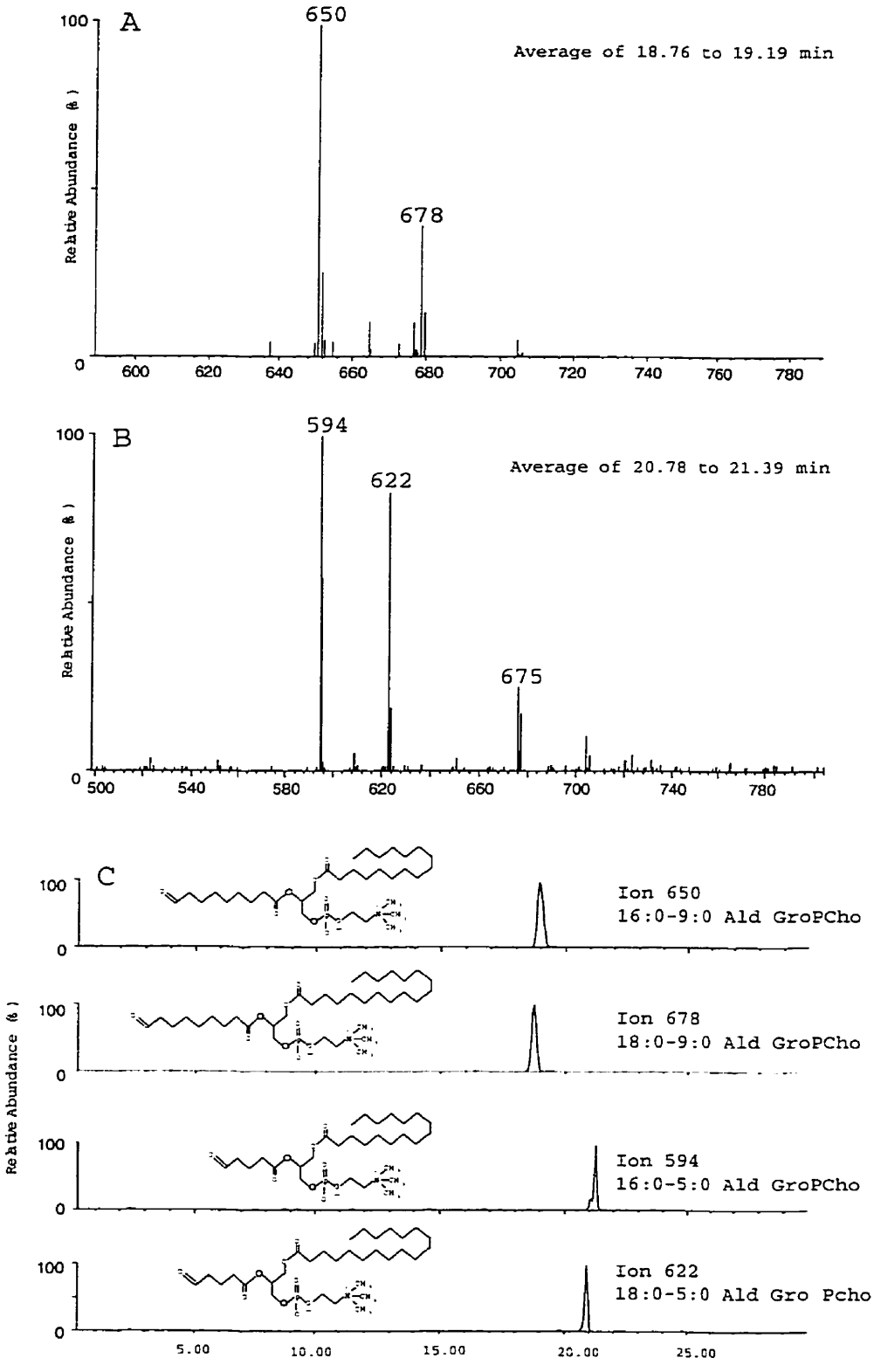


Fig. 8.4 Major PtdCho hydroperoxides in oxidized LDL. LDL was supplemented with PtdEtn (15 nmole/mg LDL protein) (○) or supplemented with Glc PtdEtn (20 nmole/mg LDL protein) (●). LDL was oxidized by dialysis against 5 μM copper sulfate solution (37 °C). At the end of the oxidation LDL lipids were extracted and analysed by LC/ES/MS in the positive mode of ionization. LC/ES/MS conditions as described in Fig 1.4.

Fig. 9. 4 Major PtdCho core aldehydes in oxidized LDL supplemented with Glc PtdEtn. (A) spectra averaged over the early eluting (C_9 aldehydes in *sn*-2) and late eluting (C_5 aldehydes in sn-2) aldehyde peaks; (B) single ion plots of the C_9 and C_5 PtdCho core aldehydes. LC/ES/MS and oxidation conditions as described in Fig.1.4.


A
650
678
Average of 18.76 to 19.19 min
Relative Abundance (%)
100
0
600
620
640
660
680
700
720
740
760
780
B
594
622
675
Average of 20.78 to 21.39 min
500
540
580
620
660
700
740
780
C
Ion 650
16:0-9:0 Ald GroPCho
Ion 678
18:0-9:0 Ald GroPCho
Ion 594
16:0-5:0 Ald GroPCho
Ion 622
18:0-5:0 Ald Gro Pcho
5.00
10.00
15.00
20.00
25.00

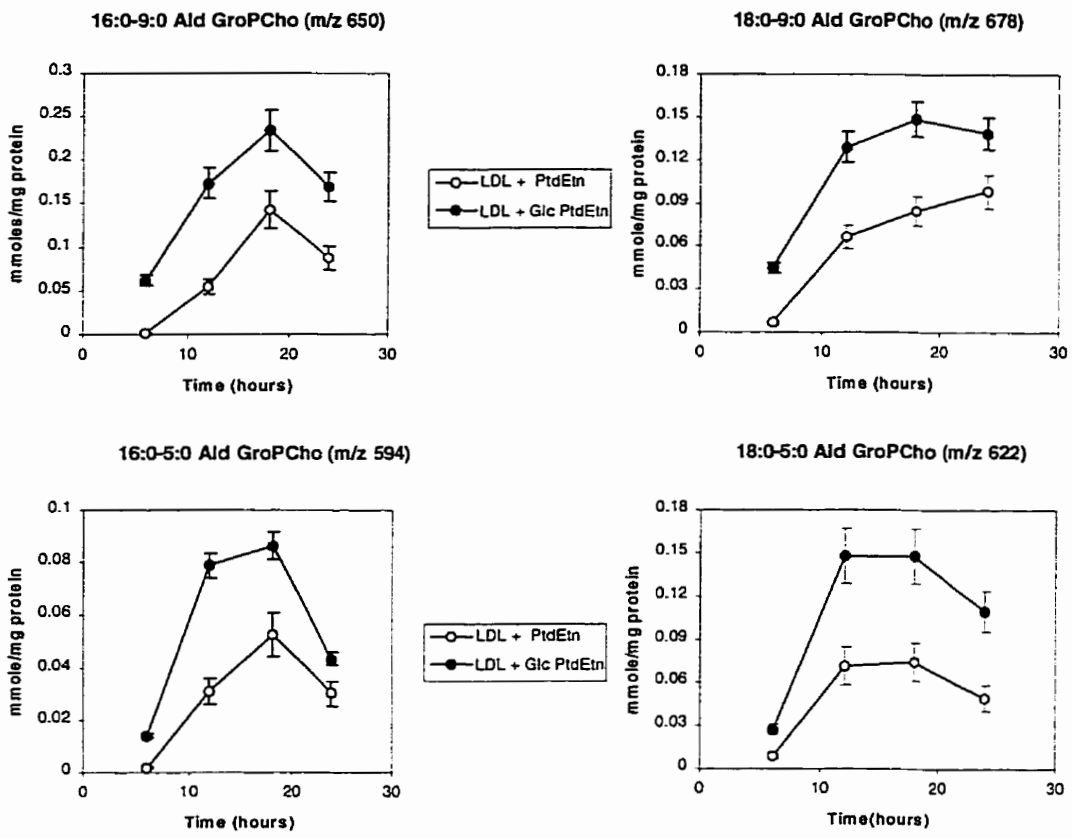


Fig. 10.4 Major PtdCho core aldehyde levels in copper oxidized LDL. LDL supplemented with PtdEtn (○) LDL supplemented with Glc PtdEtn (●).

shown). On the average the increase in the core aldehyde formation due to Glc PtdEtn supplementation was of glucosylation of the aminophospholipids in a fashion similar to that already reported for protein AGE formation in LDL and other biological systems (17, 25).

Cholesteryl ester oxidative susceptibility

The addition of Glc PtdEtn to LDL also promoted the oxidation of the cholesteryl esters in the interior of the particle. Fig. 11.4 shows a more rapid loss of the two major species of polyunsaturated cholesteryl esters during dialysis against copper ions of LDL supplemented with Glc PtdEtn, when compared to LDL supplemented with similar amounts of PtdEtn. The destruction of the arachidonoyl ester occurred at a much faster rate than that of the linoleoyl ester of cholesterol. Furthermore, the cholesteryl esters appeared to undergo peroxidation much more rapidly than the glycerophospholipids (compare Figures 9 and 11), which suggests that access to the interior of the particle was facilitated by addition of Glc PtdEtn.

Isolation of glycated PtdEtn and PlsEtn from atherosclerotic tissue

Figure 12.4 shows the total negative ion current profile (Fig. 12.4A), the single ion chromatograms for major species (Fig. 12.4B), and the mass spectrum averaged over the elution time (15.01-15.89 min) of the glycated ethanolamine phospholipid peak (Fig. 12.4C) as obtained by normal phase LC/ES/MS for a total lipid extract of an atherosclerotic plaque from a diabetic male. In order to increase the sensitivity of detection, the scanning was limited to the mass range of 850 to 1000 amu, which eliminated any overlap with the PtdEtn, PtdCho+Cl and SM+Cl ions also present in the total negative LC/MS profile. As a result, however, only the molecular species of LysoPtdCho dimers remained visible.

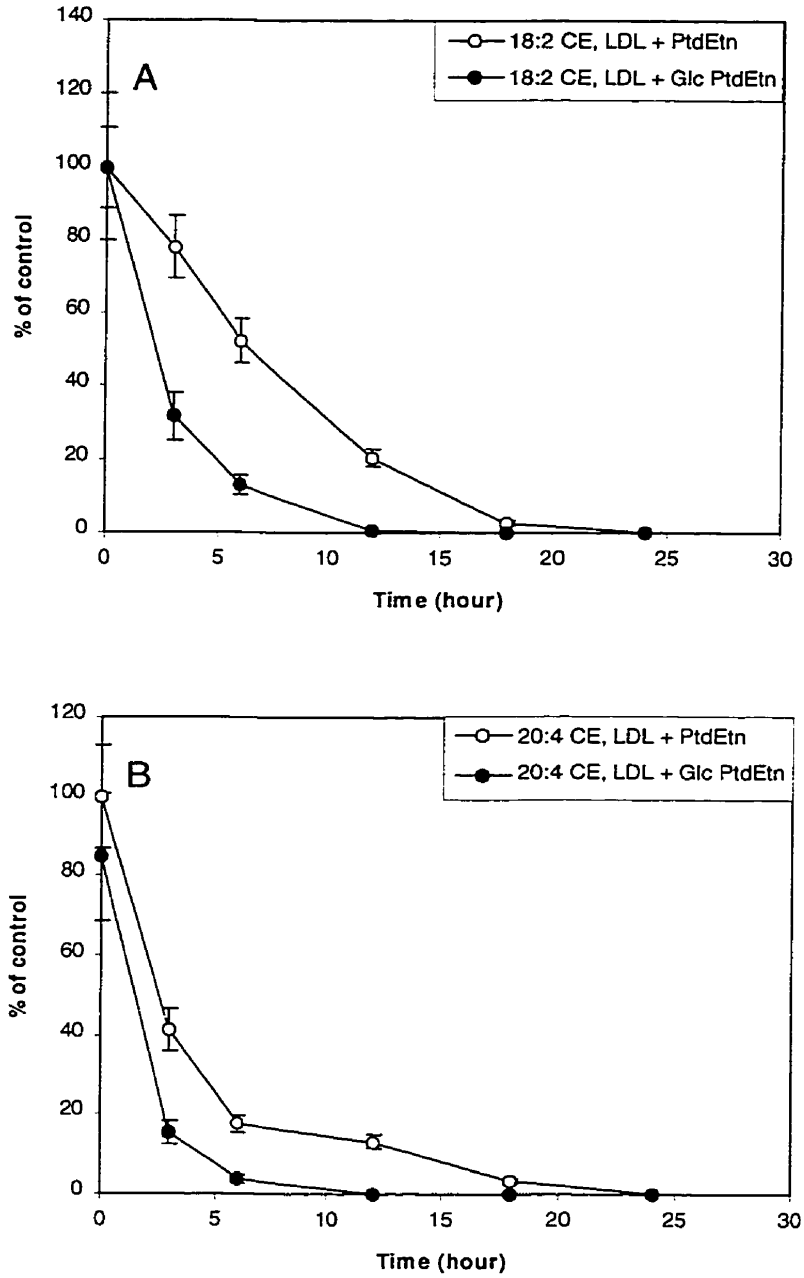


Fig. 11.4 Loss of polyunsaturated cholesteryl esters during copper catalyzed oxidation of LDL in presence of Glc PtdEtn. (A) Cholesteryl linoleate; (B) cholesteryl arachidonate. Cholesterol FAME were analysed by GLC as described in "Materials and Methods

Fig. 12.4 Identification of glycated diradylGroPEtn from atherosclerotic tissue of a diabetic male. (A) Total negative ion profile. (mass range set to 850- 1000 amu.); (B) averaged spectra from the glycated diradyl GroPEtn peak (15.01 to 15.89 min.) LC/ES/MS as described in Fig 1.4.

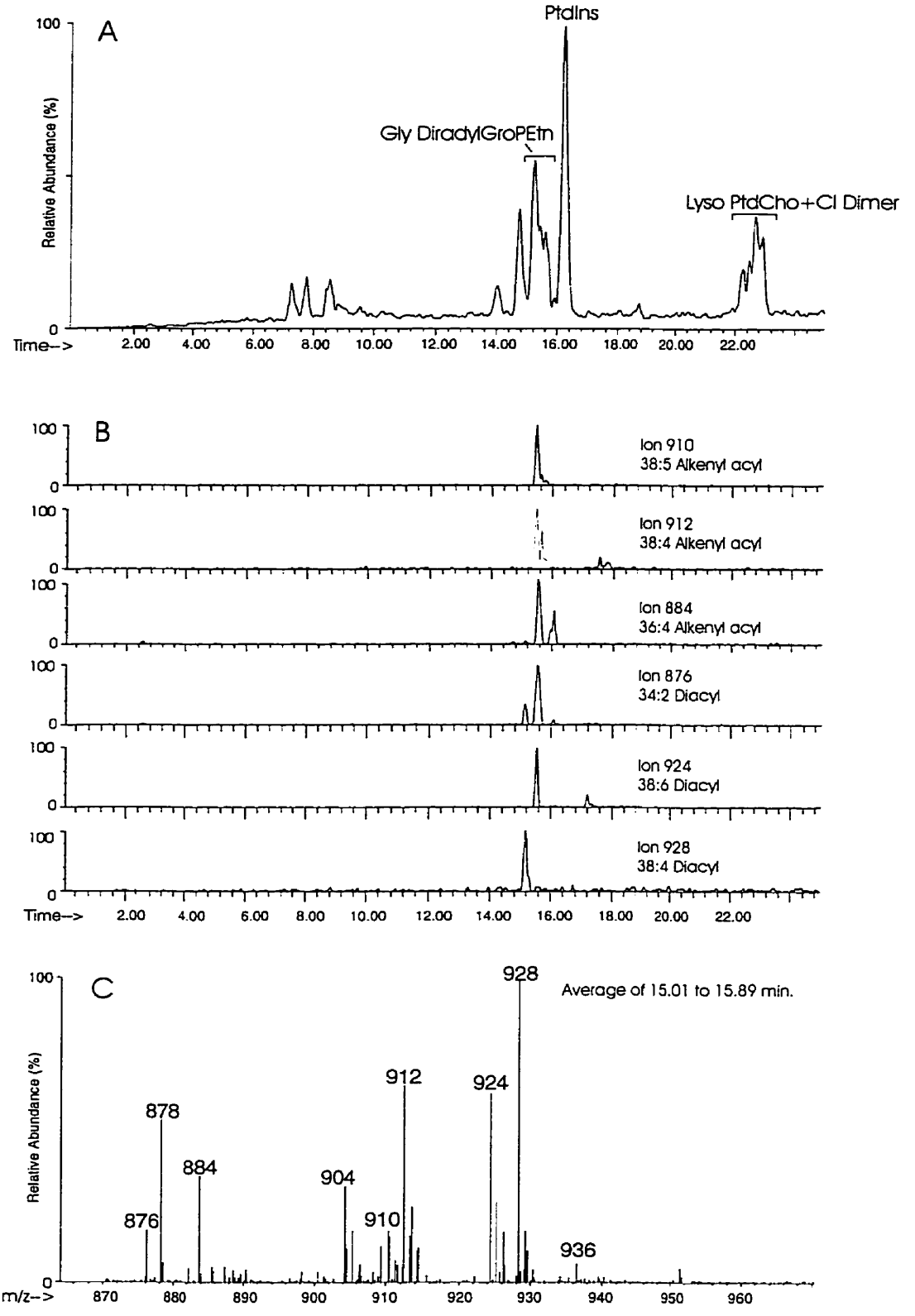

A
PtdIns
Gly DiradylGroPEtn
Lyso PtdCho+Cl Dimer
Relative Abundance (%)
Time-->
B
Ion 910
38:5 Alkenyl acyl
Ion 912
38:4 Alkenyl acyl
Ion 884
36:4 Alkenyl acyl
Ion 876
34:2 Diacyl
Ion 924
38:6 Diacyl
Ion 928
38:4 Diacyl
C
Average of 15.01 to 15.89 min.
928
912
924
878
884
904
876
910
936
m/z-->

Table 2.4

Molecular species of glycated and non-glycated diradyl GroPEtn present in atherosclerotic tissue. Lipid extracts of atherosclerotic tissue (n=6) analysed by LC/ES/MS as described under "Materials and Methods".

		Non-glycated			Glycated		
Species	**C#**	**m/z**	**mole%**	**SD**	**m/z**	**mole%**	**SD**
Alkenylacyl							
16:0-18:2	34:2	698	0.53	± 0.26	860	1.03	± 0.52
16:0-20:4	36:4	722	10.54	± 4.18	884	6.46	± 2.14
18:0-18:3	36:3	724	2.18	± 0.83	886	2.43	± 0.61
16:0-20:3							
16:0-22:6	38:6	746	2.31	± 1.14	908	0.38	± 0.14
18:2-20:4							
18:0-20:5	38:5	746	16.27	± 3.39	910	8.31	± 3.77
16:0-22:5							
18:0-20:4	38:4	750	28.09	± 4.57	912	16.27	± 3.94
18:0-20:3	38:3	752	1.36	± 0.32	914	2.13	± 0.83
18:0-22:6	40:6	774	3.63	± 0.81	938	1.32	± 1.56
Total			**64.89**	**± 4.79**		**38.33**	**± 3.21**
Diacyl							
16:0-18:2	34:2	714	1.24	± 0.25	876	9.35	± 2.89
16:0-18:1	34:1	716	1.58	± 0.47	878	7.68	± 3.13
18:0-18:2	36:2	742	2.31	± 1.25	904	4.54	± 1.22
16:0-22:6	38:6	762	8.22	± 0.54	924	10.68	± 2.35
18:2-20:4							
18:0-20:5	38:5	764	0.64	± 0.21	926	3.76	± 1.64
16:0-22:5							
18:0-20:4	38:4	766	18.34	± 2.57	928	19.73	± 3.52
18:0-20:3	38:3	768					
18:0-22:6	40:6	790	1.63	± 0.33	952	4.42	± 1.14
18:0-22:5	40:5	792	1.15	± 0.48	954	1.51	± 0.68
Total			**35.11**	**± 4.23**		**61.67**	**± 4.89**

Table 2.4 compares the composition of the molecular species of glycated and non-glycated ethanolamine phospholipids isolated from the atheroma. There is considerable disagreement between the glycated and non-glycated sets of species excluding direct *in situ* interconversion, and suggesting possible deposition of these lipids from the circulation. The composition of the molecular species of the ethanolamine phospholipids of the atheroma also differs significantly from that of human LDL (Table 1.4), with the relative proportion of the polyunsaturated PtdEtn and specially PlsEtn species being reduced. The most abundant glycated PlsEtn species in the plaque was the 38:4 (m/z 928). The possibility of selective glycation and/or oxidation is indicated by the lack of presence of glycated PtdSer species, even though PtdSer species were found in the atheroma.

DISCUSSION

LDL oxidation is claimed to play a central role in atherogenesis, which is exacerbated in diabetic hyperglycemia (1). Hyperglycemia is known to promote LDL glycation (6,17) and lipid peroxidation (6,26), but the relative importance of protein and aminophospholipid glycation in these processes has not been established. Using previously prepared standards of Glc PtdEtn (10), we have now identified the lipid glycation product of LDL as a mixture of glycated diacyl and alkenylacylGroPEtn. We had previously shown (10,11) that the glucose adduct of PtdEtn possesses a molecular weight that corresponds to either the Schiff base or its Amadori rearrangement product. In the present study we have examined the glucosyl PtdEtn isolated from Glc LDL by pseudo MS/MS with electrospray (23) and have obtained fragment ions, which are

consistent with both Schiff base and its Amadori rearrangement product. On the basis of the high relative stability of the adduct we must conclude that it is more likely to have the structure of the Amadori product rather than that of the simple Schiff base. This conclusion is consistent with the recent report of Lederer et al (24) who have unequivocally proven that Amadori products are formed from D-glucose and PtdEtn in an *in vitro* model system. Furthermore, Requena et al (25), have recently demonstrated the formation of carboxymethylEtn as an aminophospholipid adduct. Bucala et al (6,26) had shown *in vitro* that glycation of PtdEtn results in the formation of immunochemically detectable AGE. Furthermore, using ELISA assay, these authors concluded that the bulk of the AGE in LDL isolated from normal and diabetic subjects was located in the lipid phase (27). Pamplona et al (28) had obtained evidence for glycated aminophospholipids in rat liver and for their increase in animals with streptozotocin-induced diabetes. With Amadori-product formation from glucose and PtdEtn now clearly established, we undertook further investigations on the effect of Glc PtdEtn on LDL lipid oxidation.

Of special interest to LDL lipid oxidation was the presence of the PlsEtn and its fate during glycation. Previous work had attributed both antioxidant (29) and pro-oxidant (30) properties to PlsEtn in various biological systems, although the effect of PlsEtn on LDL oxidation had not been directly studied. Myher et al (31) showed that of the total diradylGroPEtn of plasma, 71.8% was due to alkenylacyl, 19.9% to diacyl and 8.3% to alkylacyl species. The bulk of these phospholipids were assumed to originate in plasma LDL. The LDL preparation used in the present studies contained 49.4% PlsEtn , while glucosylated LDL contained 35% PlsEtn of total diradylGroPEtn. Thus, only 70% of the LDL PlsEtn was glucosylated under the present working conditions. Furthermore, the

glucosylated species of both PtdEtn and PlsEtn tended to be more saturated than the respective non-glucosylated species. Analysis of diabetic plasma and red blood cells for glycated diradylGroPEtn in our earlier study (11) had indicated a near absence of glycated PlsEtn species, while aminophospholipids isolated from red blood cells and plasma were glucosylated *in vitro* in proportion to their masses. In other studies (5), it has been shown that hyperglycemic individuals have a significantly lower PtdEtn in LDL when compared to normoglycemic controls, but it has not been established whether or not this is due to decreased PlsEtn. The loss of alkenylacyl species from glycated ethanolamine phospholipids could be due to their greater susceptibility to peroxidation (29,30), as it has been demonstrated that the vinyl ether bond of alkenylacylGroPEtn is highly sensitive to peroxidation and can act as an antioxidant in protecting other phospholipids (32).

Our study shows a more rapid and more extensive glucosylation of PtdEtn compared to apo-B in LDL. The distribution of radioactive glucose suggests that PtdEtn is more susceptible to glucosylation than Apo-B possibly due to more favorable environment for trapping the initial Schiff base adduct in the lipid phase. Our measurements of radioactive glucose distribution are consistent with the results of Bucala et al (26), who showed markedly increased levels of AGE in lipid compared to the aqueous (apoprotein) phase in diabetic and end stage renal disease patients.

The present study shows that Glc PtdEtn is more easily peroxidized than non-Glc PtdEtn, and that Glc PtdEtn, included in a liposomal mixture, promotes the oxidation of PtdEtn and PtdCho. The oxidation of the phospholipids proceeds via the hydroperoxides to the core aldehydes all of which were isolated and identified as products of LDL

oxidation. Likewise, addition of Glc PtdEtn to LDL caused more extensive oxidation of PtdEtn and PtdCho than addition of an equal amount of PtdEtn. This oxidation took place in the absence of protein glycation and free glucose in the incubation medium. The increased peroxidation of glucosylated ethanolamine phospholipids can probably be attributed to an increased exposure of these molecules to the oxidizing agent, when incorporated into a liposome along with other phospholipids. The increased bulk of the polar head group in the glucosylated aminophospholipid might permit a more ready access of the oxidizing agent to the unsaturated fatty chains of all glycerophospholipids in the liposomes and in the lipid monolayer of LDL. Furthermore, addition of Glc PtdEtn to LDL in absence of protein glycation resulted in increased oxidation of cholesteryl esters which are located in the interior of the LDL particle. Clearly, Glc PtdEtn or its oxidation in the LDL lipid monolayer facilitate the access of free radicals to the cholesteryl esters in the interior of the LDL particle, which leads to a rapid destruction of the polyunsaturated cholesteryl esters.

In the past glucose has been claimed (33) to possess pro-oxidant activity in both free and bound form, but more recently increasing evidence has been obtained favoring the bound glucose pathway of oxidation of biological macromolecules. Based on albumin as a model protein, Hunt et al (34) have concluded that oxidative alterations in experimental diabetes mellitus are due to protein-glucose adduct oxidation. The promotion of LDL lipid peroxidation by Glc PtdEtn demonstrated in the present study would also be consistent with pro-oxidant action of bound glucose. However, direct measurements of the oxidation potential of aminophospholipid-bound glucose in the absence of protein glucosylation suggest that the role of lipid glycation may have been

overlooked. Hunt et al (34) did not examine the effect of PtdEtn glycation in their study. In any event, the interplay between glycation, glycoxidation and lipid peroxidation may be more complex than revealed by model studies and the separate contributions of each step of the overall process to the final result have yet to be determined. Thus, complications may arise from the interaction of the oxidation products (hydroperoxides and core aldehydes) with free amino groups of both proteins and aminophospholipids as shown elsewhere (23). The presence of Glc PtdEtn, which is negatively charged, in the particle may result in an increased LDL uptake by macrophages. Greenspan et. al (35) have demonstrated that LDL associated with negatively charged phospholipids causes a dramatic increase in uptake and deposition of cholesteryl esters in J774 macrophages. There is extensive data for the involvement of protein AGE (36-38) and lipid peroxidation in atheroma development (38). The identification of glycated PtdEtn and PlsEtn in atheroma suggests that glycated phospholipids may also be involved in plaque formation. The presence of PtdCho core aldehydes is of special interest since these compounds are found in oxidized LDL (39) and in atherosclerotic tissue (40). PtdCho core aldehydes have been specifically shown to induce increased endothelial macrophage interactions *in vitro* (40) and that PtdCho with a short chain aldehyde in the sn-2 position can mimic the platelet activating factor (41,42).

In conclusion, the present study demonstrates that Glc PtdEtn is an early product of LDL glucosylation and that it is more readily peroxidized than non-glucosylated PtdEtn. Furthermore, Glc PtdEtn promotes the peroxidation of LDL phospholipids and cholesteryl esters when included at a level of 10% of total diradylGroPEtn of LDL. These studies lend experimental support to previous speculation about the role of

aminophospholipid glycation in LDL oxidation. This is the first report of isolation and identification of glucosylated diradylGroPEtn from LDL and human atherosclerotic tissue. The study provides evidence that glucosylated PtdEtn may promote oxidation of LDL phospholipids and cholesteryl esters in hyperglycemia.

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Chapter 5: Schiff Base Adducts of Phosphatidylcholine Core Aldehydes and Aminophospholipids, Amino Acids, and Myoglobin*

* The majority of the results in this chapter have been published in the following reference: Ravandi, A., Kuksis, A., Shaikh, N.A., Jackowski, G.. (1997) *Lipids* **32**, 989-1001.

ABSTRACT

We have prepared Schiff base adducts of the core aldehydes of phosphatidylcholine (PtdCho) and aminophospholipids, free amino acids and myoglobin. The Schiff bases of the ethanolamine and serine glycerophospholipids (GPL) were obtained by reacting sn-1-palmitoyl(stearoyl) -2-[9-oxo]nonanoyl-glycerophosphocholine (PtdCho-ALD) with a two-fold excess of the aminophospholipid in chloroform/methanol 2:1 (v/v) for 18 h at room temperature. The Schiff bases of the amino acids and myoglobin were obtained by reacting the aldehyde with an excess of isoleucine, valine, lysine, carboxymethyllysine and myoglobin in aqueous methanol for 18 h at room temperature. Prior to isolation the Schiff bases were reduced with sodium cyanoborohydride in methanol for 30 min at 4°C. The reaction products were characterized by normal phase high performance liquid chromatography and on-line mass spectrometry with electrospray ionization. The monoaminoacids and aminophospholipids yielded single adducts, while lysine gave single and double adducts. A double adduct was obtained also for myoglobin, which , theoretically, could have accepted up to 23 PtdCho-ALD groups. The yields of the products ranged from 12 to 44% for the aminophospholipids and from 15-57% for the amino acids, while the Schiff base of the myoglobin was estimated at 5% level. The new compounds are used as reference standards for the detection of high molecular weight Schiff bases in lipid extracts of natural products.

INTRODUCTION

The primary products of lipid peroxidation (e. g. hydroperoxides) decompose to form secondary products, which include low and high molecular weight aldehydes (1). The aldehydes may react with cellular components or may be metabolized to inactive tertiary products (alcohols and acids). The low molecular weight bifunctional aldehydes, malonaldehyde and 4-hydroxy-nonenal, have been extensively investigated for their reactivity with various amines, amino acids and proteins, with which they yield relatively stable covalently-bound products. Specifically, malonaldehyde has been shown to undergo 1,4-addition with amino acids to form the eneamines (2), N-substituted 3-iminopropenals (3) and N,N'-disubstituted 1-amino-3-iminopropenes (4). Malonaldehyde also reacts with the amino groups in proteins (4,5) as well as with deoxynucleosides *in vitro* to produce a variety of adducts (6). The early lysine and histidine adduction chemistry of 4-hydroxynonenal has now been elucidated (7,8). It has been shown (7) that a 1:1 Michael adduct predominates in homogeneous aqueous solution and a 1:2 Michael-Schiff base adduct predominates under two-phase aqueous-organic conditions. These findings are in general agreement with recent conclusions regarding the interaction of 4-hydroxynonenal with proteins (9-11). The latter products differ from the amino acid and protein adducts formed with saturated monofunctional lipid ester core aldehydes about to be described. These adducts are more easily reversed than those resulting from the bifunctional aldehydes. Nevertheless, the formation of a Schiff base has been reported between sn-1-[9-oxo]nonanyl-2-acetylglycerophosphocholine and bovine thyroglobulin (12), which, following reduction with sodium cyanoborohydride, was suitable for the generation of antibodies that bound specifically to tritiated PAF and cross-reacted

minimally with lyso-PAF, plasmalogens and other phospholipids. The nature of the amino groups involved in the adduct formation was not established. We have previously identified the lipid ester core aldehydes among the secondary peroxidation products of glycerophospholipids and cholesteryl esters of LDL and have noted their partial retention by the apoprotein (13,14). In the following study we have used LC/MS with electrospray ionization to demonstrate that lipid ester core aldehydes readily form Schiff bases with aminophospholipids, amino acids, and myoglobin, which can be stabilized by reduction with sodium cyanoborohydride before isolation and characterization.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), sodium cyanoborohydride, horse skeletal muscle apomyoglobin, M. W. 16,950 (17), and the amino acids (valine, isoleucine, lysine and lysine methyl ester) were obtained from Sigma Chemical Co., St. Louis. MO. Since cyanoborohydride is a potential source of HCN, it should be used in a fume hood and acidification avoided. All solvents were of chromatographic purity, while all chemicals were of reagent grade or better quality and were obtained from local suppliers (Caledon Chemicals, Toronto, Canada).

Preparation of aldehydes.

The core aldehydes were prepared from egg yolk PtdCho by ozonolysis and triphenylphosphine reduction as previously described (15). The aldehydes were recovered from the reaction mixture with chloroform and were purified by preparative thin-layer chromatography (TLC) on Silica gel H (250 u thick layer, 20 x 20 cm glass plate) using a phospholipid solvent system made up of chloroform/ethanol/acetic acid/water 75:45:12:6 (by vol.) (16). The aldehyde-containing bands were located by spraying the plate with a

Schiff base reagent, which gave a purple color (18). The product (5-10 mg from 10-20 mg egg yolk PtdCho) was made up of 70% 1-palmitoyl-2-[9-oxo]nonanoyl and 30% 1-stearoyl-2-[9-oxo]nonanoyl glycerophosphocholine.

Preparation of reduced Schiff bases of aminophospholipids

Dioleoyl GroPEtn (2 mg) and the PtdCho-ALD (1 mg) were dissolved in chloroform-methanol 2:1 and the mixture kept at room temperature for 18 hr. After this time freshly prepared $NaCNBH_3$ in methanol solution (final concentration, 70 mM) was added and the reaction mixture was kept at 4 °C for 30 min. At end of reaction the excess reagent was removed by washing with water. The reduced Schiff base of phosphatidylethanolamine (I) was identified by normal phase LC/ESI/MS as shown below. An identical procedure was used for the preparation and identification of the reduced Schiff base of phosphatidylserine (II). The yields of the Schiff bases were estimated by LC/ESI/MS to range from 30% for the PtdSer adduct to 60% for the PtdEtn adduct.

Preparation of reduced Schiff bases of amino acids.

The PtdCho-ALD (1 mg) was dissolved in methanol (2 ml) and a two-fold molar excess of the amino acid was added as saturated solution in water (2 ml). The reaction mixture was shaken at room temperature for 1 hr, and then reduced with $NaCNBH_3$ as described above. The Schiff bases and the residual PtdCho core aldehydes were recovered by extraction with chloroform-methanol 2:1 and were isolated by normal phase HPLC and identified by LC/ESI/MS as described below. The yields of the Schiff bases were 15% for lysine (III), 30% for isoleucine, 47% for valine (IV) and 57% for lysine methyl ester (V). Structures I to V are shown in Scheme 1.

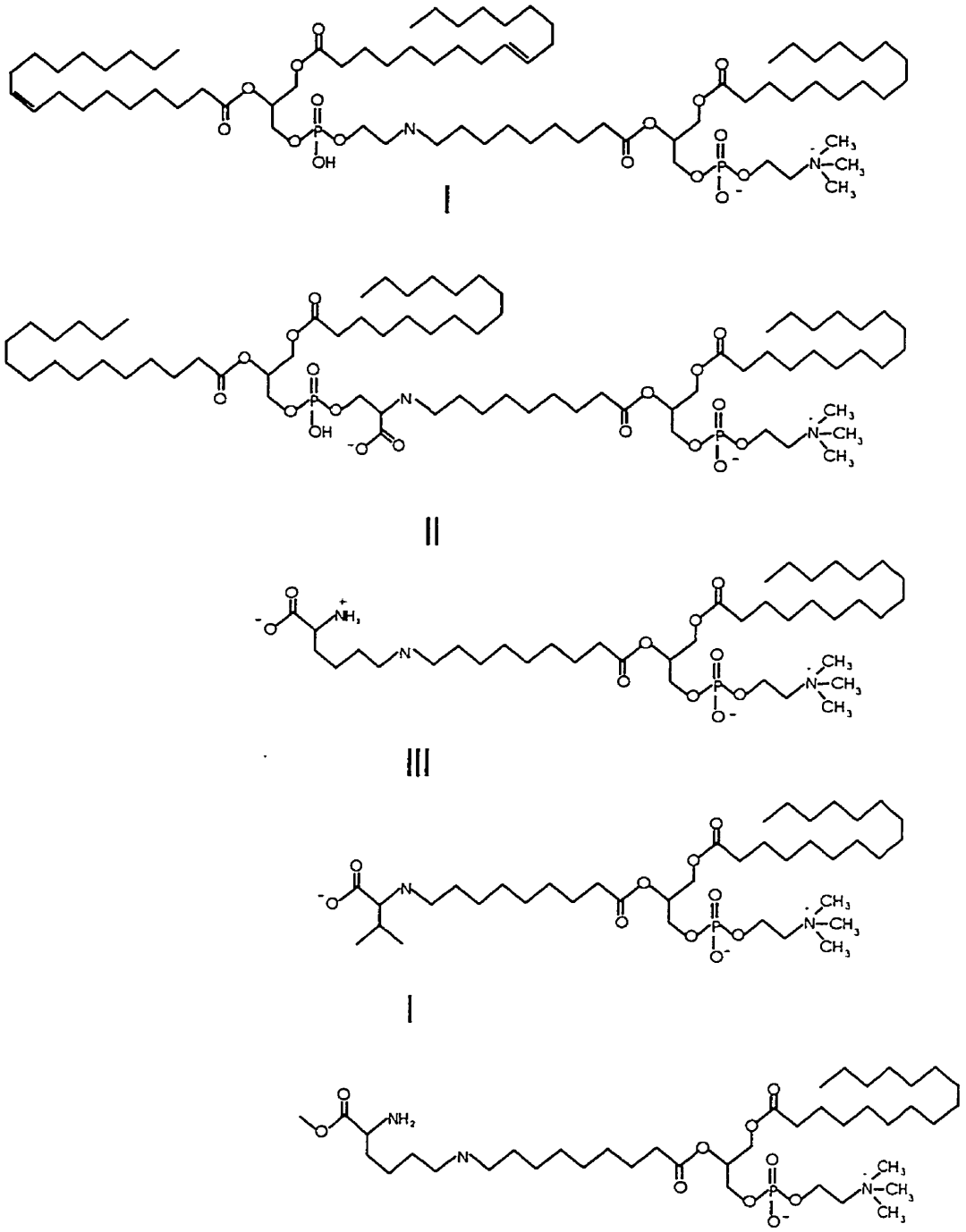


Fig1.5 Chemical structure of reduced Schiff base adducts.

Preparation of reduced Schiff base of the myoglobin

The myoglobin (0.5 mg) was dissolved in distilled water (1 ml). To this solution was added the PtdCho-ALD (2 mg) in ethanol (2 ml) (12) to give an approximate 100:1 ratio of aldehyde to protein. The reaction mixture was kept at room temperature for 1 hr. Then $NaCNBH_3$ was added to a final concentration of 70 mM and the mixture kept at 4°C for 30 min. The reaction mixture was dialyzed against distilled water for 24 hr with 5 changes of the solvent in order to remove excess reducing agent. The dialyzed sample was lyophilized and kept at -20°C until further analysis.

Normal Phase HPLC and LC/ESI/MS.

Chromatographic analysis of the reduced reaction products of the aminophospholipids or amino acids with PtdCho-ALD were performed on a silica column (Spherisorb, 3 μ, 100 x 4.6 mm ID, Alltech, Guelph, Ontario) installed in a Hewlett-Packard (Palo Alto, CA) Model 1050 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B Quadrupole mass spectrometer equipped with a nebulizer assisted electrospray ionization interface (19). The column was eluted with a linear gradient of 100% A (chloroform/methanol/ 30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% B (chloroform/methanol/water/30% ammonium hydroxide 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min (20). Positive ionization spectra were taken in the m/z range 400-1200. Selected ion chromatograms spectra were retrieved from the LC/ESI/MS data. The molecular species of the various Schiff bases were identified from the molecular masses provided by the mass spectrometer, the knowledge of the composition of the reaction mixture, and the relative order of elution (less polar species emerging ahead of more polar species) of the anticipated products from the normal phase column.

Flow ESI/MS

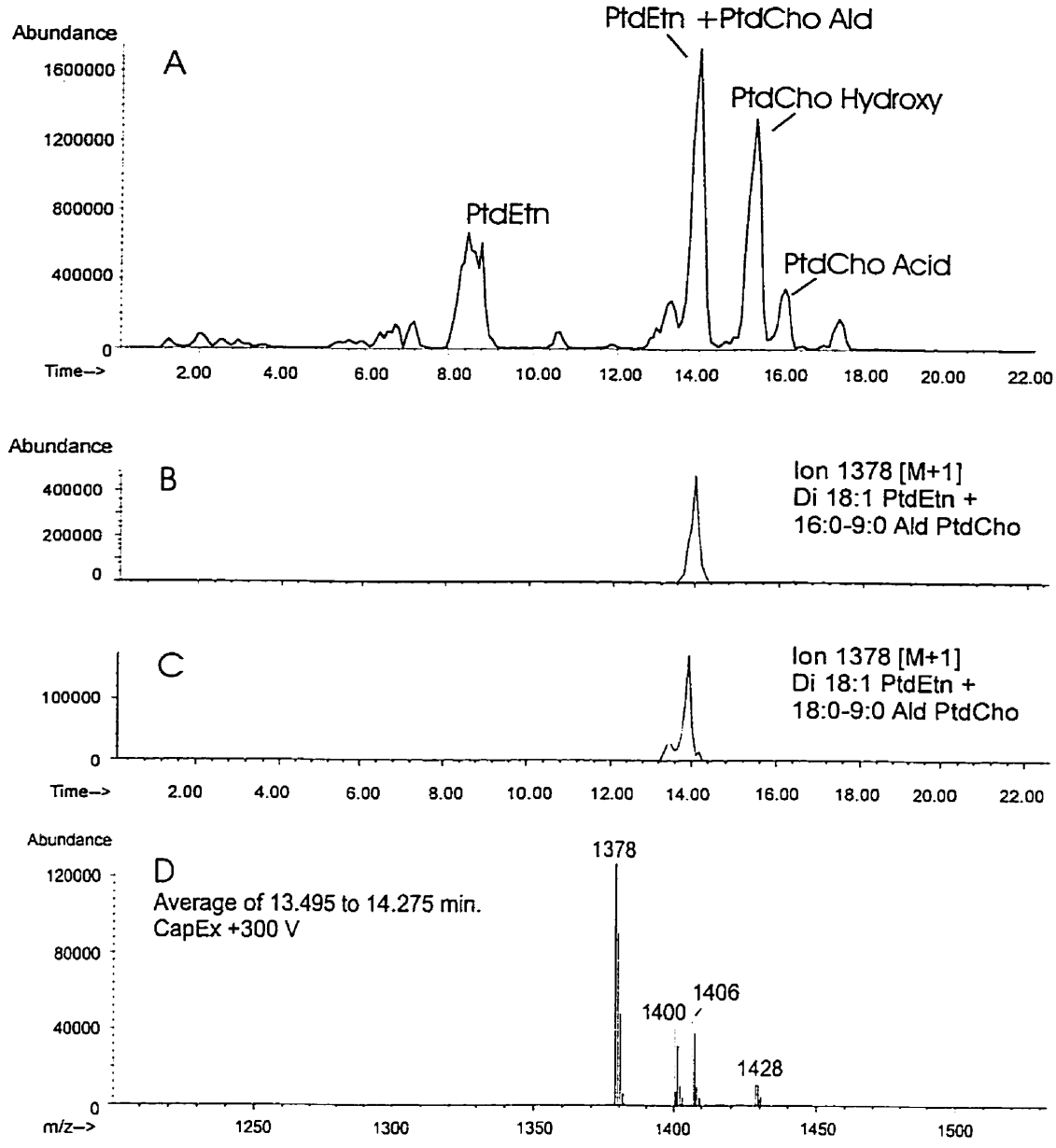
The Schiff bases of PtdCho -ALD and myoglobin were analyzed by the Hewlett-Packard Model 5988B Quadrupole mass spectrometer equipped with the ESI interface using the flow injection mode (19). The lyophilized sample was dissolved in 1 ml methanol/water/acetic acid (50:50:1, by vol) and 50 μL of the sample representing 1.5 nM protein was injected into the ESI interface at 100 μl/min. Positive ion spectra were taken in the m/z range 300-2,000. Similarly the non-reduced Schiff bases of PtdCho-ALD and amino acids were analyzed by flow ESI/MS to indicate that they could be detected as the primary product.

RESULTS

Schiff Bases of Aminophospholipids

Fig. 2.5 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dioleoyl GroPEtn along with the reconstructed single ion chromatograms (B and C) for the $[M+H]^+$ of 16:0-9:0ALD GroPCho (m/z 1378) and 18:0-9:0ALD GroPCho (m/z 1406) derivatives, respectively, and the mass spectrum (D) averaged over the entire reduced Schiff base peak (PtdEtn+PtdCho ALD). The PtdEtn-PtdCho Schiff base is clearly resolved from the unreacted PtdEtn and the excess reagent, which has been converted to its hydroxy derivative by the reducing agent, and the small amounts of the azelaoyl GroPCho and lyso GroPCho present in the original reagent. On the basis of the peak area proportions, it

Fig. 2.5 Normal phase LC/ES/MS of sodium cyanoborohydride reduced reaction products of dioleoyl GroPEtn and 1-palmitoyl(stearoyl)-2-[9-oxo)nonanoyl-sn-GroPCho. A, total positive ion current chromatogram; B and C, reconstructed single ion chromatograms for the 16:0-9:0ALD GroPCho (m/z 1378) and 16:0-9:0ALD GroPCho (m/z 1406) derivatives, respectively; D, total mass spectrum averaged over the entire Schiff base peak in A. LC/ESI/MS equipment and operating conditions are as given under Materials and Methods. PtdEtn, phosphatidylethanolamine; PtdChoAld, phosphatidylcholine core aldehyde; PtdCho Hydroxy, reduction product of PtdCho core aldehyde. Major ions are identified in figure, other ions are described in text.


Abundance
A
PtdEtn +PtdCho Ald
PtdCho Hydroxy
PtdEtn
PtdCho Acid
1600000
1200000
800000
400000
0
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Abundance
B
Ion 1378 [M+1]
Di 18:1 PtdEtn +
16:0-9:0 Ald PtdCho
400000
200000
0
C
Ion 1378 [M+1]
Di 18:1 PtdEtn +
18:0-9:0 Ald PtdCho
100000
0
Time-->
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Abundance
D
Average of 13.495 to 14.275 min.
CapEx +300 V
1378
1406
1400
1428
120000
80000
40000
0
m/z-->
1250
1300
1350
1400
1450
1500

was estimated that about 40% of the PtdCho-ALD had reacted with the PtdEtn. Reconstructed single ion chromatograms for m/z 1378 (B) and 1406 (C), corresponding to $[M+H]^+$ of the reduced Schiff base of the dioleoyl GroPEtn with 16:0-9:0 ALD GroPCho and 18:0-9:0 ALD GroPCho, respectively, document coelution of these two products. The mass spectrum averaged over the range of this peak (13.495-14.275 min). shows that the only other high mass ions are due to $[M+Na]^+$ at m/z 1400 and 1428, respectively.

Fig. 3.5 shows the fragmentation spectra of the Schiff base of dioleoyl GroPEtn and palmitoyl/[9-OXO]nonanoyl GroPCho as obtained by increasing the Cap Ex voltage to -300V in the negative ion mode (A) and to +300V in the positive ion mode (B). All the major fragment ions detected in the negative and positive ion mode can be assigned to the plausible cleavage products shown in A and B, respectively. The minor ions at m/z 125 and m/z 86 are due to loss of trimethylamine and phosphate, respectively, from phosphorylcholine (m/z 184).

Fig. 4.5 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dipalmitoyl GroPSer along with the reconstructed single ion chromatograms (B and C) for 16:0-9:0 ALD GroPCho (m/z 1370) and 18:0-9:0ALD GroPCho (m/z 1398), respectively, along with the mass spectrum averaged over the entire peak of the Schiff base (D). The reduced PtdSer-PtdCho Schiff base is only partially resolved from the PtdCho 9-hydroxynonanoates (m/z 654 and m/z 682, not shown in D), formed by reduction of the unreacted PtdCho-ALD. The reduced PtdSer-PtdCho Schiff base is preceded by a peak containing a mixture of unidentified PtdCho-ALD condensation products with m/z values ranging

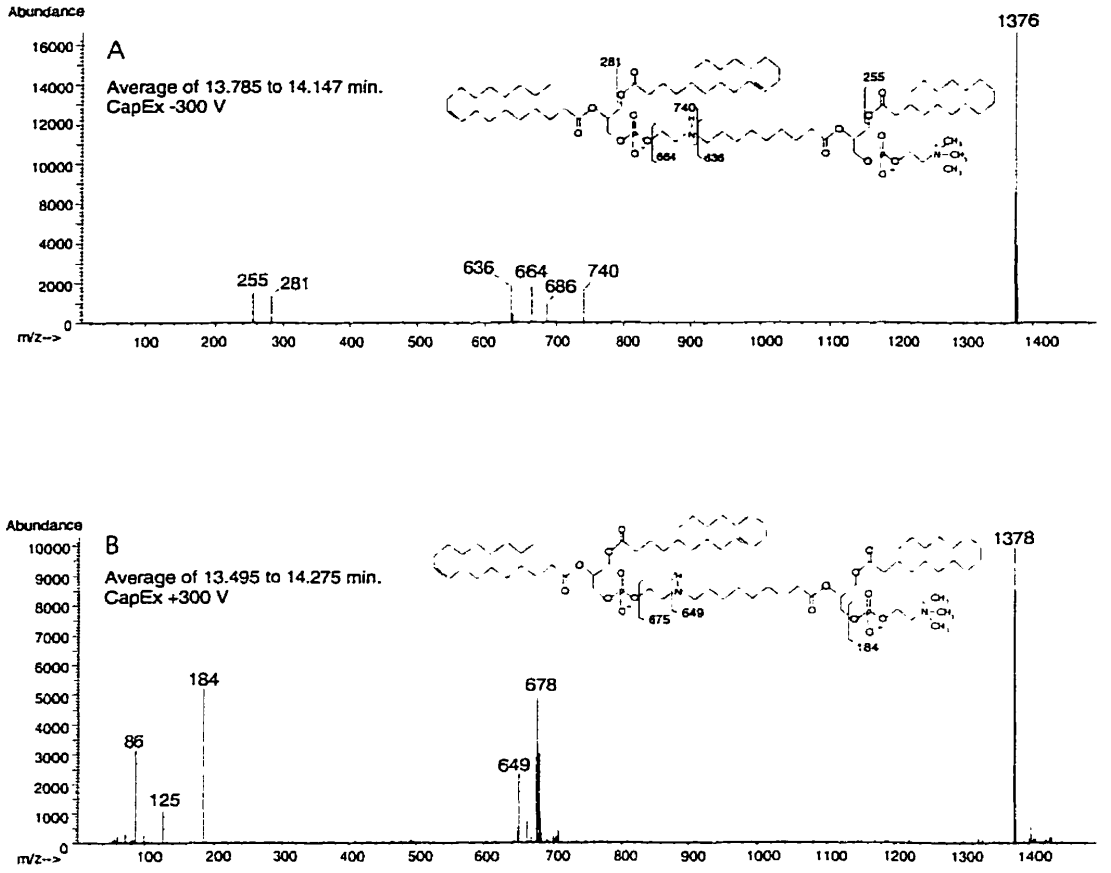


Fig. 3.5 Normal phase LC/ESI/MS fragmentation spectra of the reduced Schiff base of dioleoyl GroPEtn and 16:0-9:OALD PtdCho at negative Cap Ex voltage of -300V (A) and of positive Cap Ex voltage of +300V (B). LC/ESI/MS conditions are as given in Fig. 1.5. The generated ions correspond to the plausible bond cleavages depicted in the sketches accompanying the figures. Other chromatographic and mass spectrometric conditions are as given under Materials and Methods.

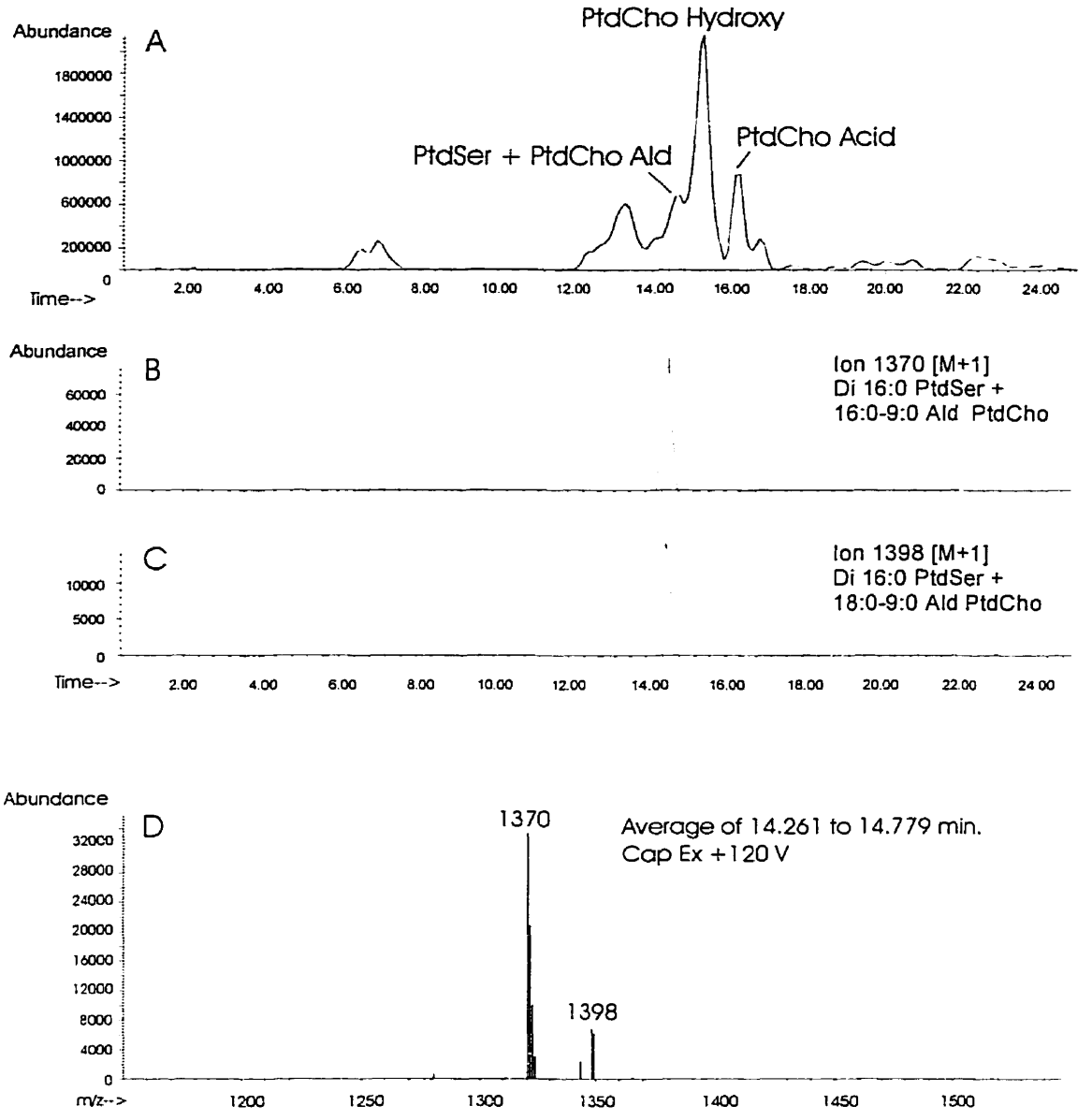


Fig. 4.5 Normal phase LC/ESI/MS of sodium cyanoborohydride reduced reaction products of dipalmitoyl GroPSer and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl - sn-GroPCho . A, total positive ion current chromatogram; B and C, reconstructed single ion chromatograms for the 16:0-9:0ALD (m/z 1370) and 18:0-9:0ALD (m/z 1398) derivatives, respectively; D, total mass spectrum averaged over the Schiff base peak in A. LC/ESI/MS equipment and operating conditions are as given under Materials and Methods. PtdSer, phosphatidylserine; PtdCho ACID, oxidation product of PtdCho core aldehyde.

from 664 to 852. On the basis of the peak area proportions, it was estimated that about 20% of the PtdCho-ALD had reacted with the PtdSer. Reconstructed single ion chromatograms for the m/z 1370 and 1398, corresponding to $[M+H]^+$ of the reduced Schiff base of the dipalmitoyl GroPSer with 16:0-9:0ALD GroPCho and the 18:0-9:0ALD GroPCho, respectively, again document coelution of the two products. Only the molecular ions for the PtdSer-PtdCho Schiff bases are seen, with no other ions being detected in the high mass range. Clearly absent are the sodium adducts that were so prominent for the PtdEtn-PtdCho Schiff base adducts. The other peaks in Fig. 3A were identified as the carboxy (m/z 666 and m/z 694) and hydroxy (m/z 652 and m/z 668) derivatives of the 16:0 and 18:0 GroPCho-ALD. Ionization of the PtdSer-PtdCho Schiff base at Cap Ex of 300V resulted in fragment ions, which closely resembled the pattern just established for the PtdEtn adduct (ion chromatograms not shown).

Schiff bases of amino acids

Fig. 5.5 shows the total ion current chromatogram as obtained by reversed phase LC/ESI/MS for the reduced reaction products of free lysine and 16:0-[9-oxo]nonanoyl GroPCho (A) and the full mass spectra averaged over the entire peak of the Schiff base (Lys+PtdCho Ald) as obtained by the use of Cap Ex -300V (B) and Cap Ex +300V (C), respectively. In both A and B, all the major ions are accounted for by the characteristic fragmentation of the PtdCho moiety indicated in the structural formulae given above. The low molecular weight ion at m/z 86 is due to loss of phosphate from phosphocholine, while m/z at 71 is due to a cleavage of the α-β carbon bond of bound lysine. The insert in Fig. 5.5A demonstrates that the nonreduced free lysine/PtdCho core aldehyde Schiff

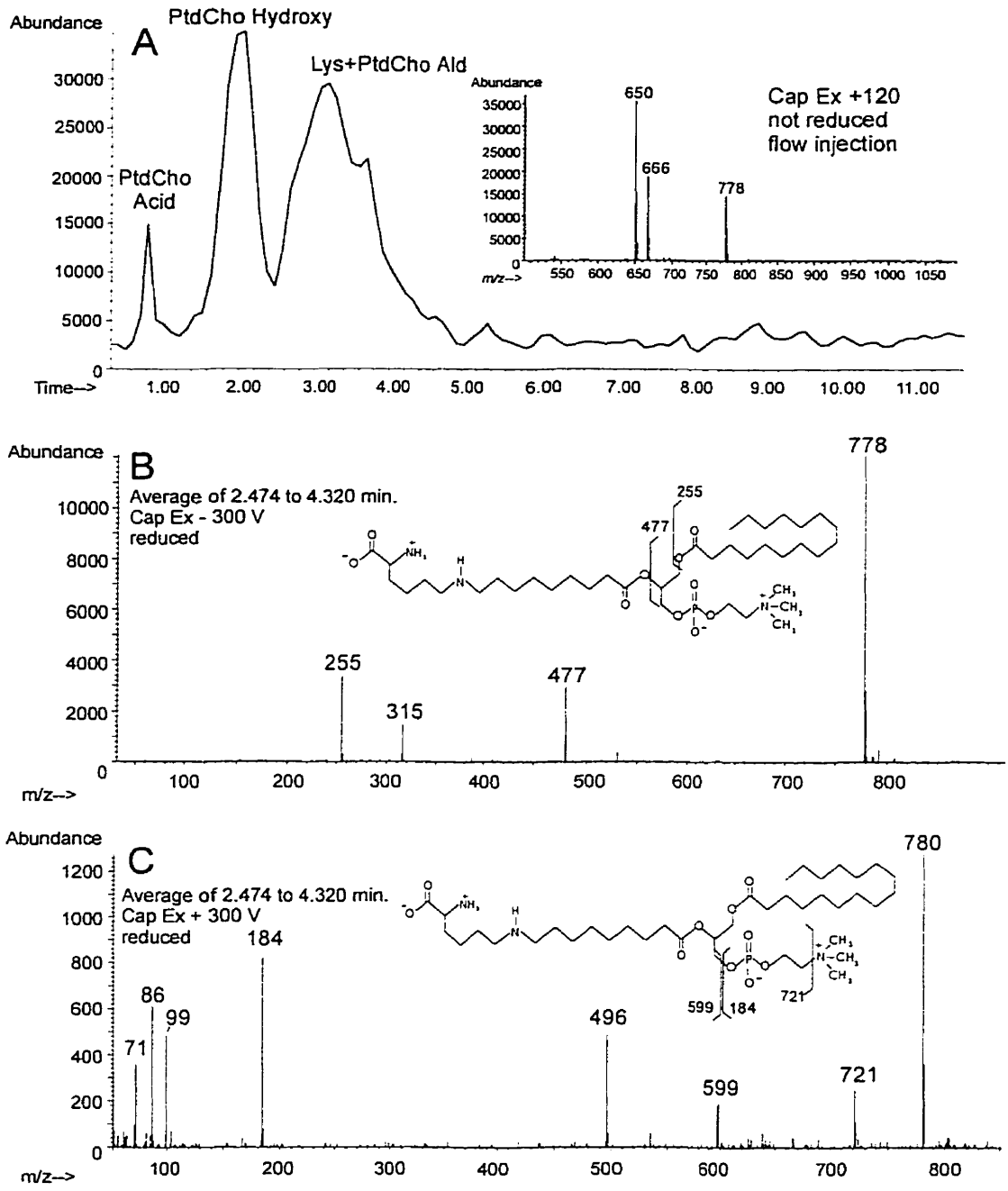


Fig. 5.5 Normal phase LC/ESI/MS fragmentation spectra of the reduced Schiff base of free lysine and 16:0-9:OALD GroPCho at negative Cap Ex voltage of -300V (A) and at positive Cap Ex voltage of +300V (B). Chromatographic and mass spectrometric conditions are as given under Materials and Methods. The generated ions correspond to the plausible bond cleavage products depicted in the sketches accompanying the figures. Other ions are as described in text.

base is stable under the conditions of flow injection yielding the molecular ion (m/z 778). The ions at m/z 650 and m/z 666 are due to the PtdCho acid and aldehyde present in the reaction mixture. It is known that the ε-amino group is more reactive towards Schiff base formation with saturated aldehydes (21).

Fig. 6.5 shows the reconstructed single ion chromatograms (A and B) corresponding to $[M+H]^+$ ions for the reduced valine-16:0-9:0ALD GroPCho (m/z 751) and reduced valine-18:0-9:0ALD GroPCho (m/z 779) adducts, respectively, along with the full mass spectrum (C) averaged over the entire peak of the Schiff base. The mass spectrum shows major ions corresponding to reduced Schiff bases of 16:0-9:0ALD GroPCho (m/z 751) and of 18:0-9:0ALD GroPCho (m/z 779) with valine. The ions at m/z 773 and m/z 801 correspond to the monosodium adducts of the two Schiff bases, respectively.

Fig. 7.5 shows the total positive ion current chromatogram (A) of the reaction mixture of the reduced isoleucine and the PtdCho-ALD along with the reconstructed single ion chromatograms (B and C) of the isoleucine-16:0-9:0ALD (m/z 765) and isoleucine-18:0-9:0ALD (m/z 793) GroPCho Schiff base adduct, respectively, along with the full mass spectrum (D) averaged over the entire adduct peak (14.464-14.954 min). The mass spectrum of the reduced Schiff base gives major ions corresponding to the adduct of isoleucine and the 16:0-9:0ALD (m/z 765) and the 18:0-9:0ALD (m/z 793) GroPCho. The ions at m/z 787 and m/z 815 are due to the monosodium adducts, respectively.

Fig. 8.5 shows the total positive ion current chromatogram (A) of the reaction mixture of the PtdCho-ALD with the methyl ester of lysine along with the single ion chromatograms (B and C) for the 16:0-9:0ALD (m/z 794) and 18:0-9:0ALD GroPCho

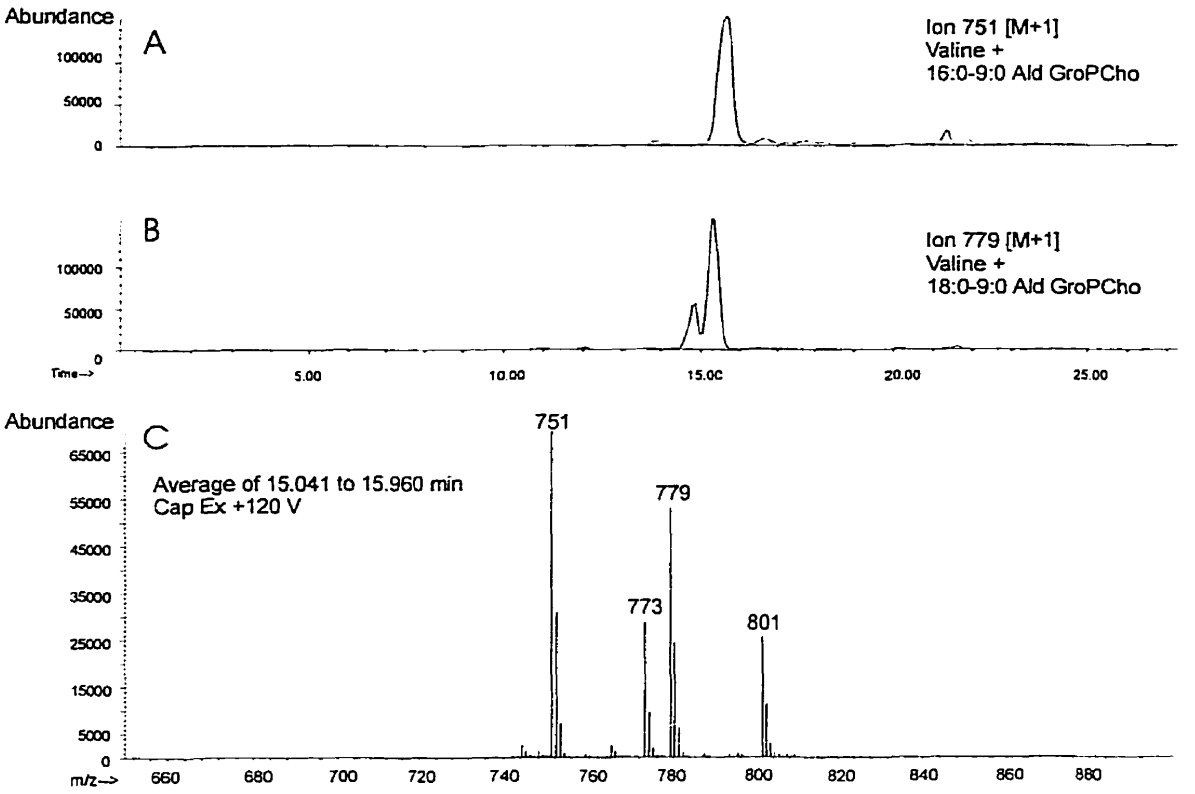


Fig. 6.5 Normal phase LC/ESI/MS of sodium cyanoborohydride reduced reaction products of valine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-sn-GroPCho. A and B, reconstructed single ion chromatograms for the 16:0-9:0ALD (m/z 751) and 18:0-9:0ALD (m/z 779) derivatives, respectively; C , total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are as given under Materials and Methods. Major ions are identified in figure; other ions are described in text.

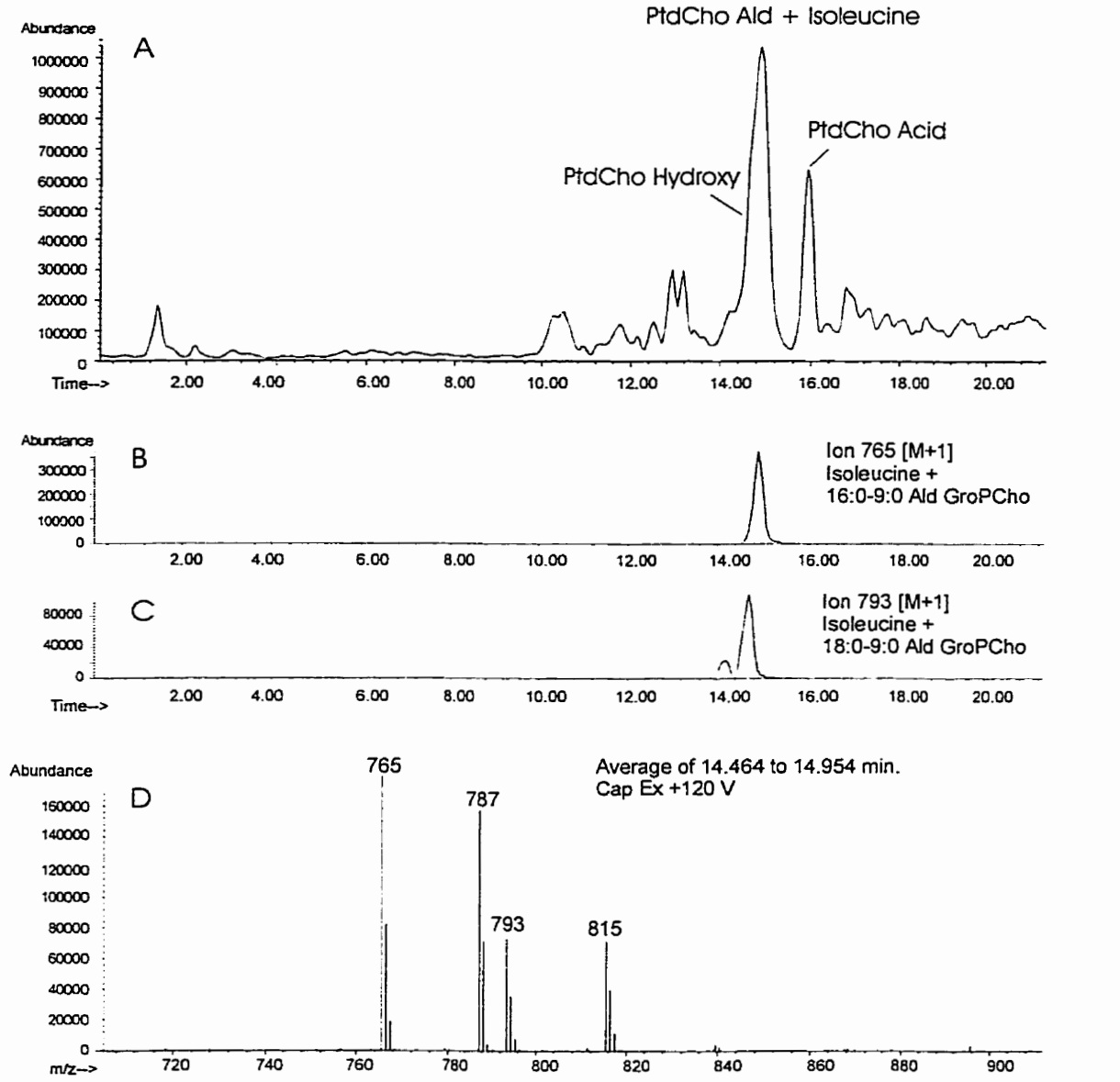


Fig. 7.5 Normal phase LC/ESI/MS of sodium cyanoborohydride reduced reaction products of isoleucine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-sn-GroPCho. A, total positive ion current chromatogram; B and C, reconstructed single ion chromatograms for the 16:0-9:0ALD (m/z 765) and 18:0-9:0ALD (m/z 793) derivatives, respectively; D, total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are given under Materials and Methods. Major ions are identified in figure; other ions are described in text.

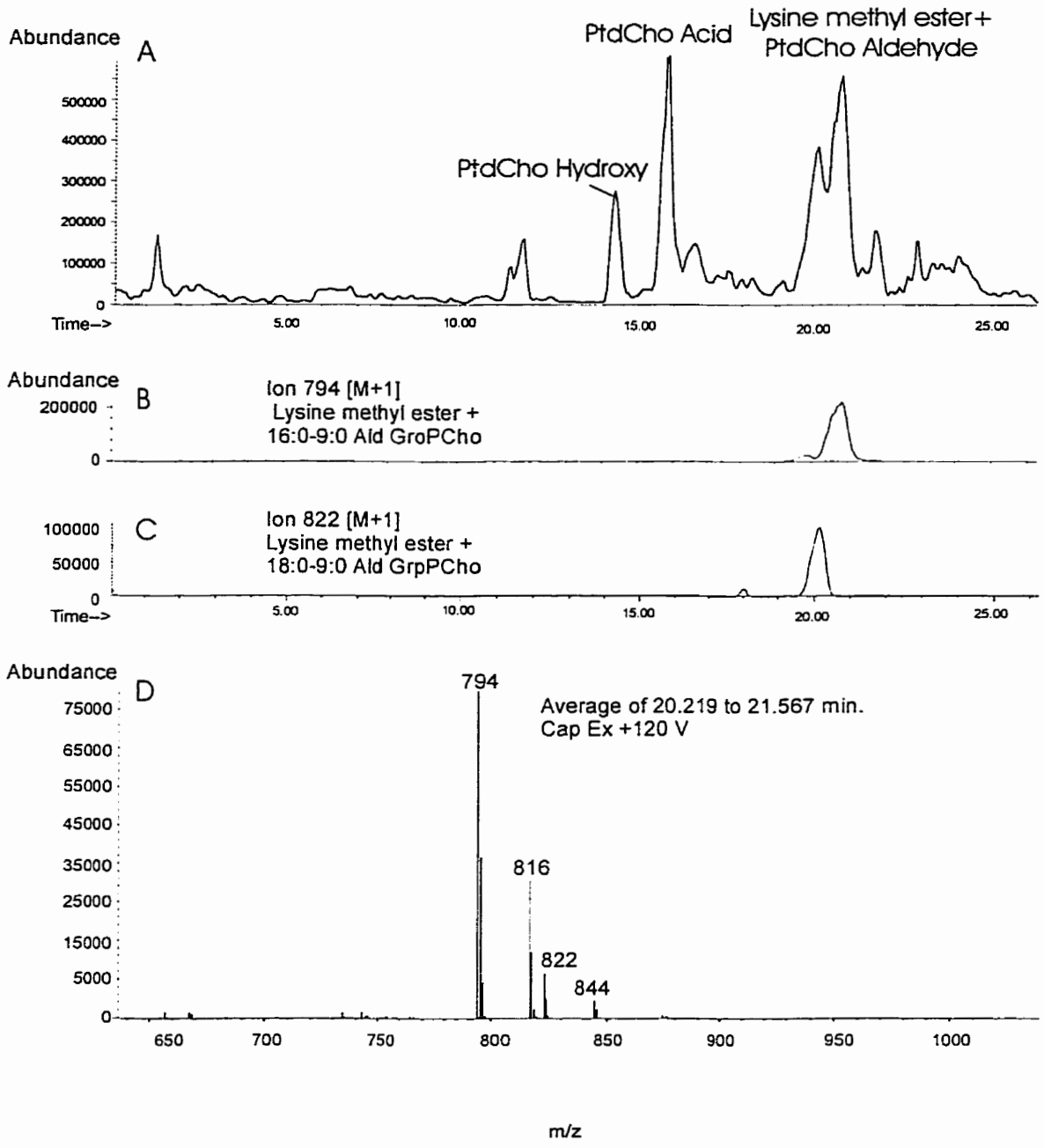


Fig. 8.5 Normal phase LC/ESI/MS spectra of sodium cyanoborohydride reduced reaction products of lysine methyl ester and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-sn-GroPCho. A, total positive ion current chromatogram; B and C, reconstructed single ion chromatograms for the 16:0-9:0ALD (m/z 794) and 18:0-9:0ALD (m/z 822) derivatives, respectively; D, total mass spectrum averaged over the Schiff base peak. LC/ESI/MS instrumentation and operating conditions are given under Materials and Methods.

(m/z 822) of the lysine methyl ester Schiff bases, as well as the full mass spectrum (D) averaged over the entire adduct peak. The ions at m/z 794 and m/z 822 correspond to the reduced Schiff bases of the C_{16} and the C_{18} homologues of the PtdCho-ALD, respectively, while the ions at m/z 816 and m/z 844 corresponded to the monosodium adducts of the two reduced Schiff bases. The structures of the reduced homologous Schiff bases were confirmed by fragmentation at Cap Ex 300V.

Schiff Bases of Myoglobin

Fig. 9.5 shows the FLOW/ESI/MS spectra and the deconvoluted molecular weights of the horse skeletal muscle apomyoglobin (A), its sodium cyanoborohydride reduction product (B), and the product of PtdCho-ALD-apomyoglobin interaction and sodium cyanoborohydride reduction (C). The original apomyoglobin gives a multicharged ion spectrum, which can be deconvoluted to give a molecular weight of 16948.73. This molecular weight corresponds to the value of 16950 reported in the literature (17). Following reduction with sodium cyanoborohydride the apomyoglobin mass spectrum shows two series of multicharged ions, one of which deconvolutes to the original horse skeletal muscle apomyoglobin (major peak) and another one, which deconvolutes to a molecular weight 80 mass units higher (minor peak). The myglobin treated with PtdCho ALD shows the presence of three series of multicharged ions, one corresponding to the original horse skeletal muscle apomyoglobin (MW 16949.89), a second corresponding to the sodium cyanoborohydride treatment product (MW 17030.74), and a third corresponding to the reduced PtdCho ALD Schiff base adduct containing two molecules of the 16:0-9:0 ALD (MW 18218.14). Other incubations gave evidence of the formation

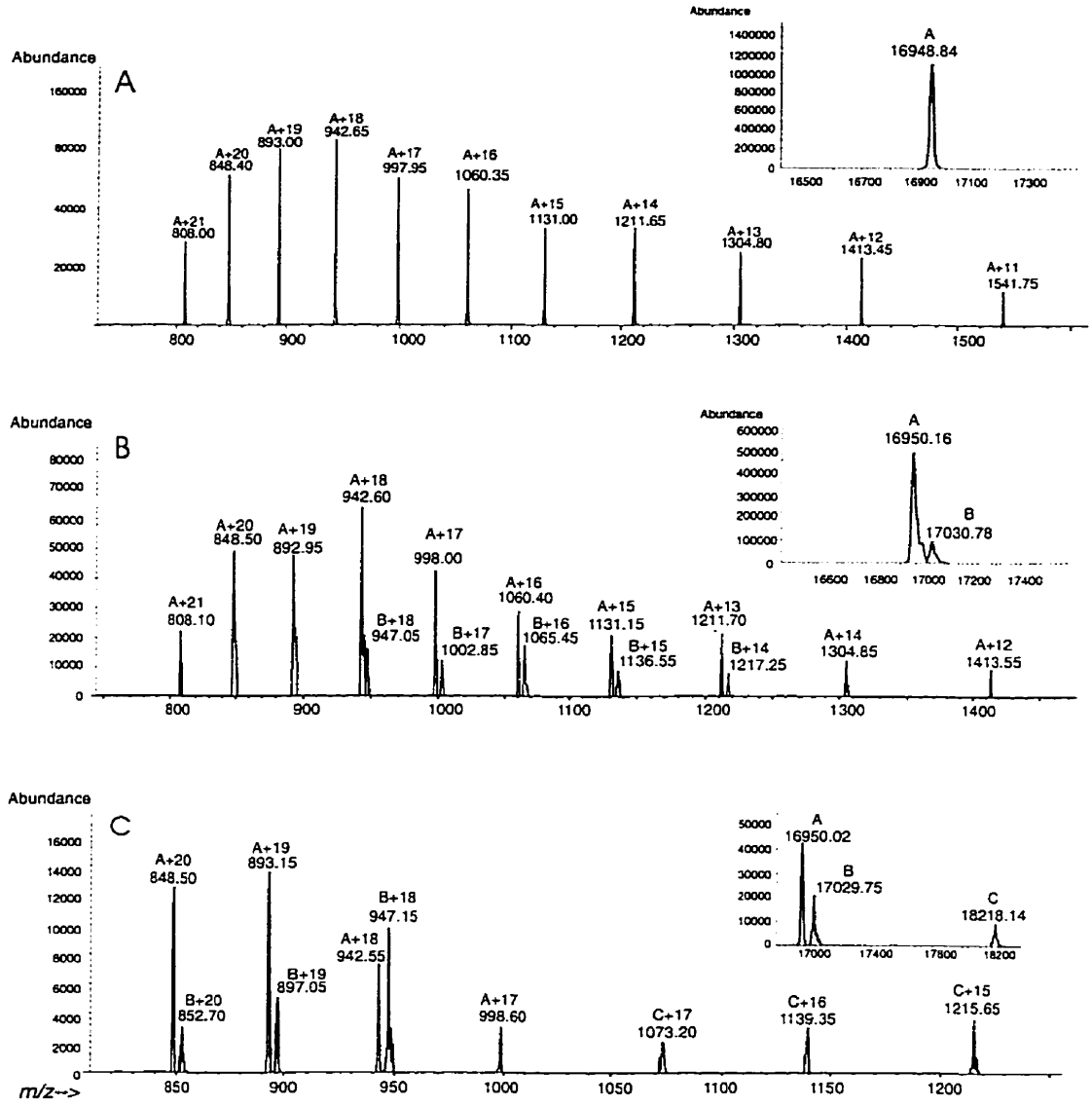


Fig. 9.5 Flow ESI/MS spectra and the deconvoluted molecular weights of the horse skeletal muscle apomyoglobin (A), its sodium cyanoborohydride reduction product (M.W. 16950.16) (B), and the product of interaction of myoglobin with two 16:0-9:0ALD (M.W. 18218.14) (C). Flow injection ES/MS instrumentation and operating conditions are given under Materials and Methods. Major ions and charge distribution are given in figure.

of Schiff bases with the mono-16:0-9:0 ALD and mono-18:0-9:0 ALD, and the di-16:0-9:0ALD and the mixed 16:0-9:0ALD and 18:0-9:0ALD adduct. The determination of the actual sites of Schiff base complexing in the apomyoglobin molecules requires further work.

DISCUSSION

The present study establishes that PtdCho ALD can react with the amino groups of aminophospholipids, amino acids and polypeptides. Aminophospholipids gave Schiff bases, which could be reduced to yield adducts of characteristic chromatographic and mass spectrometric behavior. There had been no previous demonstration of Schiff base formation between the phospholipid core aldehydes and the amino groups of aminophospholipids. Previous work had shown that the amino groups of the aminophospholipids react with simple aliphatic aldehydes, including malonaldehyde (22), but the exact structure of the products has not been established. Recently, evidence has been obtained for the glucosylation of aminophospholipids (23,24), which represent a type of Schiff base formation. The present study shows that PtdCho ALD also reacts readily with the α-amino group of free amino acids and with the ε-amino group of free and peptide-bound lysine. In a mixed phase system, the liposomal lipid ester core aldehyde reacts rapidly with the amino compounds to yield a yellow tinge, which deepens with time resulting in an extensive conversion of the aldehyde into a Schiff's base when incubated with an excess of free amino acid or polypeptide. The Schiff bases could be isolated by TLC and HPLC and could be shown to give molecular ions by flow ESI/MS

of concentrated samples in methanol/water solution. This indicated that the Schiff base was the primary reaction product in each instance. Addition of 0.5% ammonia to the HPLC mobile phase resulted in extensive dissociation of the Schiff base and loss of sensitivity of detection. In order to increase the stability of the bases and to permit the use of stronger ionizing solutions, the Schiff bases were reduced with sodium cyanoborohydride. The reduction increased the mass of the Schiff bases by two mass units without any significant effect on their TLC or HPLC migration when compared to the unreduced parent molecules.

Theoretically, free lysine could form Schiff bases either via the α- or ε- or both amino groups. Furthermore, the greater reactivity of the primary in comparison to the secondary amino group would suggest that the main product would be the Schiff base of the ε-amino group. This was confirmed by an examination of the Schiff base formed from free lysine and pure 1-palmitoyl 2-[9-oxo]nonanoyl GroPCho. Both flow injection and reversed phase LC/ESI/MS with fragmentation of the reduced adduct gave the anticipated ε-amino derivative as the sole or major product. The possibility of formation of an α-amino derivative could not be excluded. The reaction products, if both present, probably would not be separated by the chromatographic methods employed before or after reduction, and would both give the same molecular weight in the mass spectrometer. The matter was not persued further for the time being.

The Schiff bases of the amino acids, including lysine, have been previously prepared using the low molecular weight aldehydes, e. g. malonaldehyde (2-4) and 4-hydroxynonenal (7,8). In these instances, the reaction products are stabilized by secondary reactions, although the structures have not been completely established in all

instances. The simple aliphatic aldehydes yield Schiff bases that are more easily dissociated than those of the bifunctional aldehydes and require chemical reduction for stabilization (12,25).

In case of myoglobin the Schiff base formation would be expected to occur with the N-terminal amino group of glycine and any or all of the ε-amino groups of the internal lysines. Horse skeletal muscle apomyoglobin has a total of 153 amino acid residues with 1 α-amino group and 19 ε-amino groups than can react with carbonyl compounds. In the present experiments, however, only two and no more than four PtdCho ALD appeared to be involved in Schiff base formation with horse skeletal muscle apomyoglobin. It is possible that the apomyoglobin molecule contains two particularly reactive sites susceptible to Schiff base formation, which could explain the presence of only minor amounts of the single Schiff base adduct. The formation of a bis-Schiff base adduct corresponding only to the 1-palmitoyl species is probably due to the predominance of this species (80%) compared to the 1-stearoyl species (20%) in the reaction mixture. The reduced bis-Schiff base adduct of the dipalmitoyl derivative was selected for illustration.

In other studies LC/MS with electrospray has been employed to demonstrate the Schiff base formation between acetone and the ε-amino groups of horse myoglobin lysine (26). The identified protein species contained from 1 to 6 adducts of methyl isobutyl ketone or acetone. In the present experiments two to four residues of the core aldehyde were bound to horse apomyoglobin. The specific amino acid residues involved in the Schiff base formation were not determined. Since the amino acid sequence of horse myoglobin is known, the exact location of the Schiff base forming lysines could be

established in the future by trypsin digestion and reversed phase LC/ESI/MS of the released peptides, the molecular weights of which could be calculated. The peptides bearing the phospholipid moieties would be expected to be retained much longer on reversed phase columns than the corresponding peptides without the phospholipid moiety (8).

The previously prepared Schiff base of C_9 core aldehyde of 2-acetylglycerophosphocholine and thyroglobulin (12) was not characterized beyond the demonstration that an antibody could be raised to the reduced adduct to recognize in tissue extracts the platelet activating factor, which the C_9 core aldehyde resembles structurally. Uncharacterized have also remained the radioactive complexes formed between apoprotein B and oxidized 2-[1-^{14}C]arachidonoyl PtdCho (27), which must have involved the C_5 core aldehyde, because the malonaldehyde and 4-hydroxynonenal would not be labeled.

The present findings are of interest because the lipid ester core aldehydes like the short chain aldehydes would be expected to form covalently-bound complexes with proteins. Malonaldehyde and 4-hydroxy nonenal-modified lipoproteins have been found entrapped in aortic walls of both humans and animals (28,29), while lysine modification of LDL or lipoprotein (a) by 4-hydroxynonenal or malonaldehyde decreases platelet serotonin secretion (30). The generation of reactive aldehyde species next to membrane components possessing active amino groups, which may lead to Schiff base formation, could lead to membrane damage, loss of enzyme activity and protein-protein or protein-lipid cross-linking (31). Proteins in the red blood cell membrane show increase in molecular weight, cross-linking, when exposed to lipid oxidizing conditions (32). The

hydrophobicity acquired from complexing with the lipid ester core aldehydes would promote greatly the membrane association of the proteins and contribute to their resistance to proteolytic digestion and trans-membrane transport among other effects. Recent work (33) with monoclonal antiphospholipid antibodies has shown that they are directed against epitopes of oxidized phospholipids. A variety of structures that could occur in peroxidized tissues have been suggested but only a few have been experimentally demonstrated. The present work provides experimental evidence for the existence of some of these compounds as well as records LC/ESI/MS characteristics of these compounds that are required for the isolation and identification of the Schiff base adducts from natural sources.

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Chapter 6: Glycated lipid moiety of low-density lipoprotein promotes macrophage uptake and accumulation of cholesteryl ester and triacylglycerol

ABSTRACT

This study reports the specific effect of glucosylated phosphatidylethanolamine (Glc PtdEtn), the major LDL lipid glycation product, on LDL uptake and cholesteryl ester and triacylglycerols accumulation by THP-1 macrophages. LDL specifically enriched (10 nmole/mg LDL protein) with synthetically prepared Glc PtdEtn when incubated at a concentration of 100 μg/ml protein with THP-1 macrophages resulted in a significant increase in CE accumulation when compared to LDL enriched in non-glucosylated PtdEtn. After a 24 hour incubation with LDL containing Glc-PtdEtn the macrophages contained nearly two fold higher CE (10.11 ± 1.54 μg/mg cell protein) and TG (285.32 ± 4.38 μg/mg cell protein) when compared to LDL specifically enriched in non-glucosylated PtdEtn (CE, 3.97± 0.95, $P<0.01$, and TG, 185.57± 3.58 μg/mg cell protein, $P<0.01$) The corresponding values obtained with LDL containing glycated protein and lipid were similar to those of LDL containing Glc-PtdEtn (CE,11.95±1.35 and TG 281.23 ± 6.32 μg/mg cell protein). The accumulation of both neutral lipids was further significantly increased by incubating the macrophages with Glc-PtdEtn LDL exposed to copper oxidation (5μM $CuSO_4$/37°C/12h). Utilizing the fluorescent labeled probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), a 1.6 fold increase in Glc PtdEtn + LDL uptake when compared to control LDL. Competition studies revealed that AcLDL is not a good competitor for Glc PtdEtn LDL (5-6% inhibition) and that glycated LDL resulted only in a 55% inhibition of LDL + Glc PtdEtn uptake by macrophages. These results indicate that glucosylation of PtdEtn in LDL accounts for the entire effect of LDL glycation on macrophage uptake, and therefore the increased atherogenic potential of LDL in hyperglycemia.

INTRODUCTION

LDL glycation has been proposed to play central role in the atherosclerosis of diabetic hyperglycemia (1). The effect of protein glycation in LDL modification and its oxidation has been extensively investigated, but the significance of lipid glycation is unclear. Several group of investigators have shown that glycated LDL is capable of inducing foam cell formation in a variety of cell culture systems (2-4). The first study to show an altered biological activity of a glycated lipoprotein measured LDL uptake by normal human fibroblasts (5). Recognition of *in vitro* glycated LDL by these cells, which only have classical LDL receptors, was impaired. This impairment was proportional to the degree of modification of the lysine residues. Later Klein et al (6) showed that recognition of LDL from diabetic patients in poor glycemic control by human fibroblasts was also impaired, supporting the role of glycation in altering recognition of LDL by classical LDL receptor.

LDL from diabetic patients has also been shown to increase CE accumulation in macrophages and the extent of CE accumulation is correlated with the extent of LDL glycation (7). It was demonstrated by Lopes-Virella et al (4) that the scavenger receptors were not involved in glycated LDL uptake. Competition studies using acetylated LDL showed that the scavenger receptor pathway was not able to inhibit macrophage binding of gly-LDL thus a separate high affinity receptor was proposed (4). There are also *in vivo* studies (8,9) that demonstrated the diminished LDL receptor activity with glycated LDL in man. In all these studies it was presumed that the ApoB is responsible for the altered activity and the contribution of LDL lipid glycation products were overlooked.

We have recently demonstrated that not only proteins are involved in *in vivo*

nonenzymatic glycation but aminophospholipids are also involved in this process (10,11). Plasma and red blood cell aminophospholipids isolated from diabetic subjects showed a 10 fold increase in glycated PtdEtn when compared to control subjects. Utilizing advanced glycosylation end products (AGE) antibodies Bucala et al (12) has shown that the lipid component of glycated LDL contains most of the AGE present. Although no lipid glycation products were isolated or identified, the relative amount of this AGE antigen was proportional to the susceptibility of LDL to peroxidation. In this report we demonstrate directly that LDL lipid glycation products result in increased LDL uptake, and on CE and TG accumulation in macrophages.

MATERIALS AND METHODS

Cell culture

THP-1 cells were obtained from the American Type Tissue Culture Collection (TIB 202) and were propagated in RPMI 1640 /10%fetal calf serum (FCS)/penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37 °C, 5% CO_2. Cells were plated at a density of $1x10^6$ cells /ml in 10% FCS medium containing phorbol myristate acetate (10^{-7} M) for 72 hrs. The cells were then washed extensively with serum-free RPMI medium and incubated with or without lipoproteins as indicated for each experiment. In all experiments, cell viability exceeded 90% as determined by Trypan blue exclusion.

Synthesis and Isolation of Glucosylated PtdEtn

Glucosylated PtdEtn was prepared and purified as described in detail previously (10). Briefly, PtdEtn (2 mg) dissolved in 1 ml of methanol was transferred to a 15 ml test

tube and the solvent evaporated under nitrogen. Four ml of 0.1 M phosphate buffer containing 0-400 mM glucose and 0.1 mM EDTA were added and sonicated at low power for 5 min. at room temperature and the mixture incubated under nitrogen at 37 °C for various periods of time. Lipids were extracted into chloroform/methanol (2:1, v/v) as described by Folch et al (12) and the solvents evaporated under nitrogen. Samples were redissolved in chloroform/methanol (2:1, v/v) and kept at -20°C until analysis. Glucosylated PtdEtn (2 mg) was purified by preparative TLC (20 x 20 cm glass plates) coated with silica gel H (250 μ thick layer). The chromatoplates were developed using chloroform/methanol/30% ammonia (65:35:7, by vol.) as described (10). Phospholipids were identified by co-chromatography with appropriate standards and visualizing any lipid bands under ultraviolet light after spraying the plate with 0.05% 2,7-dichlorofluorescein in methanol. Both glucosylated and non-glucosylated lipids were recovered by scraping the gel from appropriate areas of the plate and extracting it twice with the developing solvent.

Lipoprotein Isolation and Oxidation

LDL (1.019-1.069 g/ml) was obtained by density gradient ultracentrifugation (14) from plasma of fasted normolipidemic individuals. LDL (2 mg protein/ml) was subsequently dialyzed against 0.1 M phosphate buffer (pH 7.4) containing 0.1mM EDTA for 24 hrs (three buffer changes). LDL samples were sterilized by passing through a 0.22 micron filter (Millipore, Milford, MA), kept at 4 °C, and used within 1 week. Lipoprotein concentration was determined by the method of Lowry et al (15) and expressed as mg/ml. Oxidation of LDL (5 mg protein/5 ml) was performed by dialysis against 5 μM $CuSO_4 \cdot 5H_2O$ in 0.1M phosphate buffer, pH 7.4, for 12 hr at 37°C in the dark. LDL (2 mg

protein/ml) in 1 mM EDTA, containing 0.1 mg/ml chloramphenicol and 3mM NaN_3 was incubated with 50 mM glucose at 37°C for 1 week under nitrogen to obtain glycated LDL. Acetylated LDL (AcLDL) was prepared by the method of Basu et al (16).

Enrichment of LDL with PtdEtn

Glucosylated and non-glucosylated PtdEtn was incorporated into LDL essentially as described by Engelmann et al. (17) for enriching human plasma lipoproteins with phospholipids. Glucosylated PtdEtn (1 mg) in chloroform-methanol (2:1, v/v) was transferred to a 15 ml test tube, the solvent evaporated under nitrogen, and the lipids dispersed by vortexing in 1.5 ml buffer containing 50 mM Tris /HCl, 1 mM dithiothreitol and 0.03 mM EDTA (pH 7.4). The solutions were sonicated in a bath sonicator for 5 min at 1 min intervals while being kept on ice under a stream of nitrogen. The liposome mixture was centrifuged at 3500 g and the supernatant collected and passed through a 0.45 μ filter. The liposomal mixture (1 ml) was added to fresh plasma (4 ml) containing 3 mM NaN_3 under gentle mixing. The mixture was incubated under nitrogen at 37 °C for 24 hours. Lipoproteins were isolated as described above.

LC/ES/MS of Lipoprotein phospholipids

Normal phase high performance liquid chromatography (HPLC) of phospholipids was performed on a 5μ Spherisorb column (250 mm x 4.6 mm i. d., Alltech Associates, Deerfield, IL). The columns were installed into a Hewlett-Packard Model 1090 Liquid chromatograph and eluted with a linear gradient of 100 % Solvent A (chloroform /methanol/ 30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% Solvent B (chloroform/methanol/ water/30% ammonium hydroxide 60:34:5.5:0.5, by vol.) in 14

min, then at 100% B for 10 min. (18). The flow was set at 1 ml/min. The peaks were monitored by on-line ES/MS. Normal phase -HPLC with on-line electrospray mass spectrometry (LC/ES/MS) was performed by splitting the HPLC flow by 1/50 resulting in 20μL/mL being admitted to a Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface (HP 59987A) (22). Tuning and calibration of the system was achieved in the mass range of 400-1500 by using the standard phospholipid mix dissolved in the HPLC solvent A and flow-injected at 50 μL/min into the mass spectrometer. Capillary voltage was set at 4 kV, the endplate voltage at 3.5 kV and the cylinder voltage at 5 kV in the positive mode of ionization. In the negative mode, the voltages were 3.5 kV, 3 kV and 3.5 kV, respectively. Both positive and negative ion spectra were taken in the mass range 100-1100 amu. The capillary exit (Cap Ex) voltage was set at 120 volts in the positive and 160 volts in the negative ion mode. Nitrogen gas was used as both nebulizing gas (40 psi) and drying gas (60 psi, 270° C). Phospholipids were quantified on basis of standard curves established for each phospholipid class. The equimolar ion intensities of different species of each phospholipid class varied by less than 5% (19) in each of the ion modes. The LC/ES/MS response to different phospholipid classes varied greatly and required the regular use of standards.

Labeling of Lipoprotein

LDL was labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) according to the method of Stephan et al (20). Briefly, to 10 ml of LDL solution (1mg/ml protein) containing 1mM EDTA in 0.1 M PBS, 300 μl of DiI solution in DMSO (30 mg/ml) was slowly added under gentle

agitation. The solution was incubated for 8 hrs at 37 °C under nitrogen and in the dark. The LDL was reisolated by ultracentrifugation. Under these conditions the average labeling efficiency was 20-25 ng of DiI/ μg of LDL protein.

Quantitative spectrofluorometry of DiI LDL uptake

To study the uptake of DiI labeled LDL, cells seeded in 24 well culture dishes and were incubated for 4 hrs at 37 °C with increasing concentrations of the lipoprotein (10-200 μg/ml) (21). Specific uptake of DiI-LDL in all preparations was determined at 10 μg lipoprotein/ml with 50-fold excess of unlabelled lipoprotein. After, the cells were washed twice with PBS containing 0.4% BSA and twice with PBS alone. To each well 1 ml of lysis buffer was added (1g/l SDS, 0.1 M NaOH). Cells were incubated at room temperature under gentle shaking for 1 hr. This allowed both direct fluorescence and protein measurement. The fluorescence of each well was measured in duplicate by a Shimadzu spectrofluorometer (RF5000U). The excitation and emissions wavelengths were set at 520 and 575 nm, respectively. The detection range for the fluorescence was linear from 0.05 to 20 μg/ml LDL protein. Protein determinations were done in duplicates with the method of Lowry et al (15) using BSA dissolved in lysis buffer as standard. Fluorescent microscopy was performed as described previously (22)

Cellular cholesterol and triacylglycerol accumulation

The cells were exposed for 24 hrs to control and modified LDL preparations. After incubation the cells were washed once with ice cold PBS containing 0.4% BSA and twice with PBS alone. Cells were scraped from the culture flask into PBS and sonicated. The cellular lipids were extracted with chloroform-methanol (2:1 v/v). The lipid extract was digested with phospholipase C (*Clostridium welchii*) as previously described (23).

The reaction mixture was extracted with chloroform-methanol (2:1 v/v) containing 100 μg tridecanoyglycerol as internal standard. The lipid extracts were then reacted for 30 min at 20 °C with SYLON BFT plus one part dry pyridine. This procedure converts the free fatty acids into silyl esters and the free sterols, diacylglycerols and ceramides into silyl ethers, leaving the cholesteryl esters and triacylglycerols unmodified. Free cholesterol, cholesterol esters and triacylglycerols were measured by gas liquid chromatography (Hewlett-Packard 5890) with a polarizable capillary column as described previously (24).

Statistical analysis

Cellular uptake and neutral lipid accumulation assays were done in triplicate and statistical significance was performed with ANOVA.

RESULTS

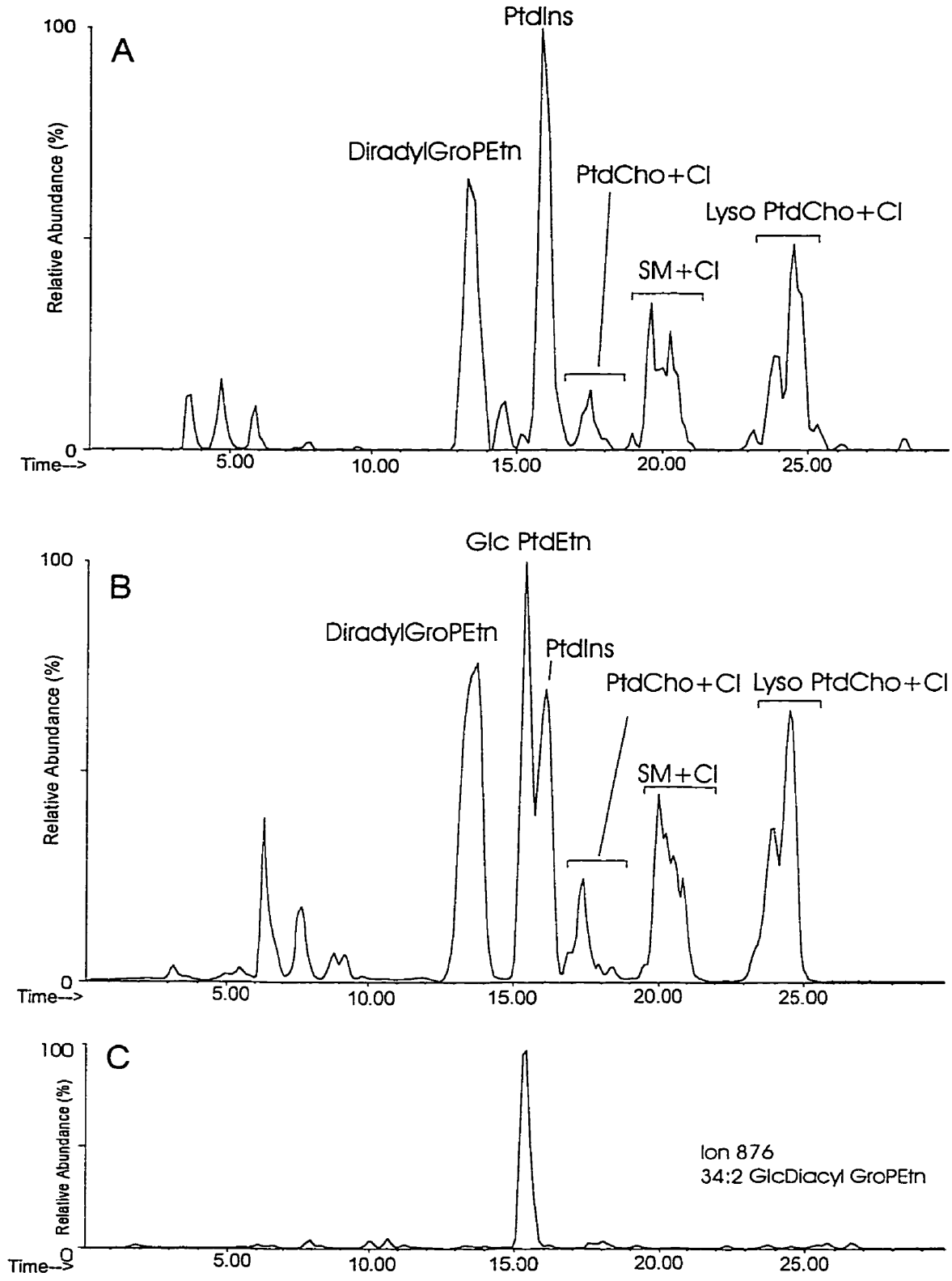
LDL phospholipid analysis

LC/ES/MS analysis of phospholipids in our LDL preparations allowed us to determine the amounts of glucosylated PtdEtn (Fig1.6). The added Glc PtdEtn was 16:0-18:2 GroPEtn with a MW of 877 giving a molecular ion in the negative ion mode of 876 m/z which was easily detected in the LDL phospholipid total ion profile (Fig1.6 A).

Glc PtdEtn effects on CE uptake

Using this technique we were able to prepare well defined LDL preparation for our cell culture studies. As shown in Fig 2.6, when LDL containing glucosylated PtdEtn (10-30 nmole/mg LDL protein) was incubated with THP-1 cells there was a significant increase in CE accumulation when compared to LDL containing non-glycated PtdEtn

Fig. 1.6 LC/ES/MS analysis of LDL phospholipids. (A) Control LDL, (B) LDL enriched with 16:0-18:2 Glc GroPEtn, (C) single ion plot of ion 876 representing the Glc PtdEtn present in LDL enriched with the glycated phospholipid.


A
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00
PtdIns
DiradylGroPEtn
PtdCho+Cl
Lyso PtdCho+Cl
SM+Cl
B
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00
Glc PtdEtn
DiradylGroPEtn
PtdIns
PtdCho+Cl
Lyso PtdCho+Cl
SM+Cl
C
100
0
Relative Abundance (%)
Time-->
5.00
10.00
15.00
20.00
25.00
Ion 876
34:2 GlcDiacyl GroPEtn

(10.11 ± 1.54 vs. 3.97 ± 0.35 μg/mg cell protein, ($P < 0.01$). As control we utilized LDL molecules enriched to same extent with non glycated form of PtdEtn. The presence or absence of PtdEtn in LDL did not have any significant effect on CE accumulation. In order to determine the contribution of LDL lipid and protein glycation on CE accumulation, macrophages were incubated with LDL that had been glycated in presence of glucose (50 mM, 7 days, 37 °C). The glycated LDL preparation resulted in CE accumulation (11.95 ± 1.38 μg/mg cell protein) which was comparable to that obtained in cells when Glc PtdEtn LDL was incubated with macrophages. As shown in Fig. 2.6 both glycated LDL and Glc PtdEtn + LDL showed increases in not only CE but TC levels compared to the control LDL TC levels. (35.85 vs. 37.49 μg/mg cell protein).

TG accumulation in THP-1 cells

When the macrophages were incubated with 100 μg/ml LDL the TG levels in these cells had a dramatic increase in both LDL + Glc PtdEtn and glycated LDL (Fig3.6). This increase in TG levels was also seen for incubations of 50 μg/ml LDL.

CE and TG accumulation due to OxLDL enriched with Glc PtdEtn

Previous done in our laboratory has shown that Glc PtdEtn is more susceptible to oxidation and also facilitates the oxidation of other LDL phospholipids and CE present in the core of the molecule. In order to investigate effects of Glc PtdEtn on LDL oxidation and foam cell formation we incubated the copper oxidized lipoproteins containing the glucosylated phospholipid. Glc PtdEtn + LDL resulted in a significant increase of CE, TC, and TG accumulation in these cells as compared to oxidized LDL lacking Glc PtdEtn + LDL (Fig 4.6). This is in accordance with previous work indicating that glycated LDL in more susceptible to oxidation and the modifications resulting from the oxidation and

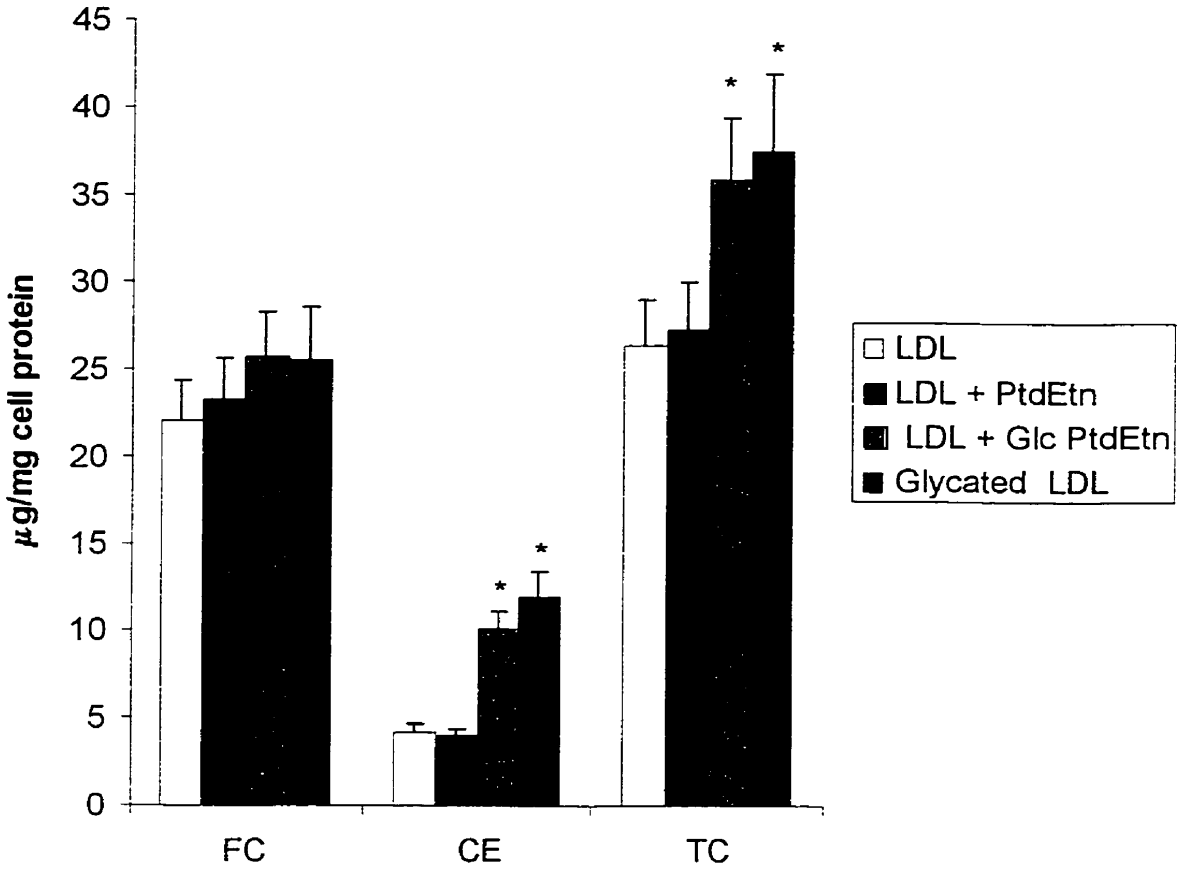


Fig. 2.6 Cellular accumulation of Free (FC), esterified (CE), and total (TC) cholesterol mass in human THP-1 macrophages incubated with 100mg/ml LDL for 24 hrs. Compared to control *P<0.01.

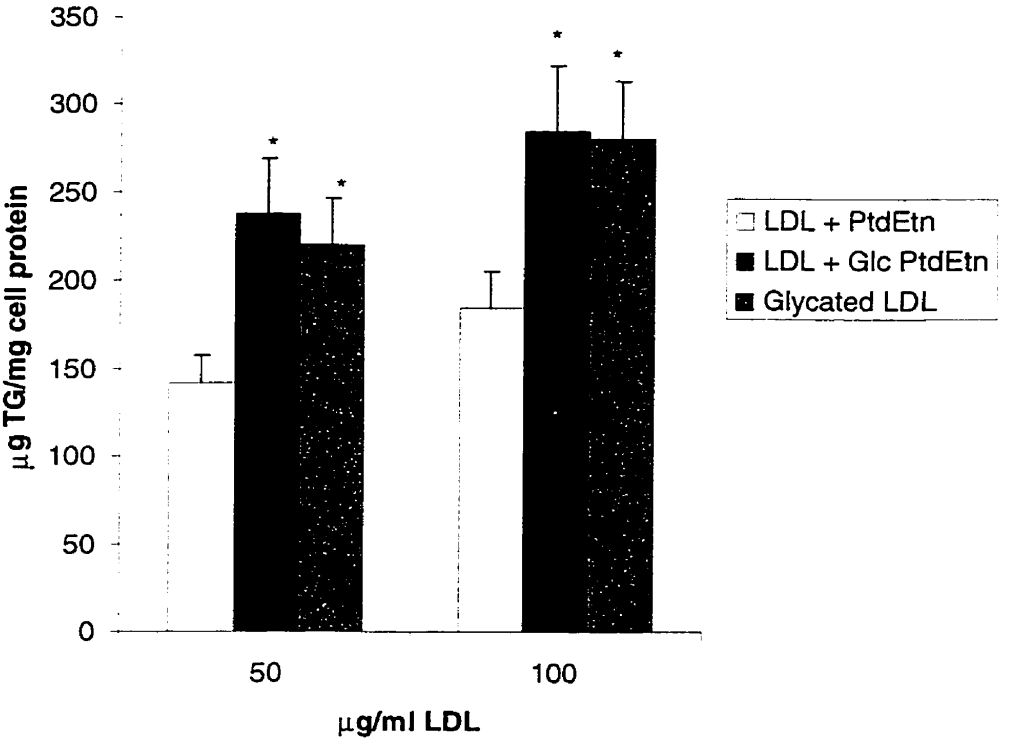
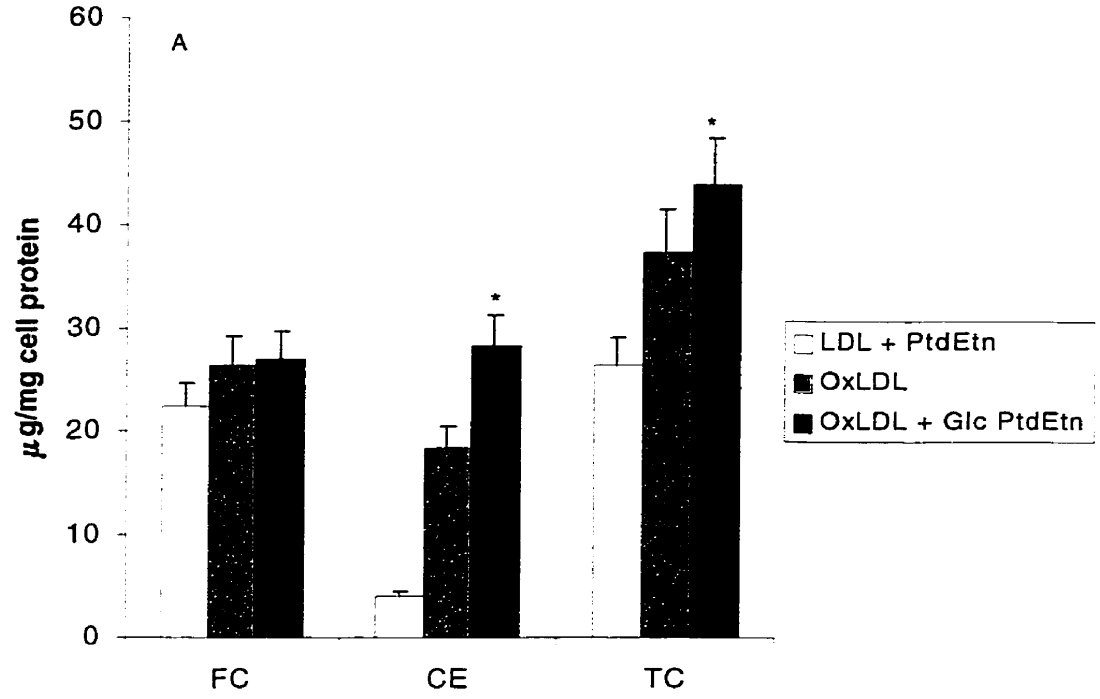
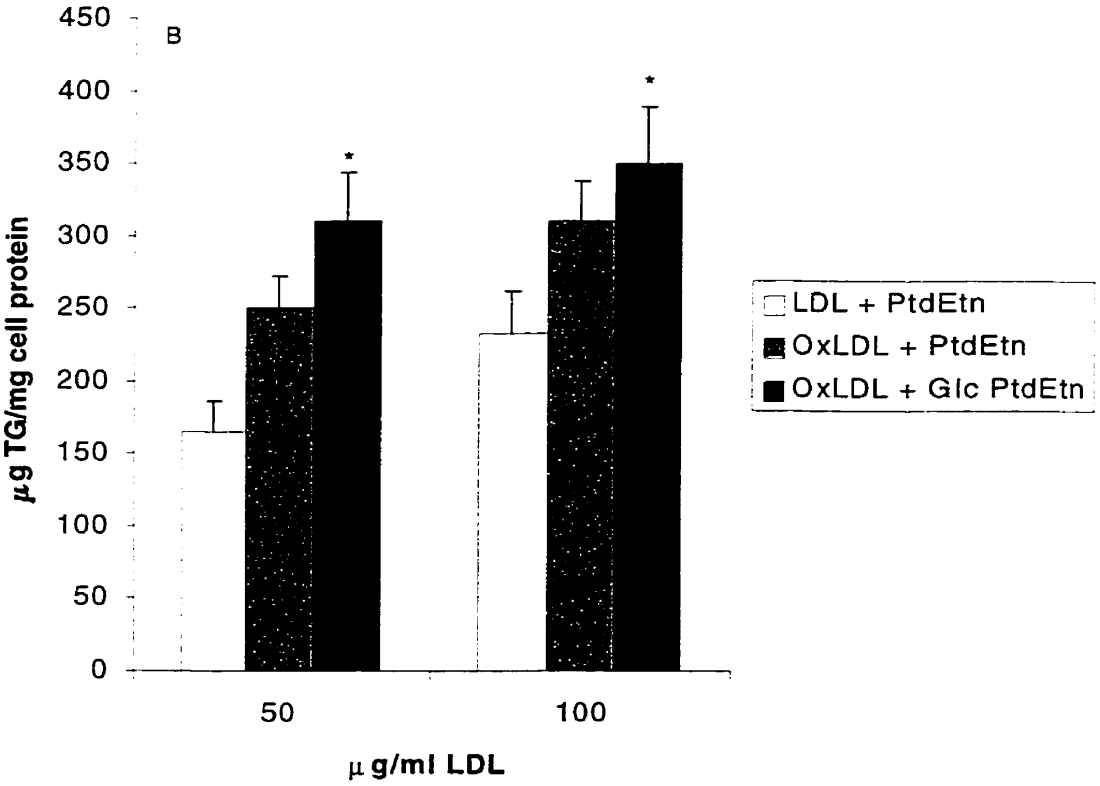


Fig. 3.6 Triacylglycerol accumulation in THP-1 macrophages incubated with LDL for 24 hrs. Compared to control *P<0.01.

Fig 4.6 Accumulation of CE (A) and TG (B) in THP-1 macrophages incubated with oxidized LDL for 24 hrs. Compared to OxLDL lacking Glc PtdEtn *P<0.01


A
60
50
40
30
20
10
0
µg/mg cell protein
FC
CE
TC
LDL + PtdEtn
OxLDL
OxLDL + Glc PtdEtn
*
*
*


B
450
400
350
300
250
200
150
100
50
0
µg TG/mg cell protein
50
100
µg/ml LDL
LDL + PtdEtn
OxLDL + PtdEtn
OxLDL + Glc PtdEtn
*
*

glycation, increase neutral lipid accumulation.

Cellular uptake of DiI labeled Glc PtdEtn LDL

Now that the foam cell formation as a result of Gluc PE has been established we set out to investigate the role of Glc PE in LDL uptake by the cell line. In order to quantitate the rate of uptake of LDL we utilized DiI labeled lipoproteins, which were incubated with cells for 4 hrs and at the end of that time the fluorescence intensity was measured as an indicator of total cell associated lipoprotein. Cells incubated with DiI Glc PtdEtn LDL (10-200 μg/ml) showed an increase in cell associated lipoprotein when compared to control and appear to reach a plateau at 200 μg/ml (Fig 5.6).

To investigate whether Glc PtdEtn LDL and control LDL were taken up at similar rates, we conducted time course studies (4,6,10 and 20 h), comparing macrophage accumulation of 50 mg/ml fluorescently labeled lipoprotein. (Fig 6.6) In all time points the average accumulation was consistently higher for LDL preparation containing Glc PtdEtn.

To explore the specificity Glc PtdEtn LDL interaction with macrophages, we investigated the ability of AcLDL, Control LDL, Glycated LDL, and LDL + Glc PtdEtn at increasing concentrations to compete with DiI labeled Glc PtdEtn (50μg/ml) for uptake by macrophages (Fig 7.6). After the 4 h incubation the AcLDL and control LDL were only able to inhibit 5-6% cell association. Glycated LDL led to 58% inhibition of Glc PtdEtn LDL uptake while the unlabeled Glc PtdEtn resulted in 66% uptake inhibition.

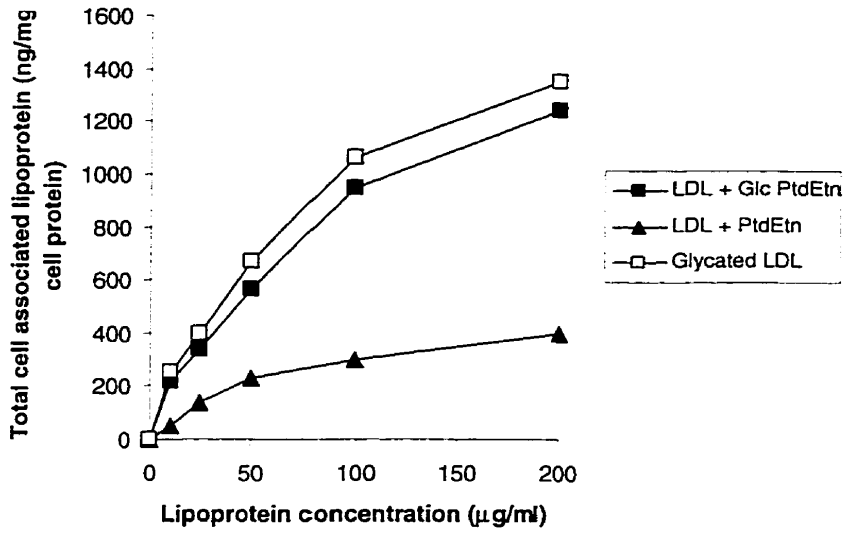


Fig. 5.6 Cellular uptake of DiI labeled LDL in presence and absence of Glc PtdEtn by THP-1 macrophages compared to glycated LDL.

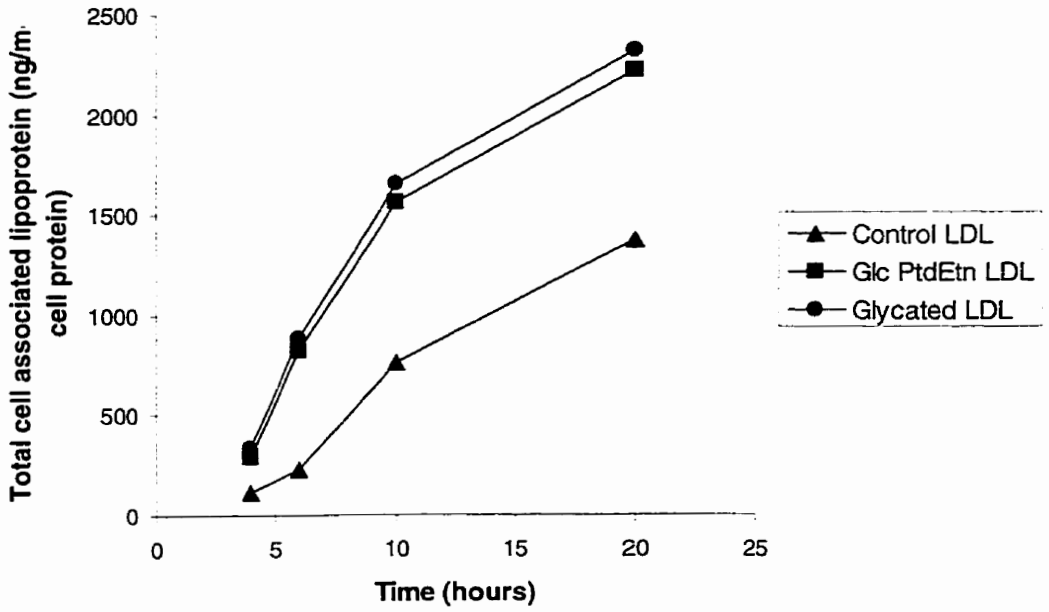


Fig. 6.6 Time course of uptake of 50 μg/ml DiI labeled Glc PtdEtn and control LDL compared to that of glycated LDL.

Fluorescent microscopy of THP-1 cells incubated with DiI labeled LDL

Fluorescent microscopy analysis of LDL uptake by THP-1 cells showed the increased fluorescence in cells incubated with Glc PtdEtn LDL as compared with the LDL containing the non-glycated LDL. Both AcLDL and Glycated LDL showed similar patterns of fluorescence a shown in Fig. 8.6.

Increase in negative charge of PtdEtn due to glycation

Due to similarity in structure of Glc PtdEtn with anionic phospholipids such as PtdIns, we utilized an LC/ES/MS method to characterize this molecule. In Fig. 9.6 the total negative ion current profile of PtdEtn and Glc PtdEtn chromatographed on a normal phase silica column is shown. The equimolar amount of each phospholipid was injected but a higher response was observed for Glc PtdEtn when compared to PtdEtn (1.5 fold). This was comparable to other anionic phospholipids in comparison to PtdIns (Table 1.6).

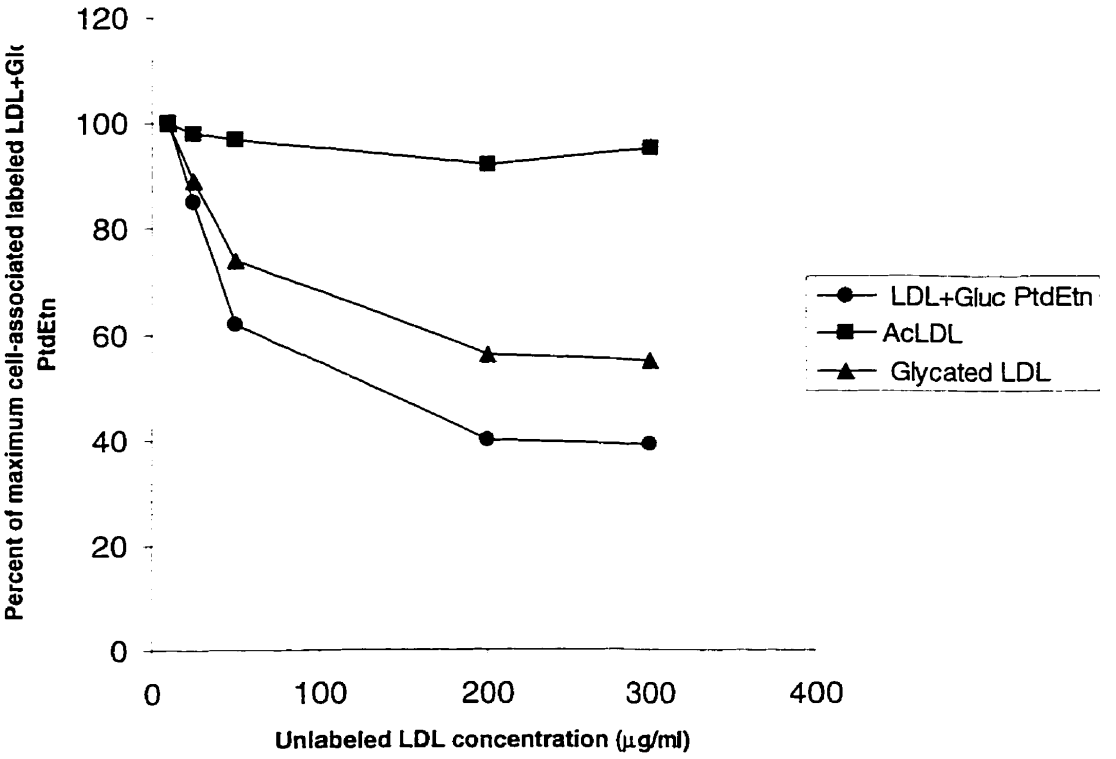


Fig.7.6 Competition of DiI Glc PtdEtn LDL with unlabeled AcLDL (■) Glycated LDL (▲) and LDL enriched with Glc PtdEtn (●)

Fig.8.6 Fluorescence microscopy of THP-1 macrophages incubated with DiI labeled LDL for 3 hrs. (A) Control LDL, (B) glycated LDL, (C) Glc PtdEtn LDL, (D) AcLDL.

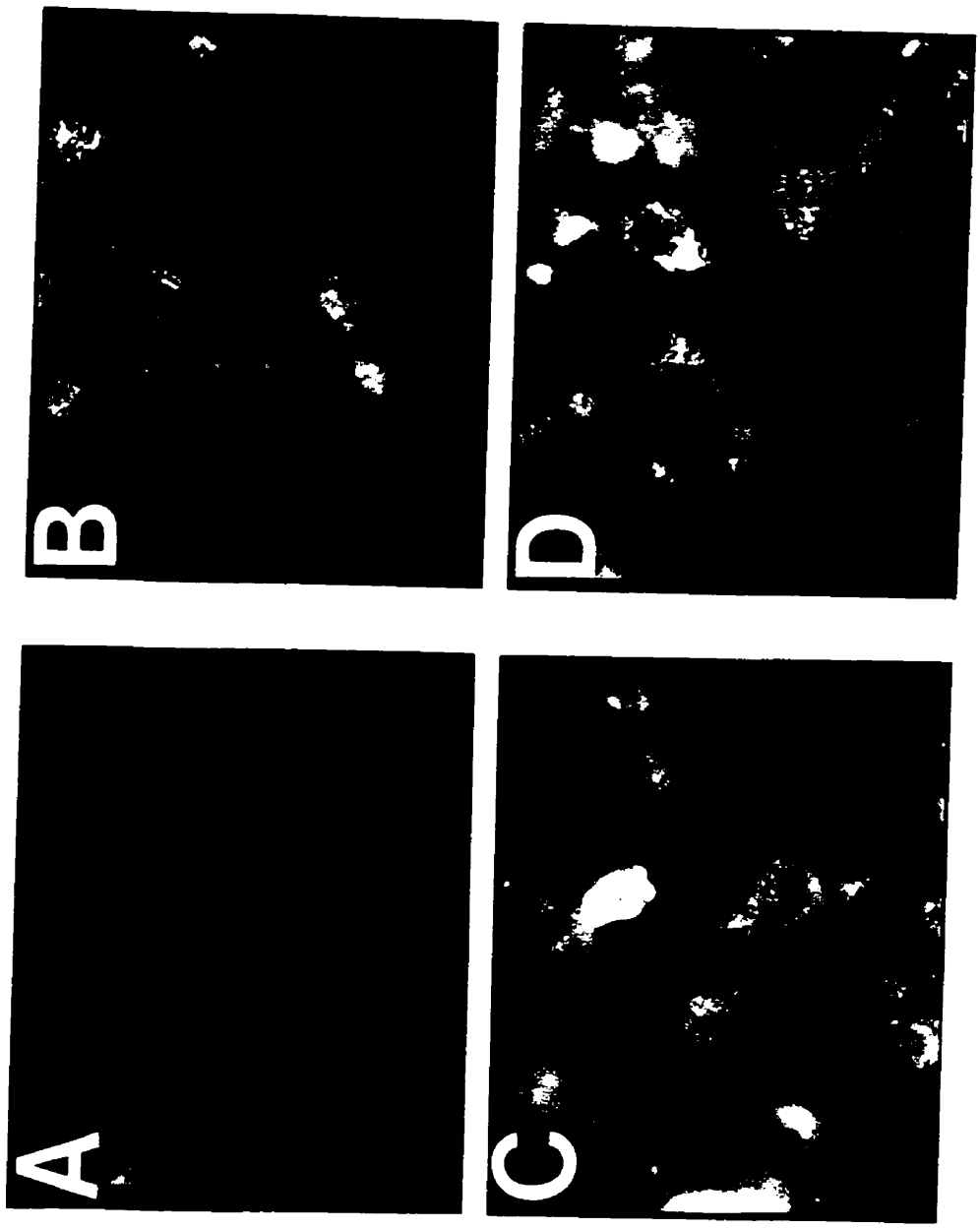

B
D
A
C

Fig. 9.6 LC/ES/MS analysis of synthetic PtdEtn and Glc PtdEtn (A) total negative ion profile for equimolar amount of glucosylated and non- glucosylated PtdEtn (B) single ion plots for 16:0-18:2 GroPEtn (m/z 714) and 16:0-18:2 Gro GlcPEtn (m/z 876), (C) Ion spectra averaged over both glucosylated and non glucosylated PtdEtn.

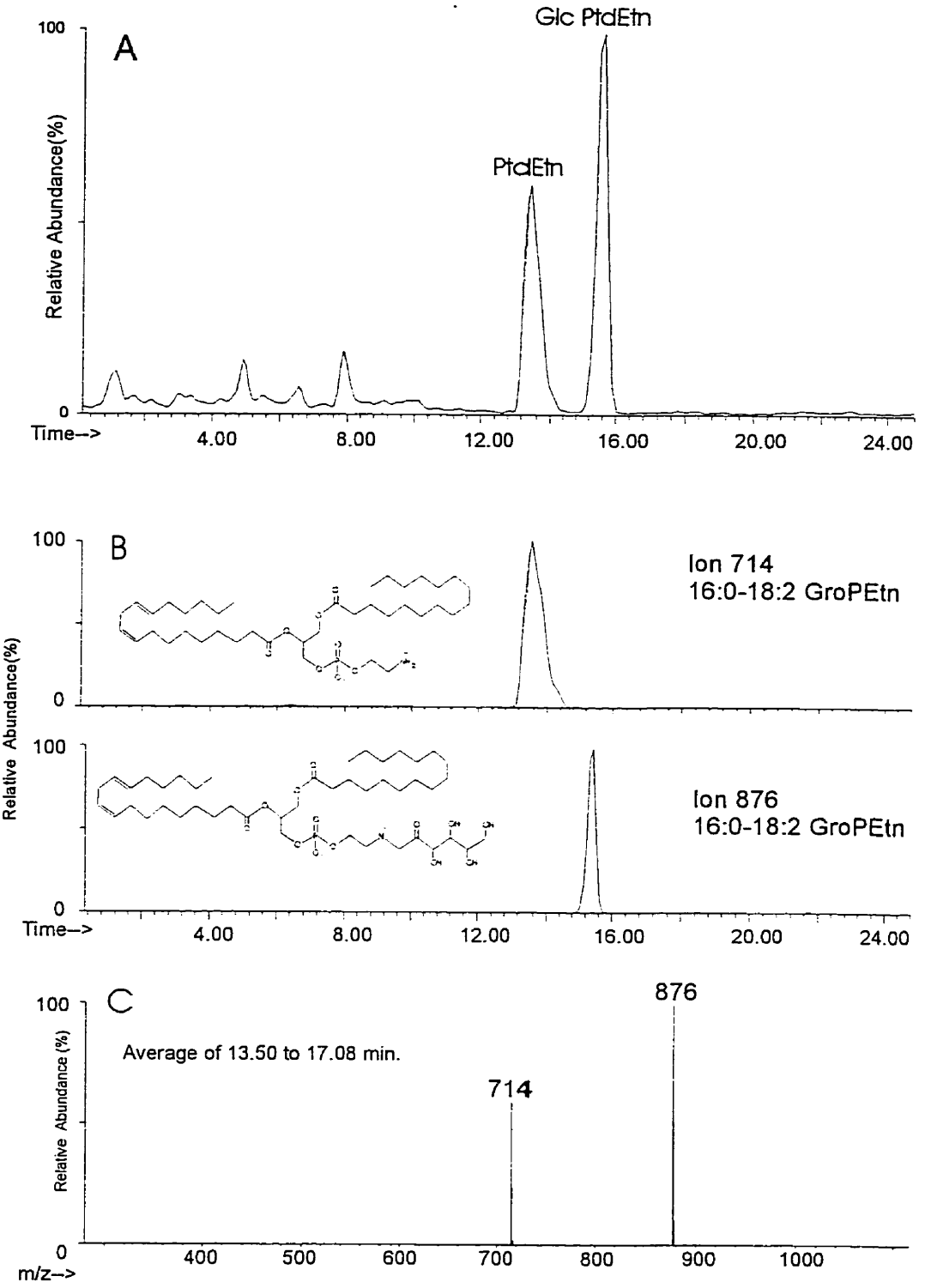

A
Glc PtdEtn
PtdEtn
100
0
Relative Abundance(%)
Time–>
4.00
8.00
12.00
16.00
20.00
24.00
B
Ion 714
16:0-18:2 GroPEtn
Ion 876
16:0-18:2 GroPEtn
Relative Abundance(%)
C
Average of 13.50 to 17.08 min.
876
714
Relative Abundance (%)
m/z–>
400
500
600
700
800
900
1000

Table 1.6

LC/ES/MS relative ionization intensities in negative ionization mode for anionic phospholipids as compared to PtdEtn.

Phospholipid	*Ionization ratio*
PtdEtn	1
Glc PtdEtn	1.53
PtdSer	1.62
PtdIns	1.57
CL	2.12

DISCUSSION

In these series of experiments we have studied the role of the glucosylated PtdEtn on cellular LDL uptake and foam cell formation. LDL glycation and its interactions with macrophages have been extensively studied. It has been shown that *in vitro* glycated LDL can stimulate CE synthesis in human monocyte-derived macrophages (25,26). Within the LDL population there is a subfraction that is glycated which is present in both normals and diabetic individuals. This subfraction which is elevated in the diabetic condition has been shown to promote CE deposition in macrophages and increased CE synthesis rate.

Nonenzymatic glycation of LDL has also been shown to increase fluidity of the phospholipid monolayer and results in an altered lipid composition (27), and possibly its increased oxidative susceptibility. Many of these altered biological and physiochemical properties have been attributed to ApoB glycation without consideration of LDL phospholipid glycation.

Recently aminophospholipid glycation products have been isolated and identified by our group. The major LDL lipid glycation product was identified as Glc PtdEtn (submitted). Using this compound we have tried to specifically elucidate the role of LDL lipid glycation on LDL interactions with macrophages. We have now shown that the presence of Glc PtdEtn in LDL, independent of any protein glycation can result in increased binding/uptake of the lipoprotein molecule. This binding was found to be competed only by glycated LDL and Glc PtdEtn LDL. Both glycated LDL and Glc PtdEtn LDL have comparable levels of uptake suggesting that lipid glycation is sufficient in increasing scavenger receptor binding. This increase in uptake also results in CE

deposition in macrophages. Foam cell formation was also partially caused by the increased TG accumulation.

Due to the increased negative charge of Glc PtdEtn compared to PtdEtn and structural similarities with PtdIns, possible receptors involved in its uptake could be the family of receptors that identify anionic phospholipids such as CD 36 (28). It has been shown that CD 36 present in photoreceptor outer segment cells that binds PtdSer and PtdIns rich liposomes and results in their uptake (29).

Macrophages have also been shown to specifically phagocytose oxidized RBC in which the outer leaflet of the phospholipid bilayer expose anionic phospholipids specifically PtdSer.

Sambrano et al (30) demonstrate that by oxidizing RBC (OxRBC) results in disruption of the asymmetry of the plasma membrane phospholipid bilayer resulting in PtdSer exposure and recognition of these OxRBC by macrophages and their subsequent phagocytosis. Inhibition of uptake of OxLDL by these OxRBC showed that the scavenger receptors on macrophages were responsible for a major part of the OxRBC recognition (31)

Recently another receptor, SRB1, has been shown to have binding capabilities to anionic phospholipids. The inhibition of the selective uptake of HDL-CE in liver paranchyma cells by modified LDL, in particular OxLDL and ionic phospholipids, suggest that in liver the SRB1 is responsible for the efficient uptake of HDL-CE (32).

The concept that negatively charged molecules can form complexes with LDL is not restricted to negatively charged phospholipids. Basu et al (16) demonstrated that a complex containing LDL and large molecular weight dextran sulfates could be avidly

metabolized by macrophages via a receptor, which appears to be distinct from the AcLDL receptor. Besides the effects of PtdSer on macrophage uptake association of other negatively charged phospholipids like cardiolipin with LDL increases its uptake and the deposition of cholesteryl esters by macrophages (33). It has also been suggested that an alteration in the composition of LDL phospholipids can influence the metabolism of LDL by macrophages such as LDL treated with phospholipase D and A_2 (34,35). A third possible receptor for Glc PtdEtn is the newly characterized AGE receptor or RAGE (36). This receptor has been demonstrated to have specificity toward protein glycation products (37). Many of the binding assays conducted for this receptor have only focused on protein glycation product and the interaction of Glc PtdEtn with RAGE is still unclear. Glycation of LDL PtdEtn has also effects on its oxidizability. In our experiments it was shown that the presence of the glycated lipid results in increased modification of the LDL molecule in presence of copper ions evident by the increase in CE and TG deposition in THP-1 macrophages. It has been shown previously that both apolipoprotein and lipid in glycated LDL and not in LDL were oxidized in the presence of naturally occurring transition metal Fe^{3+}, during *in vitro* incubation. As suggested previously Fe^{3+} could be coordinated with the endiol group in Amadori compounds and could be converted to ferryl iron with a high redox potential (38).

It has been postulated that based on the known chemistry of Schiff base and Amadori products formed during the nucleophilic addition of glucose to protein amino groups, that such early glycation products on proteins deposited in the arterial wall could themselves generate free radicals capable of oxidizing lipids (39). It was demonstrated that both Schiff base and Amadori glycation products were found to generate free

radicals in a ratio of 1:1.5 and that these radicals resulted in increased peroxidation of membrane lipids (39). In similar studies Kobayashi et al (40) demonstrated that glycated LDL when oxidized results in increase binding and degradation by cultured bovine aortic endothelial cells than normal or oxidized LDL.

The results presented in this communication demonstrate that the interaction of glucosylated phospholipids with LDL leads to the formation of a complex, which is readily phagocytosed by macrophages and glycoxidative modification of LDL.

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Chapter 7: General Discussion

Introduction

The general discussion recapitulates the main findings and assesses their significance in view of current understanding of role of glycoxidation and atherosclerosis.

The review of literature (Chapter 1) confirmed the earlier conclusions that the absence of detailed knowledge regarding lipid glycation products represents a major impediment to the advancement of knowledge in the area of LDL glycoxidation and non-enzymatic glycation in general. Lack of data regarding the structure of lipid glycation compounds has hindered detailed investigation on the role of glycated lipids in biological systems. Without the availability of glycation products in pure state it was not possible to assess the existence of these compounds *in vivo* or investigate their role in pathological processes with any certainty. Much of the basic chemistry involved in isolation, characterization and handling of non-enzymatic glycation products obtained *in vivo* or from lipoproteins such as LDL where mostly under an aqueous environment. Thus a need for basic information regarding the chemical behavior of lipid glycation products in hydrophobic medium is important. Despite much speculation, the studies linking glycation and increased oxidation, had failed to assess the specific contribution of aminophospholipid glycation products.

The work presented in this thesis describes the first identification and characterization of aminophospholipid glycation products (Chapter 2). Both PtdEtn and PtdSer were shown to be readily glycated *in vitro* and the glycation was dependent of glucose concentration. The glycation products were isolated and characterized in reduced

and non-reduced forms. Subsequently Glc PtdEtn was identified in both diabetic and normal plasma and RBC, with the diabetic levels being 10 fold higher (Chapter 3). Characteristic pro-oxidant activity of Glc PtdEtn was demonstrated in liposomes and LDL by detailed analysis of oxidized phospholipids. The isolation of Glc PtdEtn from atherosclerotic tissue supported the speculation about the role that Glc PtdEtn can play in LDL oxidation and atherogenesis (Chapter 4). The products of the most abundant phospholipid aldehydes generated during LDL oxidation were also shown to react with free amino groups both in lipids and proteins (Chapter 5). The presence of Glc PtdEtn, the major LDL glycation product, in LDL resulted in increased uptake of LDL in macrophages. In parallel to the increased uptake of LDL, there was a significant increase in CE and TG deposition in these macrophages (Chapter 6).

Evidence for aminophospholipids glycation

In the past much effort has been expended to identify and characterize the pathological role of protein glycation products (1). Knowledge regarding the identity of aminophospholipid glycation products either *in vitro* or *in vivo* has been lacking. Utilizing a LC/ES/MS system we were able to obtain for the first time direct evidence for the formation of Schiff base and/or an Amadori product between glucose and aminophospholipids (Chapter2). The key to this discovery was the mild ionization provided by the LC/ES/MS technique which allowed the identification of these molecules. The development of LC/ES/MS for phospholipid analysis allowed us to obtain detailed analysis on phospholipid modification. It also provided for complete quantitation and identification of molecular species of complex oxidized or non-oxidized phospholipid mixtures from different natural sources (2-6).

Previous methods of analysis of glycation products were too harsh and resulted in destruction of Glc PtdEtn and Glc PtdSer. Coupling the resolving power of normal phase HPLC silica column and mass spectrometric analysis allowed for the analysis of complex mixtures containing glycated aminophospholipids with minor manipulation of the sample. Previous attempts had not proven successful in the isolation of glycated PtdEtn from phospholipid-containing membranes (7). The earlier failure to isolate glycated PtdEtn may have been due to the easy dissociation of the Schiff base and Amadori compounds in the presence of mild acid, which is frequently employed in lipid extraction and TLC of phospholipids (8). However, we have confirmed the decomposition of the glycosylation products during extraction and chromatography (HPLC and TLC) with solvents containing small amounts of acetic, trifluoroacetic or phosphoric acid. In contrast, these solvent systems allowed effective extraction and chromatography of the cyanoborohydride reduction products of the glucosylated aminophospholipids (Chapter 3).

The effect of pH was investigated only over a limited range, keeping close to physiological conditions. This was done in order to approximate the non-enzymatic *in vivo* formation of Schiff bases between the aldehyde form of glucose and the aminophospholipids, and to avoid subsequent transformations. More alkaline conditions (pH > 7.4) were not used because of potential hydrolysis of the lipid ester bonds during prolonged incubation. The alkaline HPLC solvent system and the soft electrospray ionization method allowed us to demonstrate the glucosylation of both PtdEtn and PtdSer in the presence of glucose concentrations found in diabetes and hyperglycemia. Such glucose concentrations have been shown to cause membrane damage and cell death of cultured pericytes, endothelial cells, kidney cells, retinal rods, and red blood cells (9-12). Subsequent

to our study Lederer et al (13) confirmed our results which demonstrated the production of Amadori Glc PtdEtn compounds in semi organic mixtures of PtdEtn and glucose. The deduction of the structure was based on the NMR and chemical synthesis of the ethanolamine head group of PtdEtn without considering the glycerol backbone and the esterified fatty acids.

Presence of glycated aminophospholipids *in vivo*

The synthesis of reference standards of glucosylated PtdEtn allowed for the identification of Glc PtdEtn in plasma and RBC of both diabetic and normal individuals (Chapter 3). The use of LC/ES/MS allowed for the direct identification of Glc PtdEtn in blood samples. Indirect measurements of aminophospholipid glycation products *in vivo* had been carried out by Pamplona et al (14) in 1995. They had shown detectable levels 5-(hydroxymethyl)-2-furfuraldehyde (5-HMF) in the phospholipid fraction of rat liver after treatment with strong acid. The levels of 5-HMF were elevated in livers of streptozotocin treated diabetic rats when compared to controls. We have to be cautious about the conclusions of this study since lipid derivatizations were carried out under oxidative conditions and it has been shown that AGE like compounds can be generated from lipid peroxidative reactions and not from glycation reactions (15). Bucala et al (16,17) had shown *in vitro* that AGE like compounds present in phospholipids result in the formation of immunochemically detectable AGE. Using ELISA assay, these authors concluded that the bulk of the AGE in LDL isolated from normal and diabetic subjects was located in the lipid phase, without identifying any compounds (17). Our studies have shown that the major product of aminophospholipid glycation is the Amadori rearranged product (Glc

PtdEtn) representing close to 90% of all products even at longer incubation times (30 days). This shows that aminophospholipid Amadori products are more stable than the Amadori products generated from peptides. Recently a study by Lertsiri et al (18) has confirmed our studies in which Glc PtdEtn was isolated from incubations of PtdEtn with glucose. They also identified this product in incubations of human RBC and plasma with glucose. Although no Glc PtdSer was detected, due to lack of resolution of the HPLC system utilized in their study, the levels of Glc PtdEtn was correlated with glycated hemoglobin levels. Of interest was their confirmation of our *in vivo* study (Chapter 2) with their *in vitro* experiments suggesting an increased plasma Glc PtdEtn compared to RBC Glc PtdEtn. The reason for this increase was due to markedly lower PtdEtn levels in plasma than in RBC, providing a significantly higher ratio for the number of glucose molecules relative to PtdEtn amino groups in plasma than in RBC.

The presence of other forms of glycated PtdEtn has been shown to occur *in vivo*. Requena et al (19), have recently demonstrated the formation of carboxymethylEtn (CME) following the hydrolysis of the aminophospholipid adduct. *In vitro* CME was formed during glycation of both saturated and unsaturated PtdEtn under oxidative conditions. CME was also detected in incubations of PtdEtn under oxidative conditions in absence of glucose suggesting that the generation of CME is a result of oxidative reactions and less dependent on glycation. Although CME was also detected in urine and RBC from normal and diabetic individuals it only comprised a minor fraction of total phospholipids (0.01%), which cast doubt on the physiological significance of these compounds (20).

There is increasing evidence that the early glycation reaction products (Schiff base and Amadori products) play an important role in glycoxidation reactions. It should be noted that early glycation products are present *in vivo* at much higher concentrations (21). A recent report by Friedlander et al (22) suggested that early protein glycation products are present at 100 fold higher concentrations than AGEs. It has also been suggested that Fe^{3+} could be coordinated with the endiol group in Amadori compounds and could be converted to ferryl iron with a high redox potential (23).

It has been postulated, based on the known chemistry of Schiff bases and Amadori products formed during the nucleophilic addition of glucose to protein amino groups, that such early glycation products on proteins deposited in the arterial wall could themselves generate free radicals capable of oxidizing lipids (24). It was demonstrated that both Schiff base and Amadori glycation products of proteins are able to generate free radicals in a ratio of 1:1.5 and that these radicals promote increased peroxidation of membrane lipids (24).

Role of LDL lipids in macrophage scavenger recognition

Recognition of modified forms of LDL by the scavenger receptors present on macrophages has been suggested to be the primary step in foam cell formation (25). The majority of reports have assumed that oxidation of LDL lipids and their covalent bonding to Apo-B is the major step in recognition of LDL by scavenger receptors (26). Evidence suggests that the lipid moiety of LDL, without the influence of Apo-B, can account for the entire effect of OxLDL uptake by macrophages (27). Our results show (Chapter 6) for the first time that LDL PtdEtn glycation can account for the entire effect of LDL glycation in terms of macrophage uptake and neutral lipid accumulation. The

specific enrichment of LDL with PtdEtn resulted in increased LDL uptake in a manner indistinguishable from that of glycated LDL in which both protein and lipid are glycated. Others have suggested a role of oxidized and negatively charged phospholipids in scavenger receptor recognition). Terpstra et al (28) demonstrated that microemulsions prepared from lipids extracted from OxLDL are very effective in inhibiting the binding and uptake of ^{125}I-labeled OxLDL by mouse peritoneal macrophages. Since no specific oxidized lipids were identified, it is still not clear whether any specific functional group is required for this recognition. The ligand specificity may rest on some topologically specific array of negative charges or polar head groups, on the one hand , or on specific array of negative charges or polar head groups, i.e., recognition of some narrowly defined microdomain(s). The recent study by Horkko et al (29), using the mAbs that recognize oxidized phospholipids but not native phospholipids, showed that specific protein recognition of microdomaines (or clusters of them) is quite possible. The polar fatty acid degradation products, esterified to the parent phospholipids, generated as a result of oxidative reactions (ketones, acids and aldehydes) or in the case of PtdEtn glycation of the polar head group, probably re-orient themselves with the polar constituents being presented at the water-lipid interface. This could result in specific surface configurations that in terms of charge and polarity form domains recognized by scavenger receptors.

Many of the scavenger receptors identified in macrophages such as SR-AI and AII, Macrosialin, CD36, LOX-1, SREC, and SR-BI can not only bind OxLDL but also a variety of other negatively charged molecules especially PtdSer liposomes (30). These receptors have been shown to be have alternative physiological roles to that of OxLDL uptake, such as cell attachment, clearance of damaged and apoptotic cells and host

defense against infection (31). In the case of Glc PtdEtn, the possibility of a specific receptor distinct from the scavenger receptors still remains. There have been reports on the identification of AGE specific receptors on macrophages that could also be involved in PtdEtn uptake (32).

The elucidation of both chemical and physiological characteristics of aminophospholipid glycation has furthered our understanding of the role of aminophospholipid glycation in LDL modification. The glycation of ethanolamine phospholipids in LDL can lead to LDL modification in two ways. First, by increasing the oxidative susceptibility of LDL to oxidative stress and, second, by change in polarity and charge of the phospholipid monolayer. Both modes of LDL modification may synergistically promote foam cell formation and thus increase the atherogenic potential of LDL (Fig. 1.7).

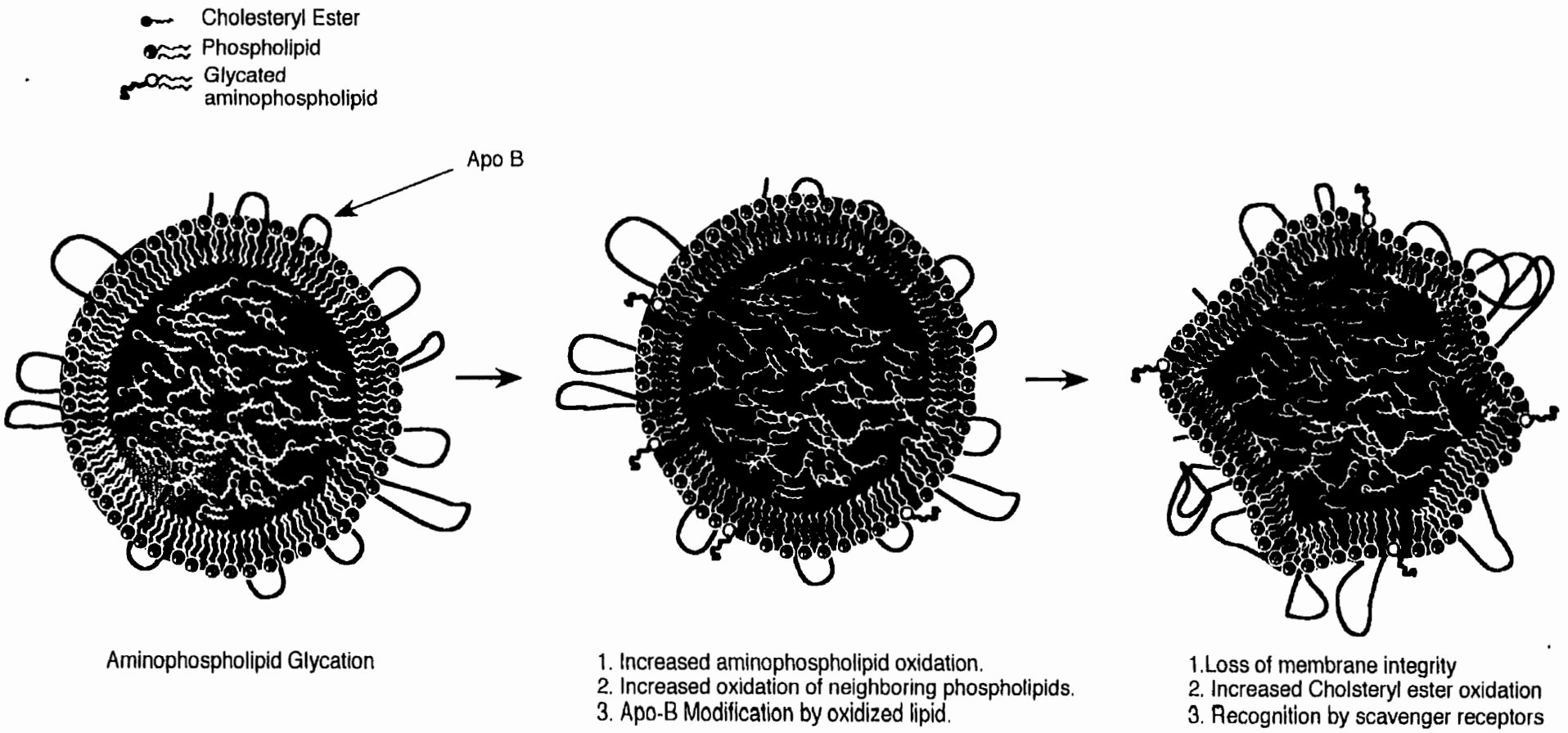

Fig. 1.7 Schematic representation of aminophospholipid glycation and LDL modification.

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Summary

This Thesis reports:

(1) The isolation and identification of the products of glucose and aminophospholipids which had not been previously accomplished. The glycation products were resolved and identified from non-glycated aminophospholipids by utilizing LC/ES/MS;

(2) The first direct evidence for the presence of aminophospholipid glycation products *in vivo*. Glycated PtdEtn was identified in plasma, RBC, and atherosclerotic tissue of diabetic and normal individuals. The levels of glycated PtdEtn in diabetics were 10 fold higher than those of normal individuals.

(3) Glc PtdEtn is the major LDL glycation product. Glc PtdEtn shows pro-oxidant properties when incorporated in liposomes or in LDL. It increases the oxidative susceptibility of LDL resulting in increased production of PtdCho hydroperoxides and aldehydes;

(4) The PtdCho aldehydes which are generated during LDL lipid peroxidation can react with amino groups of aminophospholipids, amino acids and proteins;

(5) The presence of Glc PtdEtn in LDL causes an increased uptake of LDL and neutral lipid accumulation in THP-1 macrophages. Finally, that glucosylation of PtdEtn in LDL accounts for the entire effect of LDL glycation on macrophage uptake, and therefore the increased atherogenic potential of LDL in hyperglycemia.

Future Directions

This Thesis has clarified the specific role of glycated aminophospholipids in LDL oxidation and macrophage uptake. Since oxidative processes are involved in many pathological phenomena the potential role of glycated lipids in other diseases needs to be examined. It would be of interest to see if glycated PtdEtn or PtdSer are present in tissue lesions in Alzheimer's disease, lipofuscenosis, cataracts, and nephropathy. The production of specific antibodies towards Glc PtdEtn and Glc PtdSer would appear to be potentially attractive. The presence of multiple oxo-groups in the structure will allow for a strong immunological response to these compounds. This would facilitate identification and quantification of these compounds *in vivo* and histochemical localization of the glycated aminophospholipids in specific subcellular compartments. There also remains the possibility of a distinct receptor for Glc PtdEtn and Glc PtdSer on cellular membranes. The use of antibodies will allow for investigation into possible receptors for glycated aminophospholipids on cells.

Glucose is the least reactive of the sugars towards Schiff base formation with free amino groups. Utilizing the LC/ES/MS system it would be interesting to see if the glycation products of other sugars, such as galactose, fructose, ribose, and deoxyribose, with PtdEtn and PtdSer also exist *in vivo*.

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19. Modification of amino acids, polypeptides, and aminophospholipids by Schiff base formation with core aldehydes of phosphatidylcholine and cholesteryl ester. **A. Ravandi**, A. Kuksis, N.A.Shaikh. 87th AOCS Conference (1996) Indianapolis, Indiana.

20. Isolation of glycated aminophospholipids from diabetic blood. **A. Ravandi**, A. Kuksis, Canadian Lipoprotein Conference (1995) Jasper, Alberta.

21. Preparation and characterization of glucosylated ethanolamine and serine glycerophospholipids A. Kuksis, **A. Ravandi.** 86th AOCS Conference (1995) San Antonio, Texas.

22. Increased phospholipid-bound (core) aldehydes in plasma and red cells of diabetic subjects. A. Kuksis, **A. Ravandi**, H. Kamido, L. Marai and J.J. Myher. 21st

World Congress of the International Society for Fat Research (1995) Hague, Netherlands.

23. Isolation of glycated phosphatidylethanolamine from red blood cells and plasma of diabetics. **A. Ravandi**, A. Kuksis, Clinical Research Society of Toronto (1995) Toronto.

24. Normal-phase liquid chromatography/mass spectrometry with electrospray for sensitive detection of oxygenated glycerophospholipids. J.J. Myher, A. Kuksis, **A. Ravandi**, N. Cocks (1994) INFORM 5, 478.

Scholarships and Awards resulted from the current work:

- Canadian Society for Atherosclerosis, Thrombosis and Vascular Biology Young Investigator Award, (1998).
- Ontario Graduate Scholarship. (1998-99).
- Travel Scholarship to American Heart Association vascular biology conference, Department of Laboratory Medicine & Pathobiology, 1998.
- Farber Award, Best Ph.D. oral presentation Department of Laboratory Medicine & Pathobiology, 1998.
- University of Toronto Doctoral Open Fellowship (1997-98).
- Best Ph.D. poster presentation, Department of Clinical Biochemistry, 1997.
- University of Toronto Masters Open Fellowship. (1996-97).
- American Oil Chemists' Society Eijadi Excellence in Research Award for research in the field of lipid biochemistry, 1996.
- International Honored Student Award, American Oil Chemists' Society, 1996.
- Best student presentation, Canadian Oil Chemists' Society, Guelph, 1995.
- Best abstract award, Clinical Research Society of Toronto, Toronto, 1995.
- Summer Research Studentship, Banting and Best Department of Medical Research under the supervision of Dr. A. Kuksis, 1994.
- Summer Research Studentship, Department of Biochemistry, University of Toronto under the supervision of Dr. A. Kuksis, 1993.
- Summer Research Studentship, Department of Chemistry, University of Toronto, under Supervision of Dr. S. Skolnick, 1992.

Extracurricular Activity:

- First Degree Black belt in Shotokan Karate.
- Member of the University of Toronto Karate Varsity Team since 1992.
- Gold Medallist at the Ontario Grand Prix Tournament in sparring, 1993.
- Gold Medallist at the Canadian University Championships in Montreal in Free Sparring and Kata, 1994.
- Member of the University of Toronto sparring Team winning the gold medal at the 1996 Canadian Universities Championships.
- Silver Medallist at the Karate Ontario Grand Prix in Kata, 1995.
- Instructor at the University of Toronto Karate Club, 1994-present.
- Member of the executive committee of the Banting and Best Graduate Student Union (1995-1997).
- President of the Biochemistry Undergraduate Student Union. (1993-94).