การประยุกต์ใช้เทคนิคทางเอฟทีไออาร์สเปกโทรสโคปีและแมสสเปกโทรเมทรีในการศึกษาการเปลี่ยนแปลงองค์ประกอบภายในและฟอสโฟลิพิดซับสปีชีส์ของเม็ดเลือดแดงในผู้ป่วยโรคเบาหวานชนิดที่ 2 หลังได้รับอาหารที่มีไขมันสูง

นายสุกฤต ศิริขวัญพงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์(สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

APPLICATION OF FTIR SPECTROSCOPIC AND MASS SPECTROMETRIC TECHNIQUES FOR THE INVESTIGATION OF THE ALTERATION IN ERYTHROCYTE CONTENTS AND PHOSPHOLIPIDS SUBSPECIES OF TYPE 2 DIABETIC PATIENTS AFTER ORAL HIGH FAT LOADING

Mr. Sukrit Sirikwanpong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic year 2009

Thesis Title	APPLICATION OF FTIR SPECTROSCOPIC AND MASS SPECTROMETRIC TECHNIQUES FOR THE INVESTIGATION OF THE ALTERATION IN ERYTHROCYTE CONTENTS AND PHOSPHOLIPIDS SUBSPECIES OF TYPE 2 DIABETIC PATIENTS AFTER ORAL HIGH FAT LOADING
By	Mr. Sukrit Sirikwanpong
Field of Study	Biomedical sciences
Thesis Advisor	Associate Professor Winai Dahlan, Ph.D.
Thesis Co-Advisor	Professor Thep Himathongkam, MD.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor Tewin Tencomnao, Ph.D.)

..... Thesis Advisor
(Associate Professor Winai Dahlan, Ph.D.)

..... Thesis Co-Advisor
(Professor Thep Himathongkam, MD.)

..... Examiner
(Associate Professor Polkit Sangvanich, Ph.D.)

..... External Examiner
(Professor Yvon A Carpentier, MD., Ph.D.)

..... External Examiner
(Professor Richard J Deckelbaum, MD., Ph.D.)

สุกฤต ศิริขวัญพงศ์ : การประยุกต์ใช้เทคนิคทางเอฟทีไออาร์สเปกโทรสโคปีและแมสสเปกโทรเมทรีในการศึกษาการเปลี่ยนแปลงองค์ประกอบภายในและฟอสโฟลิพิดซับสปีชีส์ของเม็ดเลือดแดงในผู้ป่วยโรคเบาหวานชนิดที่ 2 หลังได้รับอาหารที่มีไขมันสูง.(APPLICATION OF FTIR SPECTROSCOPIC AND MASS SPECTROMETRIC TECHNIQUES FOR THE INVESTIGATION OF THE ALTERATION IN ERYTHROCYTE CONTENTS AND PHOSPHOLIPIDS SUBSPECIES OF TYPE 2 DIABETIC PATIENTS AFTER ORAL HIGH FAT LOADING) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. วินัย ดะห์ลัน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ. เทพ หิมะทองคำ, 166 หน้า.

เป็นที่ทราบกันดีว่าความผิดปกติของไขมันในเลือดหลังมื้ออาหารนั้นเป็นลักษณะเด่นที่พบได้เป็นปกติอย่างหนึ่งของภาวะไขมันในเลือดผิดปกติในผู้ป่วยโรคเบาหวานชนิดที่ 2 ที่มีแนวโน้มเพิ่มมากขึ้น แม้ว่าจะมีระดับไตรเอซิลกลีเซอรอลในเลือดในภาวะอดอาหารที่ปกติก็ตาม นอกจากนี้จากการศึกษาต่างๆแสดงให้เห็นว่าการเพิ่มขึ้นของระดับไตรเอซิลกลีเซอรอลในเลือดในภาวะหลังมื้ออาหารนั้นส่งผลกระทบอันไม่พึงประสงค์ต่อเซลล์ชนิดต่างๆมากมาย เม็ดเลือดแดงเป็นเซลล์เม็ดเลือดส่วนใหญ่ในกระแสโลหิตซึ่งเป็นเซลล์ที่สัมผัสและไวต่อการเปลี่ยนแปลงต่างๆ ทางด้านชีวเคมีได้ง่าย ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ในการประยุกต์ใช้เทคนิคทางเอฟทีไออาร์สเปกโทรสโคปีและแมสสเปกโทรเมทรีเพื่อศึกษาผลของการเพิ่มขึ้นของระดับไตรเอซิลกลีเซอรอลในเลือดหลังมื้ออาหารที่มีต่อการเปลี่ยนแปลงองค์ประกอบภายในและฟอสโฟลิพิดซับสปีชีส์ของเม็ดเลือดแดงในผู้ป่วยโรคเบาหวานชนิดที่ 2 เปรียบเทียบกับกลุ่มควบคุมที่มีสุขภาพดี กลุ่มตัวอย่างประกอบด้วยผู้ป่วยโรคเบาหวานชนิดที่ 2 จำนวน 11 คนและกลุ่มควบคุมที่มีอายุและเพศเข้ากันจำนวน 10 คน ซึ่งผู้เข้าร่วมการทดลองได้รับอาหารที่มีไขมันสูงที่ประกอบด้วยไขมันปริมาณ 40 กรัมต่อตารางเมตรพื้นที่ผิวของร่างกายและมีการเก็บตัวอย่างเลือดในช่วงก่อนและหลังจากรับประทานอาหารไปแล้วสี่ชั่วโมง หลังจากมื้ออาหาร ผลจาก FTIR แสดงให้เห็นว่าพื้นที่ใต้พีคในช่วงของไขมัน โปรตีน น้ำตาลและฟอสเฟตในกลุ่มผู้ป่วยโรคเบาหวานชนิดที่ 2 มีความแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม โดยเฉพาะ Olefinic band ที่บ่งบอกถึงพันธะคู่ของไขมัน ยังคงไม่เปลี่ยนแปลงเมื่อเทียบกับภาวะอดอาหาร ผลจาก FTIR เทคนิคมีความสอดคล้องกับผลการวิเคราะห์ฟอสโฟลิพิดที่ได้จาก LC-MS ซึ่งในกลุ่มผู้ป่วยโรคเบาหวานชนิดที่ 2 พบการเปลี่ยนแปลงเล็กน้อยของฟอสโฟลิพิดสปีชีส์ในเม็ดเลือดแดงในขณะกลุ่มควบคุมมีการเปลี่ยนแปลงที่เห็นได้ชัดเจน โดยเฉพาะการเพิ่มขึ้นอย่างมีนัยสำคัญของ PC ที่มีกรดไขมันอิ่มตัวและกรดไขมันที่มีพันธะคู่หนึ่งตำแหน่งเกาะอยู่ ($p<0.05$) ผลการทดลองจากการศึกษานี้บ่งชี้ถึงความผิดปกติทางเมตาบอลิซึมที่พบในองค์ประกอบที่เป็นโครงสร้างหลักในเม็ดเลือดแดงของผู้ป่วยโรคเบาหวานชนิดที่ 2 โดยเฉพาะการเปลี่ยนแปลงเล็กน้อยของกรดไขมันในฟอสโฟลิพิดในช่วงหลังมื้ออาหาร ซึ่งเป็นกระบวนการที่สำคัญในการปรับเปลี่ยนของฟอสโฟลิพิดในเม็ดเลือดแดงปกติ ความบกพร่องของการเปลี่ยนแปลงโครงสร้างทางชีวเคมีในเม็ดเลือดแดงของผู้ป่วยโรคเบาหวานชนิดที่ 2 นั้นอาจอธิบายไปถึงความยืดหยุ่นที่ลดลงของเซลล์เม็ดเลือดในผู้ป่วยโรคเบาหวานที่พบได้เป็นประจำ ดังนั้นความรู้ที่พบจากการศึกษานี้อาจจะเป็นเป้าหมายที่เป็นไปได้สำหรับการรักษาในอนาคตที่จะช่วยแก้ไขความผิดปกติดังกล่าว อันจะเป็นการป้องกันความเสี่ยงในการเกิดภาวะแทรกซ้อนต่อหลอดเลือดขนาดเล็กในผู้ป่วยโรคเบาหวานชนิดที่ 2 อีกทางหนึ่ง

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

468 97000 20 : MAJOR BIOMEDICAL SCIENCES

KEYWORDS : FTIR spectroscopy, Mass spectrometry, erythrocyte, phospholipids subspecies, type 2 diabetes, High fat meal

SUKRIT SIRIKWANPONG: APPLICATION OF FTIR SPECTROSCOPIC AND MASS SPECTROMETRIC TECHNIQUES FOR THE INVESTIGATION OF THE ALTERATION IN ERYTHROCYTE CONTENTS AND PHOSPHOLIPIDS SUBSPECIES OF TYPE 2 DIABETIC PATIENTS AFTER ORAL HIGH FAT LOADING. THESIS ADVISOR: ASSOC. PROF. WINAI DAHLAN, Ph.D., THESIS CO-ADVISOR: PROF. THEP HIMATHONGKAM, MD., 166 pp.

It is well recognized that postprandial dyslipemia is an inherent feature of diabetic dyslipidemia highly prevalent in type 2 diabetic patients (T2DM) even with normal fasting triacylglycerol (TG) level. In addition, several studies also demonstrated that the postprandial TG increase provided adverse affect to many cell types. Since erythrocyte is the major circulating blood cell being easily exposed and susceptible to biochemical changes, thus the aim of the present study was to apply the FTIR spectroscopic and Mass Spectrometric (MS) techniques for investigating the effect of postprandial TG increase on the changes of biochemical contents and phospholipids (PL) subspecies in erythrocytes of T2DM patients compared to healthy control subjects. The study was done in 11 T2DM patients and 10 of age- and sex-matched control volunteers. All subjects received high fat meal contained 40 g fat/m^2 body surface area and blood was drawn at before and four hours after the meal. FTTR results revealed that the integrated areas of bands in lipid, protein and sugar-phosphate regions in T2DM group were significantly different from those in control group, especially the olefinic band, which indicates double bonds in lipid structure, remained unchanged relative to fasting state. These findings were in agreement with the results of PL analyzed by LC-MS. Mild alteration of erythrocyte PL molecular species were observed in T2DM group whereas control group showed marked changes particularly the significant increase in total saturated and monounsaturated fatty acid-containing phosphatidylcholine (p<0.05). The results of the present study indicated that there were metabolic abnormalities found in major structural substances of erythrocytes of T2DM patients, especially of the rare remodeling of PL fatty acids during the meal period which was an important process for PL turnover in normal erythrocytes. Impaired alterations occurred in biochemical structure of erythrocytes of T2DM probably explain the less flexibility regularly observed in blood cells of diabetic patients. In conclusion, these findings could be a potential target for future therapeutic treatment in order to correct these abnormalities that would prevent the risks of microvascular complications in T2DM patients.

Field of Study : Biomedical Sciences

Academic Year : 2009

Student's Signature .....

Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest sincere and gratitude to my advisor, Associate Professor Dr. Winai Dahlan, for his meaningful supervision, creative guidance, meticulous revision of dissertation and manuscript, and full encouragement which enable me to carry out this dissertation successfully.

My special gratitude is also extended to my co-advisor, Professor Thep Himathongkam, MD. from Theptarin Hospital, for his valuable comments, suggestions and guidances throughout this study, to Associate Professor Dr. Polkit Sangvanich from Department of Chemistry, Faculty of Science, Chulalongkorn University, for valuable guidance and knowledge regarding mass spectrometry.

I would like to thank Professor Dr. Yvon A Carpentier, MD. and Professor Dr. Richard J Deckelbaum, MD. for his valuable comments and serving as my external thesis examiners and also to Assistant Professor Dr. Sirichai Adisakwattana for his valuable comments and revision of my manuscript.

I would like to thank Professor Dr. Tony Postle, Dr. Grielof Koster and all staffs from Lipidomic research group, University of Southampton, UK for their kind supports, guidances and techniques regarding lipid mass spectrometry.

I wish to thank Mrs. Soontaree Nakasatien and all staffs from Theptarin Hospital for their assistances in recruitment of type 2 diabetic patients and to all subjects including volunteers from Chulalongkorn University for their participations in this study.

My acknowledgements are also extended to the financial supports provided from several sources: The Commission of Higher Education, Ministry of Education, The Halal Sciences Center (HSC), The Lipid and Fat Sciences Research Center (LiFARC) and Faculty of Allied Health Sciences, Chulalongkorn University.

Special thanks are also expressed to all friends in Doctor of Philosophy Program in Biomedical Sciences (Interdisciplinary Program), Faculty of Graduate School and all staffs in HSC for their sincerity, assistance and friendship.

Last of all, but the most of my mind, I would like to express my deepest appreciation to my father, mother and members of my family for their infinite love, great attention, understanding and full encouragement which never be forgotten.

CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BMI	Body Mass Index
BSA	Body Surface Area
CETP	Cholesteryl Ester Transfer Protein
CID	Collision-induced dissociation
EDTA	Ethylene diamine tetra-acetic acid
ESI	Electrospray Ionization
FTIR	Fourier Transform Infrared
FTIR-HTS-XT	Fourier Transform Infrared spectrometer coupled with High Throughput Screening
Hb	Hemoglobin
Hb-Ch	Hemoglobin-lipid adduct
HDL	High Density Lipoprotein
HL	Hepatic lipase
HOMA-IR	Homeostasis Model Assessment Insulin Resistance
HUFA	Highly unsaturated fatty acid
LAT	Lysophosphatidylcholine Acyl-CoA Transferase
LC	Liquid Chromatography
LCAT	Lecithin Cholesterol Acyltransferase
LC-ESI-MS	Liquid Chromatography coupled with Electrospray Ionization-Mass Spectrometer
LDL	Low Density Lipoprotein
LPC	Lysophosphatidylcholine
LPL	Lipoprotein Lipase
m/z	Mass to Charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry

LIST OF ABBREVIATIONS (Cont.)

MUFA	Monounsaturated fatty acid
NIDDM	Non Insulin-Dependent Diabetes Mellitus
PC	Phosphatidylcholine
PC-TP	Phosphatidylcholine-Transfer Protein
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI-TP	Phosphatidylinositol-Transfer Protein
PLA2	Phospholipase A2
PL-FA	Phospholipid-Fatty acid
PL-TP	Phospholipid-Transfer Protein
pPE	Plasmalogen Phosphatidylethanolamine
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RBC	Red Blood Cell
RCT	Reverse Cholesterol Transport
Sd-LDL	small dense-Low Density Lipoprotein
SEM	Standard Error of Mean
SFA	Saturated fatty acid
SM	Sphingomyelin
STZ	Streptozotocin
TG	Triacylglycerol
TLC	Thin Layer Chromatography
TRLs	Triacylglycerol-rich Lipoproteins
VLDL	Very Low Density Lipoprotein
ω3	Omega 3 fatty acid
ω6	Omega 6 fatty acid
WHR	Waist to Hip ratio

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Type 2 diabetes mellitus is the one leading causes of morbidity and mortality in the world. It has the biggest impact on the people in developing countries. Recent studies have demonstrated that the prevalence of diabetes for all age-groups worldwide is estimated to be 2.8% in 2000 A.D. and 4.4% in 2030 A.D. and the total number is likely to rise from 171 million in 2000 to 366 million in 2030 (Leahy, 2005). In Thailand, the prevalence of type 2 diabetes was 9.6% in 2001, an increase of 20% over a period of 5 years (Aekplakorn et al, 2003). In type 2 diabetes, insulin resistance generally develops as the first indicator and manifested by a decrease in biological response to normal level of circulating plasma insulin. The indicators of insulin resistance include impaired glucose tolerance, hyperglycemia, and elevated plasma insulin levels to compensate for the decrease of insulin response (Avramoglu et al, 2006). Insulin resistance may be caused by several general factors such as circulating insulin antagonist(e.g. counterregulatory hormones), abnormal circulating form of insulin, decreasing in the number of receptors or affinity for insulin, chronic hyperglycemia, etc (Bloomgarden, 1998). Moreover, insulin resistance has been reported to be associated with the diabetic dyslipidemia which is the single most common complication and the leading cause of mortality in diabetic patients (Yoshino et al, 1996). Individuals with diabetes have a two to fourfold greater risk of developing CVD than those without (Haffner et al, 1998). The common diabetic dyslipidemia in type 2 diabetes is hypertriacylglycerolemia. Several studies have shown that diabetes are associated with metabolic disorder of lipid or fatty acids in phospholipids (Tana et al, 1995). As phospholipids are a class of important constituents in the biological membrane and play important role in many biological processes (Milne et al, 2006), the alteration or

modification of phospholipids may result in an impairment of cell function, structure, and may affect cell metabolism (Labrouche et al, 1996).

Recently, it is well recognized that postprandial dyslipidemia is an inherent feature of diabetic dyslipidemia and highly prevalent in diabetic patients even with normal fasting triacylglycerol level. The postprandial increase in triacylglycerol has been reported to be proinflammatory, prothrombotic and can adversely affect several cell types such as endothelial cell (Tushuizen et al, 2005). Many studies of postprandial hypertriacylglycerolemic state have shown that postprandial hypertriacylglycerolemia was strongly associated with many risk factors for cardiovascular disease. (Groot et al, 1991; Ahmad et al, 2005; Tushuizen et al, 2005). Additionally, during postprandial period, the exchange of lipids between cells, especially lipoprotein particles, is an important process of lipid remodeling mechanism. The enhancing or defect of capacity in this process can affect their lipid composition which have more atherogenic potential (Tan et al, 1995).

In type 2 diabetes, the alteration of blood cell membranes, especially erythrocyte has been reported. Erythrocyte is the major blood component in the circulation and plays important role in supplying oxygen to the cells or tissues. In general, erythrocyte is unable to carry out *de novo* lipid biosynthesis but it regulates the lipid contents in the cell by uptake or exchange of lipoproteins in the plasma (Renooij and Van Golde, 1976). Since such uptake or exchange processes of erythrocyte mainly occur in postprandial period, the impairment of this process of diabetic patients in postprandial period may affect the erythrocyte membrane lipid composition that leads to the alteration of its membrane properties.

To the best of our knowledge, very little is known about the alterations or changes of erythrocyte contents and phospholipids composition in type 2 diabetes during high fat meal period. Therefore, the aim of the present study was to apply the FTIR spectroscopic and Mass Spectrometric (MS) techniques for investigation of the effect of postprandial increase in triacylglycerol on the changes of erythrocyte contents and phospholipids in type 2 diabetic patients compared to healthy control subjects. The

results from the present study would provide the better understanding of the metabolic abnormalities found in major structural substances of erythrocytes of T2DM patients, especially of the remodeling of PL fatty acids during the meal period which was an important process for PL turnover in erythrocytes.

1.2 Objective of the study

- To study the characteristics of the postprandial biochemical response in type 2 diabetes compared to healthy subjects after oral high fat meal loading
- To investigate the high fat meal-induced postprandial triacylglycerolemia on the changes of erythrocyte contents in type 2 diabetes by using FT-IR spectroscopic technique
- To investigate the postprandial changes of erythrocyte phospholipids molecular species in type 2 diabetes by using LC-ESI-MS technique

CHAPTER II
LITERATURE REVIEW

2.1 Type 2 diabetes

2.1.1 Insulin resistance and diabetes

Numerous sources including the World Health Organization (WHO) and International Diabetes Federation (IDF) have reported a worldwide diabetes epidemic, with a parallel rise in obesity and insulin resistance. It is estimated that approximately 5% of the global population is diabetic, with 85–95% of this being attributed to non-insulin dependent diabetes mellitus or type 2 diabetes (King et al, 1998).

There are many factors that involve the development of type 2 diabetes. Insulin resistance is accepted as a major cause of the diseases, as well as causing hypertension, dyslipidemia, and atherosclerotic vascular disease (Howard, 1999). A number of factors that increase the risk for insulin resistance is genetic predisposition, obesity and inactivity, aging, medications, polycystic ovary syndrome, and rare disorders such as partial lipodystrophy (Rader, 2007). Additionally, insulin resistance may be also caused by several general factors, for instance , circulating insulin antagonist (ie, counterregulatory hormones), abnormal circulating form of insulin, decreasing in the number of receptors or affinity for insulin, chronic hyperglycemia, etc (Bloomgarden, 1998). As shown in figure 1, the concomitant conditions that are associated with insulin resistance are type 2 diabetes, hypertension, dyslipidemia, atherosclerosis and polycystic ovary syndrome.

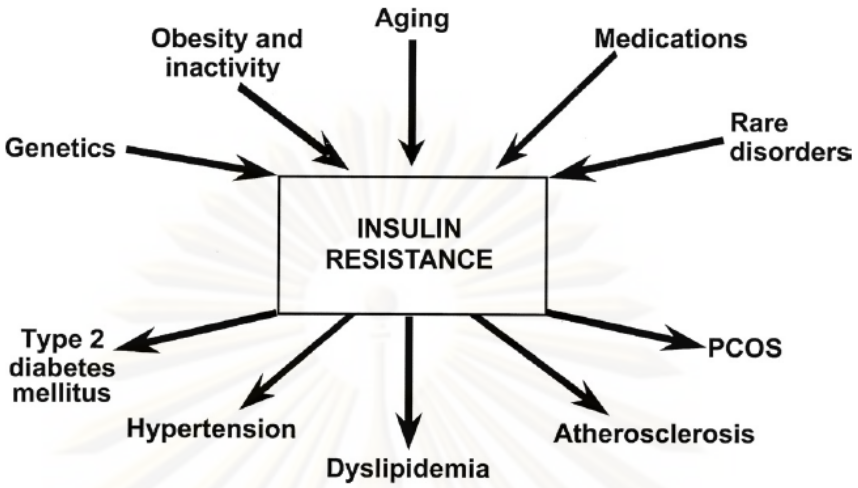


Figure 1 Causes and associated conditions of insulin resistance (Rader, 2007)

In addition, the other mechanism that is a major contributor to the development of insulin resistance is an overabundance of circulating fatty acids. Plasma albumin-bound free fatty acids are derived mainly from adipose tissue triacylglycerol stores, released through the action of the cyclic AMP-dependent enzyme hormone sensitive lipase. Fatty acids are also derived through the lipolysis of triacylglycerol-rich lipoproteins in tissues by the action of lipoprotein lipase. Insulin is important to both antilipolysis and the stimulation of lipoprotein lipase. Of note, the most sensitive pathway of insulin action is the inhibition of lipolysis in adipose tissue. Thus, when insulin resistance develops, the increased amount of lipolysis of stored triacylglycerol molecules in adipose tissue produces more fatty acids, which could further inhibit the antilipolytic effect of insulin, creating additional lipolysis. Insulin resistance can occur by the added substrate availability and by modifying downstream signaling pathway (Eckel et al, 2005). The generation of excess acyl CoAs or acyl-CoA derivatives such as ceramide can diminish Akt1 activation and interfere with insulin signaling pathway through maintaining protein kinase B (PKB)/Akt in an inactive dephosphorylated state (Teruel et al, 2001).

As mentioned, the primary biochemical abnormalities in type 2 diabetes appear to be insulin resistance, the onset of insulin resistance commonly leads to relative insulin deficiency with a slow decline in the ability to regulate blood glucose together with hyperinsulinemia and elevated circulating free fatty acid (FFA) levels (Nigro et al, 2006). The indicators of insulin resistance include impaired glucose tolerance, hyperglycemia, and elevated plasma insulin levels to compensate for the decrease of insulin response.

Regarding the relationship between insulin resistance and type 2 diabetes, several studies have clearly concluded that insulin resistance is a major risk factor for development of type 2 diabetes. Lillioja and coworkers (Lillioja et al, 1993) conducted a prospective study to determine the extent to which insulin resistance and insulin secretory dysfunction predicted the development of type 2 diabetes in a group of Pima Indians followed yearly for an average of 5.3 years. The six-year cumulative incidence of NIDDM was 39 percent in persons with values below the median for both insulin action and acute insulin response, 27 percent in those with values below the median for insulin action but above that for acute insulin response, 13 percent in those with values above the median for insulin action and below that for acute insulin response, and 0 in those with values originally above the median for both characteristics. Moreover, the relative importance of insulin resistance and abnormal insulin secretion as risk factors for the development of type 2 diabetes was also examined by Haffner and colleagues (Haffner et al, 1995), who used fasting insulin as a marker of insulin resistance and the ratio of change in insulin to change in glucose during the first 30 minutes after glucose ingestion as a marker of insulin secretion. These two markers were then used as predictors of the 7-year likelihood of 714 Mexican Americans with normal glucose tolerance developing type 2 diabetes. This finding has confirmed that the relative risk of type 2 diabetes increased with higher levels of fasting insulin and lower insulin secretion.

2.1.2 Insulin resistance and diabetic dyslipidaemia

It has been well known that insulin resistance is a major risk factor of type 2 diabetes and other conditions that include diabetic dyslipidaemia. Insulin resistance causes increased flux of free fatty acids, and thus enhanced VLDL apolipoprotein B (apo B) synthesis in the liver. Together with chylomicron and VLDL remnant competition for the common removal mechanisms, the increased substrate input results in exaggerated and prolonged postprandial lipemia. In Type 2 diabetes, dyslipidaemia is an important and common risk factor for coronary heart disease (CHD) that is the leading cause of morbidity and mortality worldwide (Isomaa et al, 2001). Recent developments have recognized the complex nature of diabetic dyslipidaemia that is a cluster of potentially atherogenic lipid and lipoprotein abnormalities. Diabetic dyslipidemia is characterized by high serum fasting triacylglycerols (TG), especially very low density lipoprotein (VLDL) and low serum fasting high density lipoprotein (HDL) cholesterol (Mero et al, 1998). In addition, the recently recognized features are small dense LDL and the proposed mechanisms behind the generation of small dense LDL and small dense HDL in type 2 diabetes are shown in Figure 2 (Taskinen, 2003). Moreover, study in normotriacylglycerolemic type 2 diabetes has found that type 2 diabetes was able to be modified and yield more atherogenic modified LDL(sd-LDL) than non-diabetic normotriacylglycerolemic donors (Sittiwicheanwong, 2006).

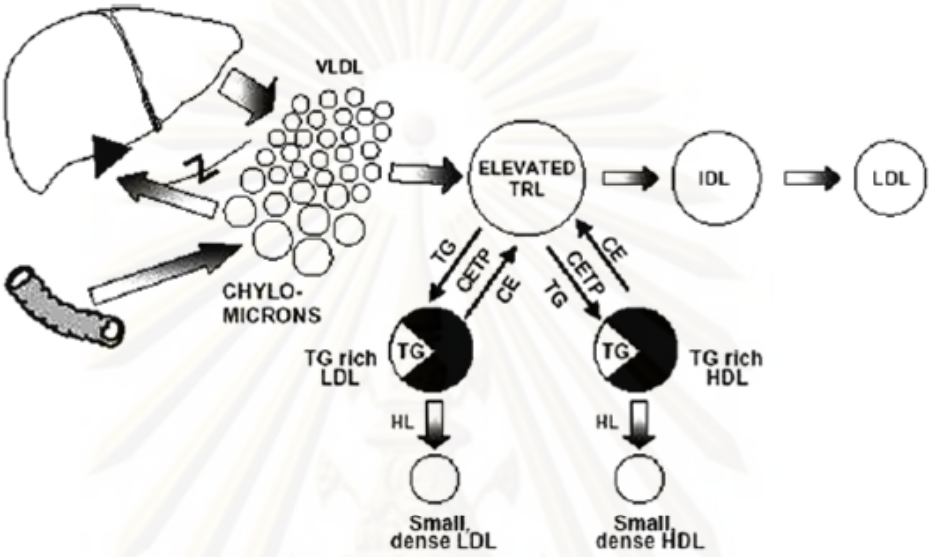


Figure 2 Proposed mechanisms behind the generation of small dense LDL and small dense HDL in type 2 diabetes (Taskinen, 2003)

The molecular mechanisms of insulin resistance and metabolic dyslipidemia are shown in figure 3. Insulin induces signal transduction via two major signaling pathways: the mitogenic, MAP-kinase pathway and the metabolic, PI 3-kinase pathway. Under normal conditions, insulin acutely reduces apoB secretion. Under conditions of insulin resistance, there is reduced sensitivity to inhibitory action of insulin on apoB resulting in enhanced VLDL secretion. Enhanced expression of PTP-1B, a key negative regulator of insulin signaling, may be a key initiating factor in inducing hepatic insulin resistance and consequently increased VLDL synthesis and secretion found in diabetes (Avramoglu et al, 2006).

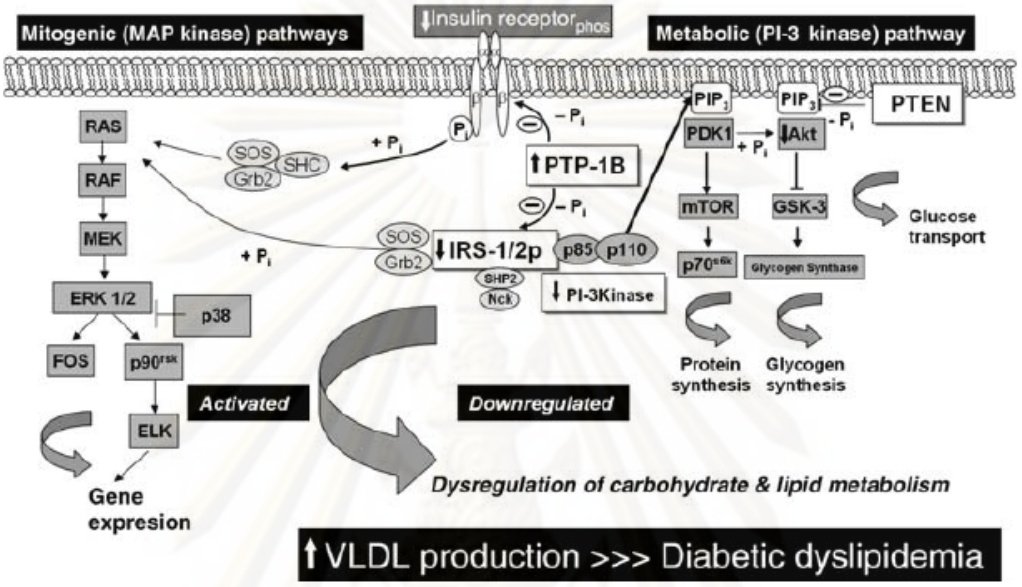


Figure 3 Postulated link between hepatic insulin signaling and downstream VLDL-apoB secretion (Avramoglu et al, 2006)

2.2 Postprandial metabolism of triacylglycerol-rich lipoproteins

2.2.1 Digestion and absorption of dietary lipid

The usual dietary intake of fat is ranging from 50 to 100 g daily. Most of it is in the form of triacylglycerols, with cholesteryl esters and phospholipids constituting only a small fraction. Ingested triacylglycerols normally contain saturated fatty acids such as palmitic and stearic acids, and unsaturated fatty acids such as oleic and linoleic acids. Absorption of dietary triacylglycerols is more than 95 % while that of cholesterol is about 40 % The pancreatic enzymes involved in lipid digestion include pancreatic lipase, cholesterol esterase and phospholipase. The hydrolysis of triacylglycerols occurs sequentially, one of the end fatty acids being removed at a time, yielding in turn sn-1 and sn-3 diacylglycerols and then sn-2 monoacylglycerol. Less than 5% of the ingested fat remains in the form of di- and triacylglycerols. Hydrolysis of sn-2 monoacylglycerols occurs only after isomerization of the fatty acid to the sn-1 position, but this is unusual, so that most sn-2 located fatty acids are absorbed in their monoacylglycerol form. Polyunsaturated fatty acids (PUFA) are usually located at the sn-2 position. Part of the dietary cholesterol is in the form of cholesteryl esters which are hydrolysed in the intestinal lumen by the pancreatic cholesterol esterase to form cholesterol and fatty acids. Phospholipids are hydrolyzed by phospholipases. Fatty acids, glycerol, monoacylglycerols, some di- and triacylglycerols, cholesterol and lysophospholipids, the end products of lipid hydrolysis, are then made available for absorption through micellar solubilization brought about by bile acids. Within the enterocytes, the subsequent fate of the fatty acids depends on their length. Fatty acids containing less than 1-12 carbon atoms (small and medium chain fatty acids) pass from the mucosal cells directly without reesterification and bound to albumin, they are transported as non-esterified fatty acids to the liver and other tissues. Readily soluble free glycerol from lipid hydrolysis is also transported by portal blood. Fatty acids with

more than 12 carbon atoms and the absorbed cholesterol are re-esterified to triacylglycerols and cholesteryl esters, respectively within the enterocyte.

2.2.2 Chylomicron formation and metabolism during postprandial state

Chylomicrons contain a core of triacylglycerols and cholesteryl esters covered by a surface layer of phospholipids, cholesterol and apoproteins. Within the intestinal epithelial cells, absorbed free fatty acids, monoacylglycerols and cholesterol are re-esterified and packed within a monolayer of phospholipids and apoproteins to form the chylomicrons. After crossing the enterocyte membrane, free fatty acids are bound to a specific fatty acid binding protein with a high affinity for long chain unsaturated fatty acids.

In the postprandial phase, chylomicrons are poured into the intestinal lymph, pass through the thoracic duct and enter the general circulation through the subclavian vein to cause postprandial lipemia. Chylomicrons, which are the largest lipoprotein compared to the others, have diameters ranging from 80 to 1,000 nm and density < 0.95 g/ml (Table1). Chylomicrons begin to appear in the plasma within 60 min of ingestion of fat with the major part of the triacylglycerol component usually removed from circulation within 6 - 8 hours after meal ingestion. After secretion from the enterocyte and following their entry into the systemic circulation, chylomicrons acquire apo CII, CIII and apo E. These surface components are transferred to chylomicrons from HDL along with free and esterified cholesterol and phospholipids. Some apo A1 and A-IV is also transferred from chylomicrons to HDL at this stage. Apo CII and CIII are transferred rapidly, followed by apo E. The C apoproteins are synthesized in the liver and to a lesser extent in the gut. Apo CII and CIII are intimately involved in the postprandial processing of both chylomicrons and very low density lipoproteins (VLDL) but unknown role has yet been assigned to apo CI in lipoprotein metabolism. Apo E is mostly synthesized in the liver but also in extrahepatic tissues, including the intestine, brain, muscle, ovaries, kidney, spleen and adrenals. Its major role seems to be the targeting of the chylomicron

remnant (the product of chylomicron triacylglycerol hydrolysis) for the receptor, serving as a ligand for remnant removal by the liver. The first step in the catabolism of chylomicrons involves the action of lipoprotein lipase located in extrahepatic tissues, resulting in the formation of triacylglycerol depleted particles termed ' remnants '. The subsequent step in chylomicron catabolism involves a hepatic receptor mediated removal of chylomicron remnants (Sethi et al, 1993).

Table 1 Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins*

	Chylomicrons	VLDL	LDL	HDL
Protein (%)	2	7	20	50
Triacylglycerol (%)	83	50	10	8
Cholesterol (%)	8	22	48	20
Phospholipids (%)	7	20	22	22
Particle mass (Daltons)	0.4-30 $x10^6$	10-100 $x10^6$	2-3.5$x10^6$	1.75-3.6$x10^5$
Density range (g/ml)	<0.95	0.95-1.006	1.019-1.063	1.063-1.210
Diameter (nm)	80-1000	30-90	18-22	5-12
Apolipoproteins	AI,AII,AIV,B-48 CI,CII, CIII, E	B-100, CI, CII, CIII, E	B-100	AI,AII,AIV,CI,CII, CIII, D, E

* from Gurr et al, 2002

2.2.3 Chylomicron hydrolysis and lipoprotein lipase

Once the chylomicron has acquired apo CII, it is capable of activating lipoprotein lipase, the enzyme responsible for the hydrolysis of triacylglycerols in chylomicron and VLDL particles. Apo CII acts as a co-lipase increasing the interaction of the enzyme with the substrate at the interface of the lipoprotein. Not all lipoprotein lipase found in tissues participates in triacylglycerol catalysis. Only that fraction of enzyme protein, the so-called functional lipoprotein lipase which is localized on endothelial surfaces of blood vessels, has contact with circulating triacylglycerol-rich lipoproteins. Because this enzyme is the rate limiting step in triacylglycerol hydrolysis, its activity is an important determinant of the extent and duration of postprandial lipaemia. Since hydrolysis by lipoprotein lipase is the initial event in the uptake of triacylglycerol fatty acid by tissues, the enzyme also controls the availability of an important cellular fuel. Lipoprotein lipase is located on the vascular endothelium of tissues with a high requirement for fatty acids, such as skeletal and cardiac muscles, adipose tissue and mammary gland. Insulin has a profound effect on lipoprotein lipase activity in adipose tissue, promoting both synthesis and release of the enzyme (Spooner et al, 1979)

Hydrolysis of triacylglycerols in chylomicrons results in smaller, relatively protein enriched particles with a redundant surface coating of free cholesterol and phospholipids. As the core triacylglycerol component becomes progressively smaller, surface materials (phospholipids, cholesterol and apoproteins CII and CIII) are transferred to HDL to maintain the stability of the chylomicron particle. The transfer of apo CII, together with increasing inaccessibility of core triacylglycerols for the lipoprotein lipase active site, results in cessation of further triacylglycerol removal. The apo B-48 present from the time of synthesis in the enterocyte remains tightly bound within the core of the chylomicron particle. Apo E also remains within the chylomicron and regions of its structure are exposed allowing recognition of the particle by two hepatic lipoprotein receptors. These are the remnant receptor, also termed the apo E receptor, and the low density lipoprotein (LDL) or apo B-100/E receptor.

2.2.4 Chylomircron clearance

Extraction of chylomicron remnants from the circulation occurs in the liver and is accomplished mainly by high affinity receptor mediated processes. There is evidence for a specific hepatic receptor which binds remnants that contain apo E (chylomicrons and VLDL), which is separate and distinct from the LDL or B-100/E receptor. Factors which appear to be important in regulating receptor mediated remnant removal include: the apo E phenotype, the activity of the hepatic triacylglycerol lipase, and the presence of apo CIII. Three isoforms of apo E are commonly found in human populations and are referred to as E2, E3 and E4. The E3/E3 phenotype is the most commonly found (60%) with E2/E3 having a prevalence of approximately 23 %. The avidity of binding of apo E isomers to receptors is in the order E4 > E3 > E2. Subjects homozygous for E2 have elevated levels of chylomicron remnants (Type III hyperlipidemia) that have an increase of risk of coronary artery disease.

There is increasing interest in the role of hepatic triacylglycerol lipase in mediating removal of chylomicron and VLDL remnants and in the hormonal and dietary factors which may determine the activity of this enzyme. Griglio and colleagues have elucidated the role of hepatic triacylglycerol lipase in chylomicron remnant removal and suggested that phospholipolysis of chylomicron remnants may be an obligatory metabolic process prior to uptake by the remnant receptor (Griglio et al, 1992).

It is therefore of interest that apo CIII has been shown to inhibit hepatic triacylglycerol lipase and also to impair remnant recognition by the hepatocyte remnant receptor (Van Berkel et al, 1983). Other suggestions are that Apo CIII appears either to displace apo E or to interact with and obscure the apo E ligand. For these reasons, it is proposed that the binding of the chylomicron remnant to hepatic receptors requires prior dissociation of apo CIII. Since removal of apo CIII occurs onto HDL, this provides a further example of the close interaction between the pathways of metabolism of HDL and triacylglycerol-rich lipoproteins and one which may be of significance to the clearance of chylomicron remnants.

2.3 Abnormal postprandial lipoprotein metabolism in diabetes

In the insulin-resistant condition and type 2 diabetes, the production of very low density lipoprotein (VLDL) by the liver is inappropriately high. Together with a reduced lipoprotein lipase activity, this results in high triacylglycerol concentrations, especially in the postprandial period (Ginsberg and Illingworth, 2001). The large amount of TRLs and their prolonged residence time in the circulation may lead to increased exchange of the core lipid cholesteryl ester for triacylglycerols between TRL and LDL and HDL particles mediated by cholesteryl ester transfer protein. This process enriches LDL and HDL with triacylglycerol, and these particles are subsequently more readily hydrolyzed by hepatic lipase resulting in smaller, denser LDL particles and lower concentrations of HDL. Moreover, the small dense LDL generated during postprandial state has also reported not only in diabetes but also in hypertriglyceridemic and healthy subjects (Noto et al, 2006). These abnormalities may explain the characteristic diabetic dyslipidaemia, which is now recognized to be very atherogenic and may result in cardiovascular complications.

The mechanisms underlying fat intolerance in type 2 diabetic subjects were not fully clear. It may be from a combined effect of both increased VLDL1 production in the liver and competition of chylomicron remnants and VLDL remnants for the common removal mechanisms. Malmstrom and colleagues have recently demonstrated that suppression of hepatic VLDL1 apoB production by insulin is impaired in type 2 diabetes, resulting in inappropriate release of VLDL1 particles (Malmstrom et al, 1997).

In type 2 diabetes, LPL activity was decreased and, hence, this pathway may be more easily contributing to the delay of TRL clearance. As further evidence for the lipolytic defect, diabetic patients lack the normal inverse correlation between postprandial lipemia and postheparin LPL activity (Syvanne et al, 1993). Other potential factors impairing TRLs metabolism in diabetic subjects include glycation of apolipoproteins, especially of apoE (Mamo et al, 1990), and accelerated rate of cholesteryl ester transfer (Bagdade et al, 1993).

Recently, postprandial hypertriacylglycerolemia is known to be an inherent feature of diabetic dyslipidemia and highly prevalent in diabetic patients even with normal fasting triglyceride level. In general practice, serum lipid concentrations including triacylglycerol are measured in the morning after an overnight fast. However, the fasting value should be considered the nadir of the 24 hours triacylglycerol profile and could therefore be misleadingly low (Tushuizen et al, 2005). Many studies have suggested that high postprandial TRLs may be related to coronary heart and/or carotid artery disease in nondiabetic and diabetic subjects (Groot et al, 1991; Stampfer et al, 1996; Teno et al, 2000). Additonally, recent study has shown that non-fasting triacylglycerol levels were associated with incident cardiovascular events, independent of traditional cardiac risk factors, levels of other lipids, and markers of insulin resistance (Bansal et al, 2007).

Moreover, in the postprandial period of type 2 diabetes, the plasma lipid, especially triacylglycerol, is above optimal concentrations for several hours after meals as shown in figure 4 (van Wijk et al, 2003; Iovine et al, 2004). These postprandial metabolic derangements increase the production of reactive oxygen species causing oxidative stress (Saxenaa et al, 2005) and functional abnormalities of the vascular endothelium at several levels, for instance, the elevation of endothelial cell microparticles (Ferreira et al, 2004), carotid intima–media thickness (IMT) (Ahmad et al, 2005), Impairment of Endothelial Function (Bae et al, 2001; Gaenzer et al, 2001; Bae et al, 2003; Giannattasio et al, 2005; Westphal et al, 2006) and also including of impairment of vasoreactivity, increased coagulation, endothelial and inflammation activation (Nappo et al, 2002; Esposito et al, 2003; Esposito et al, 2007).

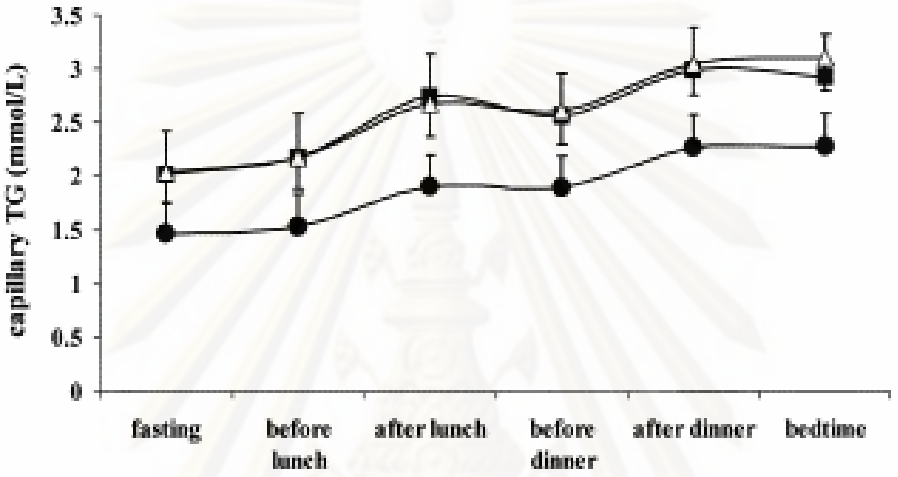


Figure 4 Mean daylong capillary triacylglycerol profiles in diabetic subjects; (■), obese nondiabetic subjects (△), and lean subjects (●) (Iovine et al, 2004)

2.4 Phospholipids metabolism

2.4.1 Chemistry of phospholipids

Phospholipids structure

Phospholipids are a class of important constituents in the biomembranes (Figure 5). Both the physical and chemical properties of the membrane bilayer can be affected by the variation of phospholipids compositions. Typical structure of phospholipids contains the glycerol hydroxyl in the sn-3 position that is linked to a polar phosphate-containing group and the other two hydroxyls are linked to acyl chains. There is usually a saturated acyl chain esterified at the sn-1-position of the glycerol backbone, whereas the acyl-chain at the sn-2-position usually is unsaturated. In eukaryotic membranes, phosphatidylethanolamine and phosphatidylserine are more unsaturated than other phospholipids (Rekila et al, 2002).

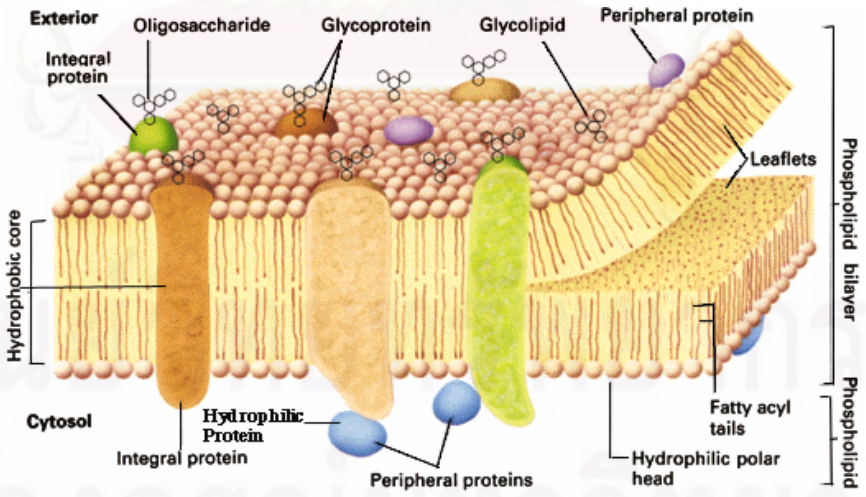


Figure 5 Phospholipids are a class of important constituents in the biomembranes (http://www.tutorvista.com/content/biology/biology-iii/cell-organization/plasma-membrane.php , accessed 23 October 2009)

As shown in figure 6, the phospholipids are classified according to the specific hydrophilic head groups that include choline, ethanolamine, serine and inositol that yield phosphatidylcholine(PC), phosphatidylethanolamine(PE), phosphatidylserine(PS) and phosphatidylinositol (PI), respectively (Serhan, 2005).

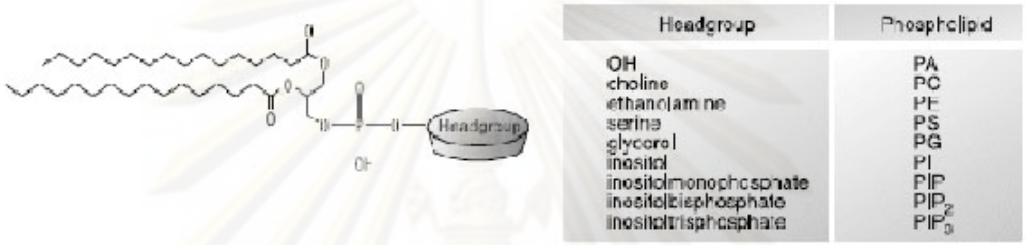


Figure 6 Phospholipid structure (Serhan, 2005 with modification)

Membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl and headgroup compositions. In addition to their structure , some phospholipids play important role in membrane fluidity, permeability, transport and signal transduction (Exton, 1994). Phospholipids serve as a reservoir for arachidonic acid (20:4 n-6) and other polyunsaturated fatty acids that can be metabolized to biologically active eicosanoids such as prostaglandins, thromboxanes, leukotrienes and lipoxins (Marcus and Hajjar, 1993).

2.4.2 Phospholipids biosynthesis (King, 2009)

Phospholipids can be synthesized by two mechanisms. One utilizes a CDP-activated polar head group for attachment to the phosphate of phosphatidic acid. The other utilizes CDP-activated 1,2-diacylglycerol and an inactivated polar head group. PE, PC and PS synthesis pathway were shown in figure 7.

A. Phosphatidylethanolamine (PE)

Phosphatidylethanolamine (once given the trivial name 'cephalin') is usually the second most abundant phospholipid in animal and plant lipids and it is frequently the main lipid component of microbial membranes. As such, it is obviously a key building block of membrane bilayers. It is a neutral or zwitterionic phospholipid at physiological pH. They contain primarily palmitic or stearic acid on carbon 1 and a long chain unsaturated fatty acid (e.g. 18:2, 20:4 and 22:6) on carbon 2. Synthesis of PE can occur by two pathways. The first requires that ethanolamine be activated by phosphorylation and then by coupling to CDP. The ethanolamine is then transferred from CDP-ethanolamine to diacylglycerol to yield PE. The second involves the decarboxylation of PS.

B. Phosphatidylserine (PS)

Phosphatidylserines will carry a net charge of -1 at physiological pH and are composed of fatty acids similar to the phosphatidylethanolamines. The pathway for PS synthesis involves an exchange reaction of serine for ethanolamine in PE. These exchanges occur when PE is in the lipid bilayer of the membrane. As indicated in figure 7, PS can also serve as a source of PE through a decarboxylation reaction.

C. Phosphatidylcholine (PC)

Phosphatidylcholine (once given the trivial name 'lecithin') is usually the most abundant phospholipid in animal and plants, often amounting to almost 50% of the total phospholipids and it is obviously the key building block of membrane bilayers. In particular, it makes up a very high proportion of the outer leaflet of the plasma membrane. Phosphatidylcholine is also the principal phospholipid circulating in plasma, where it is an integral component of the lipoproteins, especially the HDL. On the other hand, it is less often found in bacterial membranes, perhaps approximately 10%. It is a neutral or zwitterionic phospholipid over a pH range from strongly acid to strongly alkaline. In animal tissues, some of its membrane functions appear to be shared with the structurally related sphingolipid – sphingomyelin. They contain primarily palmitic or stearic acid at carbon 1 and primarily oleic, linoleic or linolenic acid at carbon 2. The lecithin dipalmitoyllecithin is a component of lung or pulmonary surfactant. It contains palmitate at both carbon 1 and 2 of glycerol and is the major (80%) phospholipid found in the extracellular lipid layer lining the pulmonary alveoli.

Choline is activated first by phosphorylation and then by coupling to CDP prior to attachment to diacylglycerol as shown in figure 7. Moreover, the other pathway to PC synthesis, involves the conversion of either PS or PE to PC. The conversion of PS to PC first requires decarboxylation of PS to yield PE; this then undergoes a series of three methylation reactions utilizing S-adenosylmethionine (SAM) as methyl group donor.

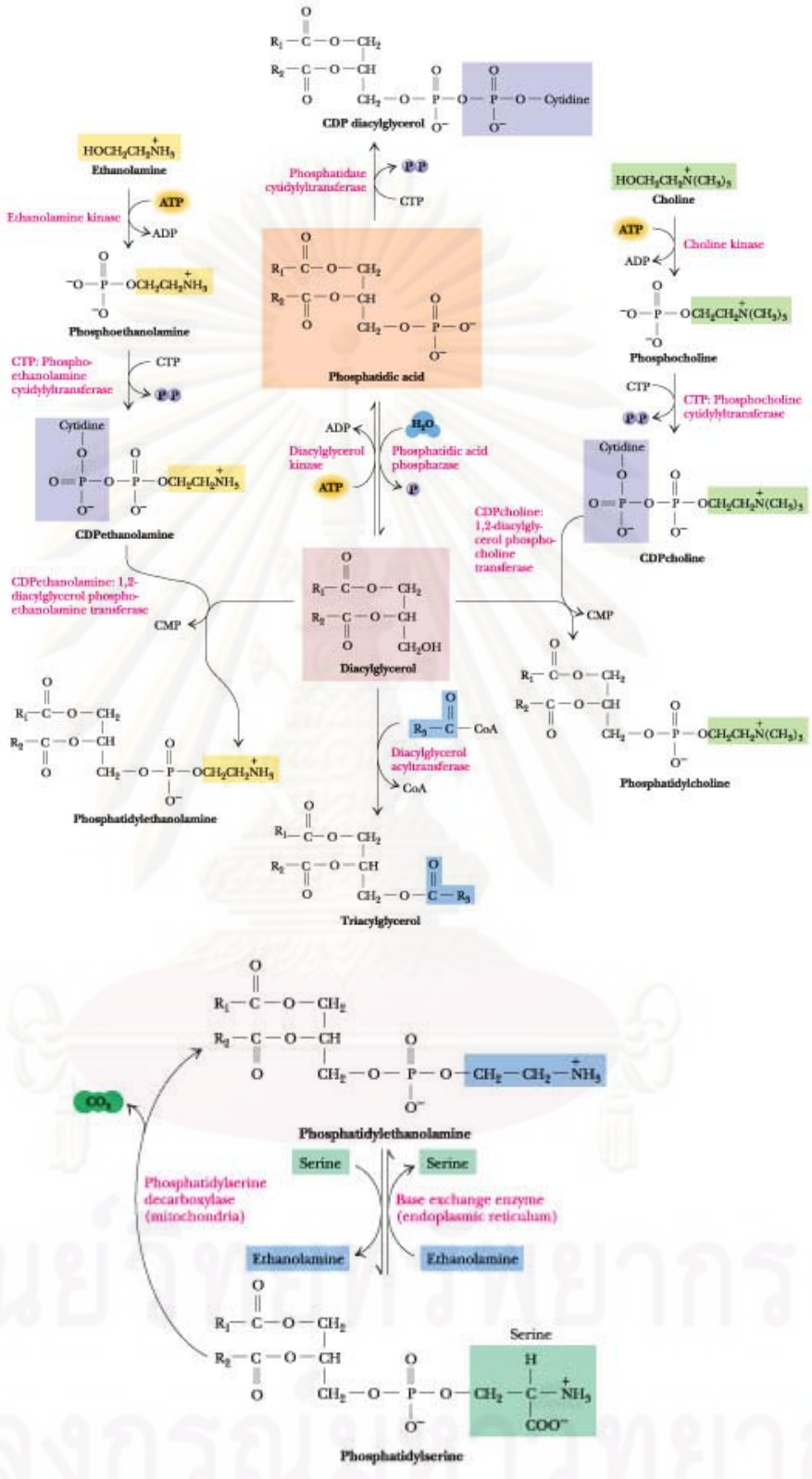


Figure 7 PE, PC and PS biosynthesis

(http://web.virginia.edu/Heidi/chapter25/chp25.htm, accessed 23 October 2009)

E. Phosphatidylinositol (PI)

Phosphatidylinositol is an important lipid, both as a key membrane constituent and as a participant in essential metabolic processes in all plants and animals, both directly and via a number of metabolites. It is an acidic (anionic) phospholipid that consists of a phosphatidic acid backbone, linked via the phosphate group to inositol (hexahydroxycyclohexane).

PI molecules contain almost exclusively stearic acid at carbon 1 and arachidonic acid at carbon 2. Phosphatidylinositols composed exclusively of non-phosphorylated inositol exhibit a net charge of -1 at physiological pH. These molecules exist in membranes with various levels of phosphate esterified to the hydroxyls of the inositol. Molecules with phosphorylated inositol are termed polyphosphoinositides. The polyphosphoinositides are important intracellular transducers of signals emanating from the plasma membrane.

The synthesis of PI, as shown in figure 8, involves CDP-activated 1,2-diacylglycerol condensation with myo-inositol. PI subsequently undergoes a series of phosphorylations of the hydroxyls of inositol leading to the production of polyphosphoinositides. One polyphosphoinositide (phosphatidylinositol 4,5-bisphosphate, PIP2) is a critically important membrane phospholipid involved in the transmission of signals for cell growth and differentiation from outside the cell to inside.

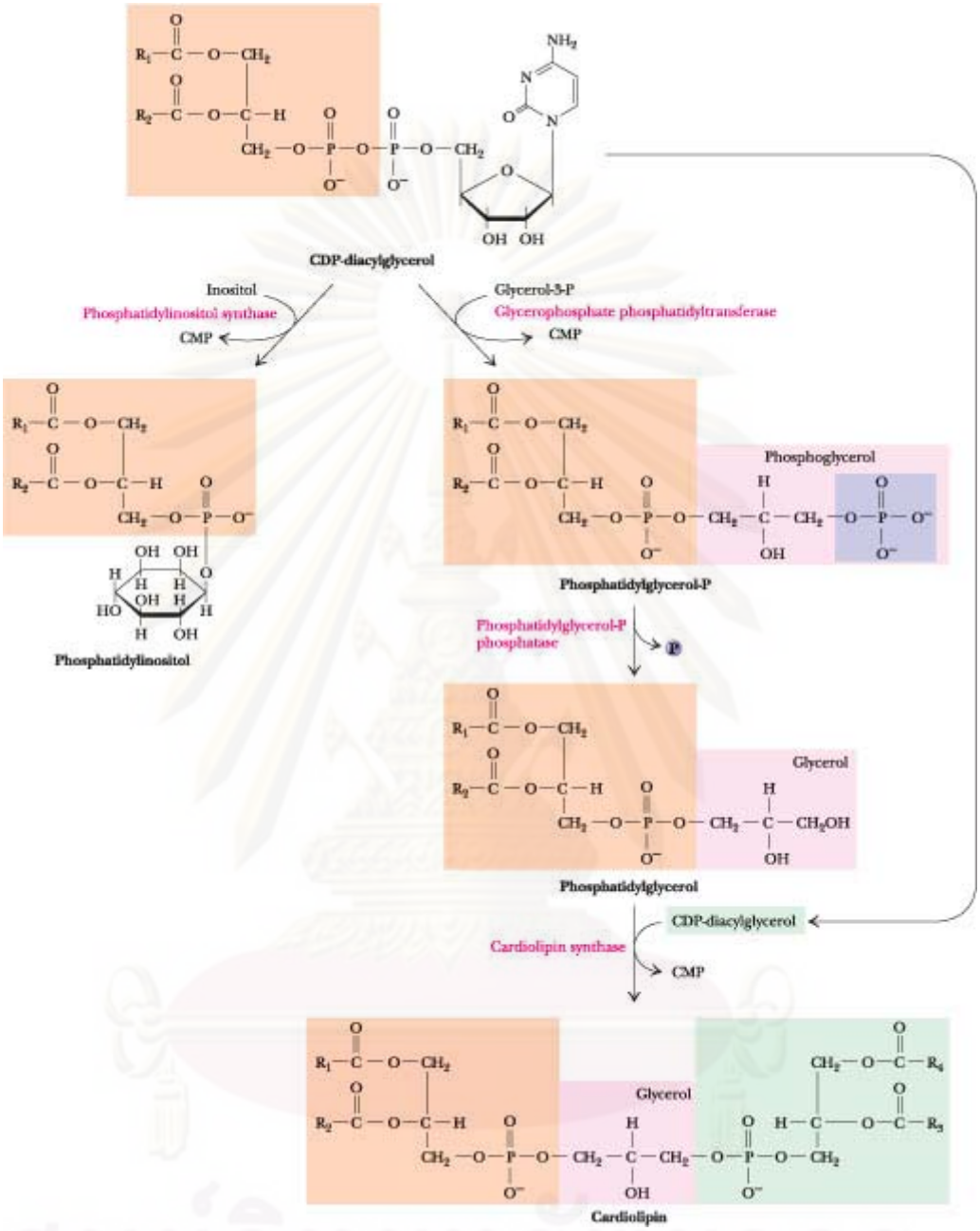


Figure 8 PI, PG and cardiolipin biosynthesis

(http://web.virginia.edu/Heidi/chapter25/chp25.htm, accessed 23 October 2009)

F. Sphingomyelin (SM) (Christie, 2009)

Sphingomyelin (or ceramide phosphorylcholine) consists of a ceramide unit with a phosphorylcholine moiety attached to position 1. It is thus the sphingolipid analogue of phosphatidylcholine. The d18:1/16:0 molecular species is shown in figure 9. It is a ubiquitous component of animal cell membranes, where it is by far the most abundant sphingolipid. Indeed, it can comprise as much as 50% of the lipids in certain tissues, though it is usually lower in concentration than phosphatidylcholine. For example, it makes up about 10% of the lipids of brain. It is the single most abundant lipid in erythrocytes of most ruminant animals, where it replaces phosphatidylcholine entirely. In this instance, there is known to be a highly active phospholipase A that breaks down the glycerophospholipids but not sphingomyelin. Like phosphatidylcholine, sphingomyelin tends to be in greatest concentration in the plasma membrane, and especially in the outer leaflet of the cells.

The biosynthesis of sphingomyelin is distinct from that of phosphatidylcholine. Indeed, it involves transfer of phosphorylcholine from phosphatidylcholine to ceramide, liberating diacylglycerols, and catalysed by a ceramide choline-phosphotransferase (sphingomyelin synthase) (Figure 10). The reaction takes place in the plasma membrane and Golgi with distinct integral enzymes in each organelle.

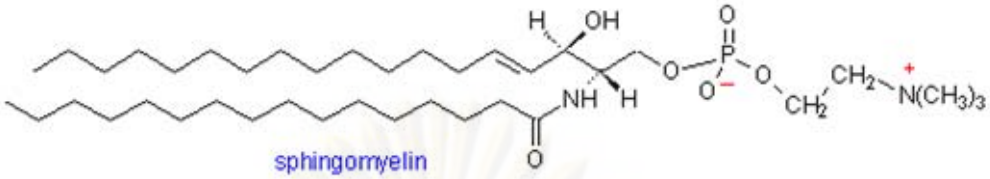


Figure 9 SM structure (Christie, 2009)

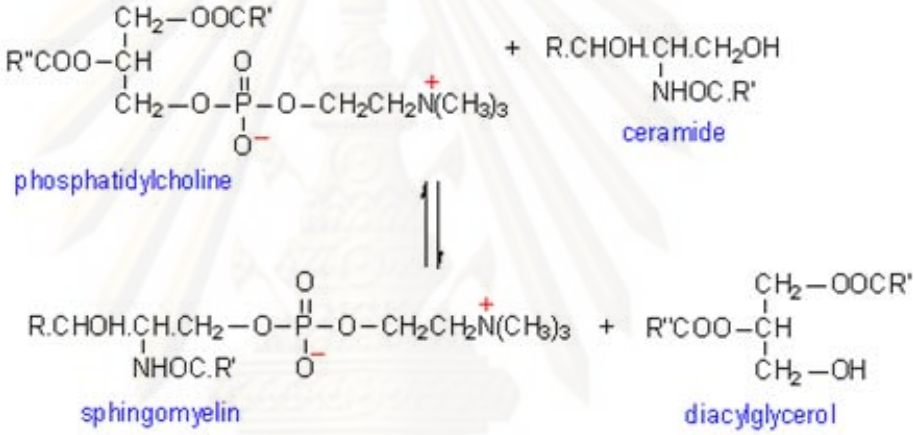


Figure 10 SM biosynthesis (Christie, 2009)

2.4.3 Digestion and absorption of phospholipids

Digestion of phospholipids occurs in the small intestine. In the bile, phospholipid is found in mixed micelles along with cholesterol and bile salts. Once in the intestinal lumen, the luminal PC will distribute between the mixed micelles and the TG droplet but PC tends to favor the micellar phase over the oily phase. It is phospholipid in micelles that serves as substrate for hydrolysis. Hydrolysis of phospholipid is largely brought about phospholipase A2, which is secreted by pancreas as prophospholipase A2 and then activated by tryspsin within the lumen of small intestine. Phospholipase A2 releases the fatty acid from sn2 position of PC to yield a fatty acid and lyso PC. Although the bulk of luminal intestinal phospholipase A2 activity is derived from pancreatic juice, there is propably some minor contribution from intestinal mucosa, which has an intensic membrane enzyme that has phospholipase and retinyl ester hydrolase activity and is known as retinyl ester hydrolase, or phospholipase B (Stipanuk, 2000).

2.4.4 Turnover and fatty acid remodeling of phospholipids

The molecular species of phospholipids presented in various tissues and cells are considerably different from those of their precursor molecule, phosphatidic acid. These results suggested that the fatty acid remodeling of phospholipids occurs after de novo synthesis. It has been widely accepted that arachidonic acid is introduced into phospholipids mainly through the remodeling pathway. Similarly, it has been suggested that a large portion of stearic acid is also incorporated into phospholipids through remodeling. This reactions were involved with the action of acyltransferases and transacylases (Yamashita et al, 1997). Phospholipids are the main constituents of membranes and are easily modified by the exchange of fatty acids in the sn-2 position. This is performed by the deacylation-reacylation pathway described by Lands (Land, 1960). Enzymes involved in this cycle are: acyl-CoA synthetase, acyl-CoA-

lysophosphatidylcholine acyltransferase and phospholipase A_2. For the deacylation/reacylation process of fatty acids in the sn-1 position of glycerophospholipids, a phospholipase A_1 is required instead of phospholipase A_2. Once a phospholipid e.g., phosphatidylcholine (PC) or phosphatidylethanolamine (PE) is made, its fatty acids in the sn-2 position can be modified according to the cycle of Lands as shown in Figure 11.

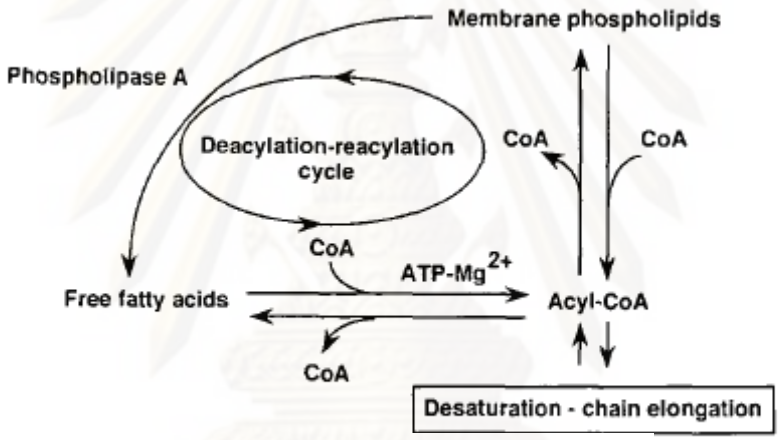


Figure 11 Deacylation-reacylation cycle (Land, 1960)

Additionally, Stoll has studied the incorporation of 14C fatty acids in phospholipids of plasma membrane sarcoplasmic reticulum of rat heart and found that the incorporation of unsaturated fatty acids (oleic and linoleic acid) was higher than for saturated fatty acids (palmitic and stearic acid). Incorporation in phosphatidylcholine was higher than in phosphatidylethanolamine and phosphatidylcholine has been showed an increasing sequence for the following fatty acids: C18:0 < C16:0 < C18:1 < C18:2 (Stoll, 1999).

2.5 Erythrocyte membrane phospholipids

2.5.1 Lipid composition and structure of erythrocyte membrane

One half of the human erythrocyte membrane is made of lipids contained within the bilayer of the membrane. It composes of 71% water, 28% protein, 7% lipid and 3% carbohydrate (Harris and Kellermeyer, 1972). Phospholipids and free cholesterol account for 95% of the total lipids. Erythrocyte membrane lipids are divided into two main classes: glycerophospholipids and sphingophospholipids. The typical distribution of phospholipids in human erythrocyte plasma membrane is PC about 30% , PE 28%, SM 25% ,PS and PI 14% measured by TLC (Figure 12). In general, the proportion of PC and SM is rather constant within range of 0.55 – 1.0.

Membrane lipid structure is characterized by the asymmetry between outer and inner leaflet of the phospholipids bilayers. In eythrocytes, the choline-containing phospholipids are predominant in outer half of the bilayer whereas the amino-containing phospholipids predominantly in inner half facing the cytosol. Additionally, the erythrocyte membrane has compositional asymmetry within its hydrophobic phase. PC, SM and glycolipids which are the major phospholipids of the outer leaflet contain very few polyunsaturated fatty acid residues. By contrast, PE, PS and PI are rich in these components, particularly arachidonic acid.

The basic property of phospholipids bilayer is its fluidity. Phospholipids can exist in two distinct physical states, a gel phase and a liquid crystalline phase. The temperature of the transition between both states depends on the class of phospholipids, the length of PL-FA chain and the degree of unsaturation. Unsaturated bonds that exist in the cis configuration tend to increase the fluidity of the bilayer by decreasing the compactness of the side chain packing. Most cell membranes including erythrocyte membranes show intermediate characteristics between these two phases. Such physical property is consistent with the fact that most membranes contain roughly equal amounts of saturated and unsaturated fatty acid (Dahlan, 1989).

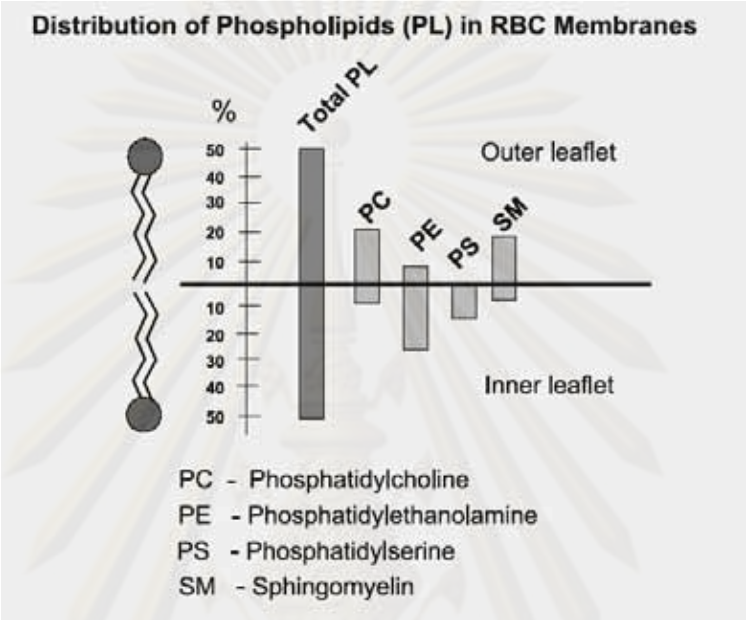


Figure 12 Distribution of phospholipids in erythrocyte membranes (Tanford, 1973)

2.5.2 The metabolism of erythrocyte membrane phospholipids

The membrane phospholipids of mature human erythrocytes are continuously renewed during the circulation of the cell. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which together comprise over 60% of the red cell membrane phospholipids are the molecular species which are primarily involved in this process. Since the red cell lacks acetyl Co-A carboxylase, this renewal occurs without the de novo synthesis of fatty acids.

Two major pathways for this renewal have been demonstrated. The first involves a passive exchange of intact phospholipids (phosphoglyceride backbone plus fatty acids) between serum lipoproteins and red cell membranes. The second involves an energy-dependent acylation within the membrane of either endogenous erythrocyte or plasma lysophospholipids by free fatty acids derived from plasma (Shohet, 1970). The erythrocyte does not increase its total content of phospholipids during its life-span. While it is clear that the first phospholipids renewal mechanism (exchange) would result in no net change in membrane phospholipids content, the second mechanism (acylation) would, in the absence of a parallel catabolic process, cause an increase in membrane phospholipids content. That such a catabolic process may exist is suggested by studies which have shown that the fatty acid moiety of red cell phospholipids may leave the cell independently of the phosphoglyceride backbone.

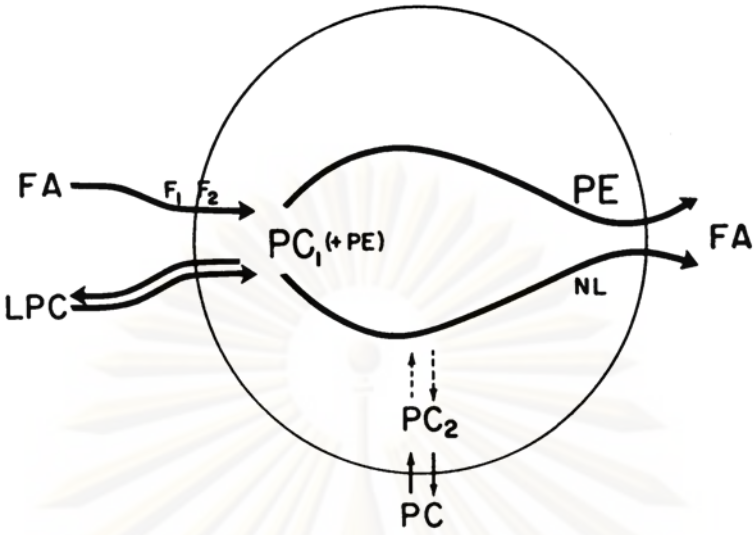


Figure 13 Working scheme of phospholipid metabolism in the mature human erythrocyte (Shohet, 1970)

A schematic summary of phospholipid metabolism in the mature human erythrocyte is shown in Figure 13. In the horizontal series of steps, a distinct cellular pool of phospholipid (PC1+PE) is assembled by an ATP-dependent acylation of lysophosphatide and fatty acid. The fatty acid is delivered to the acylation site from serum through a series of discrete stages (F1 + F2) (Shohet et al, 1968). The predominant phospholipid found at this state is PC though PE is also formed. The fatty acid moiety of the actively produced phosphatidylcholine (PC1) can then be removed and transferred to other cellular acceptors to produce additional phosphatidylethanolamine and "neutral lipids". This "transferase" activity is apparently facilitated by the presence of unheated autologous serum. Part of the final return of fatty acid back to serum is derived from these secondary acceptors.

As shown by the vertical arrows, there is also a passive equilibration of serum phosphatidylcholine with another cellular pool of phosphatidylcholine (PC2). During catabolism, there is only slight mixing between the actively and passively established pools of phosphatidylcholine and the phosphatidylcholine introduced into each can be distinguished by its subsequent metabolic behavior (Shohet, 1970).

2.5.3 Dynamic processes of membrane phospholipids

Since the membrane phospholipids of mature human erythrocytes are continuously renewed during the circulation, lipid molecules in liquid bilayers are extremely lively and undergo a range if different dynamical processes. They are constantly changing intra-molecular conformations, they are wobbling, they are protruding out of the layer and they are moving around. Figure 14 shows some of the motion that lively individual lipids perform. These motions range over an enormous time span, from picoseconds to hours. Conformation changes can be fast, since they involve rotations around C-C bonds, which typically take a few seconds. The rotation of the lipid molecules are also fast and occur on a timescale of nanoseconds, whereas lateral diffusion is in the range of tens of nanoseconds. A typical lipid will on average rotate once around its axis while it travels a distance corresponding to its own size. The wobbling of the fatty acid chain, which leads to changes in its direction within the bilayer, is much slower, typically of the order of tens of milliseconds.

The fast lateral mobility of lipids in the plane of the membrane is a typical liquid property. Over time, lipids will be able to explore the entire lipid bilayer or membrane. For a typical cell size, a lipid molecule can travel across the cell membrane within less than half a minute. Lipid molecules furthermore undergo substantial excursion perpendicular to the cell membrane plane in the form of single molecule protrusions that take place over time scales of tens of picoseconds. The motion of lipid molecules from one monolayer leaflet to the other, the so-called flip-flop process, is on the other hand, extremely slow, being of the order of hours, possibly day. In real biological membranes, special membrane proteins, so-called flippases, facilitate the redistribution of lipid molecules between the two monolayer leaflets. The actual values of the rates of the different dynamical processes depend on the type of lipid molecule. There is some temperature dependence as well as a significant dependence on the state of matter of the lipid bilayer. If the lipid membrane is taken into a solid phase, all dynamical processes slow down significantly (Mouritsen, 2005).

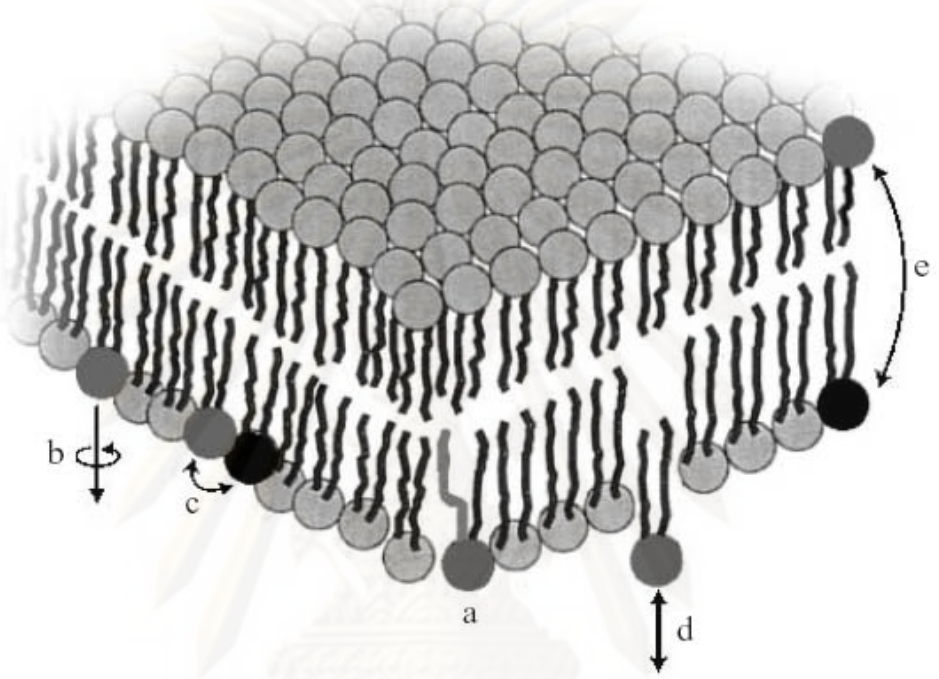


Figure 14 The many kinds of motions that lipid molecules in lipid bilayer can perform. (a) Conformation change (b) Rotation around the molecule axis (c) Lateral diffusion (d) Protrusion out of bilayer plane (e) Flip-flop between lipid monolayers (Mouritsen, 2005)

2.6 Alteration of phospholipids in erythrocyte of type 2 diabetes

Since lipid components of biological membranes play a key role in membrane fluidity and functions, the alteration or modification of such lipids may affect to cell membrane properties, for instance, the impairment of erythrocyte deformability can make oxygen delivery through the capillaries difficult, create tissue hypoxia, and contribute to some complications seen in diabetes.

Blood cells have been reported to participate in any vascular complications and diabetics present disorders in the function of blood cells. For instance in erythrocytes, Increased blood viscosity (Lowe et al, 1980), altered red blood cells (RBC) aggregation (Satoh et al, 1984), increased adhesion of erythrocytes to endothelial cells (Wautier et al, 1986). In 1996, Labrouche and colleagues have studied the blood cell membrane phospholipids in different types of diabetes and found some classes of phospholipids in diabetes were significant difference when compare to control group (Labrouche et al, 1996). In that study, erythrocyte in type 2 diabetic has low in phosphatidylserine (PS) but high in phosphatylethanolamine (PE) as compared to control group. In addition, recent study in type 2 diabetes has shown that there were some differences in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) of erythrocyte obtained from type 2 diabetic patients with racial differences. The plasma membrane of whites showed higher of PE and lower PC levels than those in blacks. Moreover, the RBC rheology properties (deformability) were lower in diabetics and black subjects, and the saturated nature of RBC was the lowest in white control subjects (Allen et al, 2006).

Some classes of erythrocyte phospholipids was also reported to be correlated with insulin resistant condition, type 2 diabetes and hyperinsulinemic nondiabetic subjects (Folsom et al, 1996; Zeghari et al, 2000; Younsi et al, 2002). Otherwise, it is not only erythrocyte phospholipid was changed in diabetes but fatty acid composition was also changed as well. Some fatty acid composition of erythrocyte phospholipids was reported to be correlated with fasting insulin and insulin resistance in obese type 2

diabetes (Enriquez et al, 2004 ; Bakan et al, 2006) and in diabetic children (Decsi et al, 2002).

It is not clear whether the alteration of erythrocyte phospholipid or fatty acid result from insulin-resistant state. Younsi and colleagues have proposed two possible explanations. First, an insulin-resistant state could affect the phospholipids composition of cell membranes through 3 mechanisms: (1) alterations in the exchanges between cells and plasma lipoproteins; (2) the presence in the plasma of molecules stimulating the cellular import of phospholipids; or (3) an effect on the synthesis of phospholipids. The movement of free cholesterol and phospholipids between cells and lipoproteins is a bidirectional process. The extent and the direction of net movement depend on the ratio of influx to efflux and are determined by both cell and acceptor properties. Both can be altered by insulin-resistance. Since the composition of lipoproteins is abnormal in insulin-resistant subjects (Stewart et al, 1993), changes in plasma PC or SM may be rapidly transmitted into erythrocyte membranes. Therefore, the phospholipids composition of erythrocyte membranes in the insulin-resistant subjects could reflect the altered composition of lipoproteins. The second explanation is that insulin resistance is a consequence of membrane phospholipids abnormalities. The membrane SM and PE could effectively have a role in the insulin action. SM is a rigidifying membrane phospholipid that can affect the insulin signal pathway. The role of changes in PE membrane content is less clear, but PE content may be an important regulator of insulin action. A high membrane PE content leading to an increase in the head group spacing of phospholipids in lipid bilayers was closely correlated with decreased protein kinase C activity, a key enzyme in insulin action (Younsi et al, 2002).

2.7 Mass spectrometric analysis of erythrocyte phospholipids

2.7.1 Overview of mass spectrometer (Siuzdak, 1995)

The history of mass spectrometry began with Sir J.J. Thomson of the University of Cambridge. He has discovered the electron in 1897, for which he was awarded the 1906 Nobel Prize in Physics. In the first decade of the 20th century, he went on to construct the first mass spectrometer, in which ions were separated by their different parabolic trajectories in electromagnetic fields and detection occurred when the ions struck a fluorescent screen plate. Later, many scientists have developed and improved the mass analyzer and ionization as shown in table 2.

Table 2 Some historical and developments in mass spectrometry *

Investigators	Year	Contribution
J.J. Thomson	1899-1911	First Mass spectrometer
Dempster	1918	Electron Ionization and Magnetic focusing
Aston	1919	Atomic Weights using MS
Mattauch & Herzog	1934	Double focusing instruments
Stephens	1946	Time-of-Flight Mass Analysis
Hipple, Sommer & Thomas	1949	Ion Cyclotron Resonance
Johnson & Nier	1953	Reverse Geometry Double Focusing
Paul & Steinwedel	1953	Quadrupole Analyzers
Beynon	1956	High Resolution MS
McLafferty & Ryahe	1959 - 1963	GC-MS
Dole	1968	Electrospray Ionization
Comisarow & Marshall	1974	FT ICR MS
Yost & Enke	1978	Triple Quadrupole MS
Fenn	1984	ESI on Biomolecules
Tanaka, Karas & Hillenkamp	1985 -1988	MALDI

* Modified from Siuzdak, 1995

A. Some basics of mass spectrometer

Mass spectrometer is an analytical device that determines the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). The ions are generated by inducing either the loss or gain of charge from a neutral species. Once the ions are formed they can be separated according to m/z and finally detected. The result of molecular ionization, ion separation and ion detection is a spectrum that can provide molecular mass and even structural information. Four basics components of MS are a sample inlet, an ionization source, a mass analyzer and an ions detector. Some instruments combine the sample inlet and the ionization source, while the others combine the mass analyzer and the detector. Sample molecules are introduced into the instrument though a sample inlet. Once inside the instrument, the sample molecules are converted to ions in the ionization source, before being electrostatically propelled into mass analyzer. Ions are then separated according to their m/z within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer.

B. Ionization source: Electrospray ionization (ESI)

ESI is a method used to produce gaseous ionized molecules from a liquid solution (Figure 15). This is done by creating a fine spray of highly charged droplet in the presence of a strong electric field. The sample solution is sprayed from a region of a strong electric field at the tip of a metal nozzle maintained at approximately 4000 V, and the highly charged droplets are then electrostatically attracted to the mass spectrometer inlet. Either dry gas, heat or both are applied to the droplets before they enter the vacuum of the mass spectrometer, thus causing the solvent to evaporate from the surface. As the droplet decrease size, the electric field density on its surface increases. The mutual repulsion between like charges on this surface become so great that it exceeds the forces of the surface tension and ions begin to leave the droplet (Figure16). The ions are directed into an orifice through electrostatic lenses leading to the mass analyzer.

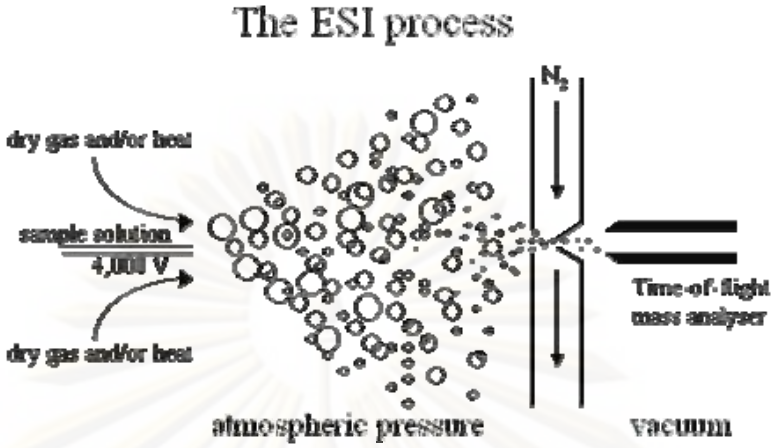


Figure 15 Diagram of electrospray ionization in Mass Spectrometer (Siuzdak, 1995)

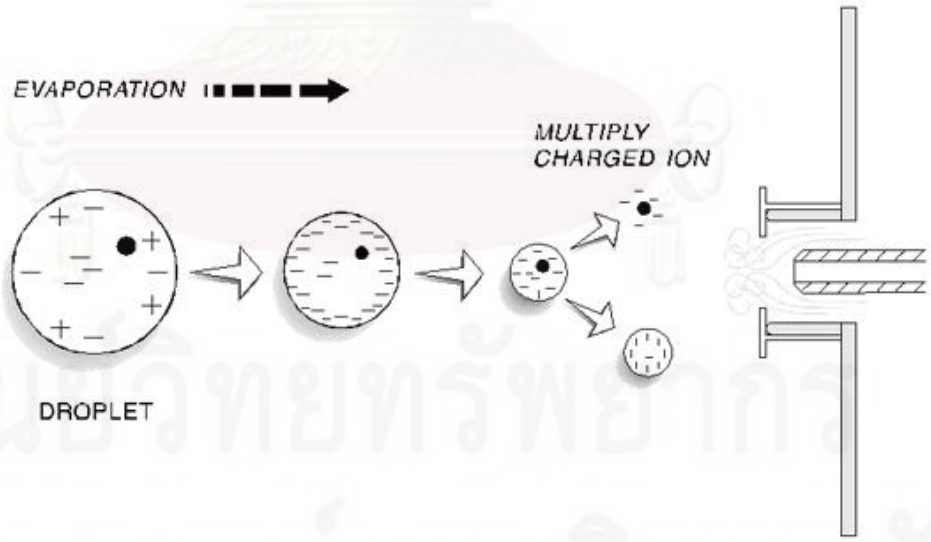


Figure 16 Ion evaporation mechanism within the ESI chamber
(EsquireControl software version 5.2, Bruker Daltonik GmbH, Oct 2009)

C. Mass analyzer

Quadrupole Mass Analyzer

Quadrupole are four parallel rods with the direct current (DC) voltage and a superimposed radio frequency (RF) potential. The field on the quadrupoles determines which ions are allowed to reach the detector. The quadrupoles have function as mass filter. As the field is imposed, ions moving into this field region will oscillate depending in their m/z and on the radio frequency field. Only ions of a particular m/z can pass through the filter. The m/z of an ion is therefore determined by correlating the field applied to the quadrupoles with the ion reaching the detector. A mass spectrum can be obtained by scanning of RF field.

Time-of-Flight Mass Analyzer

A Time-of-Flight (TOF) analyzer is one of the simplest mass analyzing devices and is commonly used with MALDI ionization. Time-of-Flight analysis is based on accelerating a set of ions to a detector with the same amount of energy. Because the ions have the same energy, a different mass of the ions reach the detector at difference times. The smaller ions reach the detector firstly because of their greater velocity and the larger ions take longer, thus the analyzer is called time-of-flight because the m/z is determined from the ions' time of arrival. The arrival time at the detector is dependent upon the mass, charge and kinetic energy of the ion.

Ion Trap Mass Analyzer

The ion trap mass analyzer is developed at the same time as quadrupole mass analyzer. The physics behind both of these analyzers is very similar. A schematic of the basic set up of the ion trap mass analyzer is shown in Figure 17. The ions, produced in the source of the instrument, enter into the trap through the inlet and are trapped through action of the three hyperbolic electrodes: the ring electrode and the entrance and exit end cap electrodes. Various voltages are applied to these electrodes which results in the formation of a cavity in which ions are trapped. The ring electrode RF potential, an AC potential of constant frequency but variable amplitude, produces a 3D quadrupolar potential field within the trap. This traps the ions in a stable oscillating trajectory. The exact motion of the ions is dependent on the voltages applied and their individual mass-to-charge (m/z) ratios. For detection of the ions, the potentials are altered to destabilize the ion motions resulting in ejection of the ions through the exit endcap. The ions are usually ejected in order of increasing m/z by a gradual change in the potentials. This 'stream' of ions is focused onto the detector of the instrument to produce the mass spectrum.

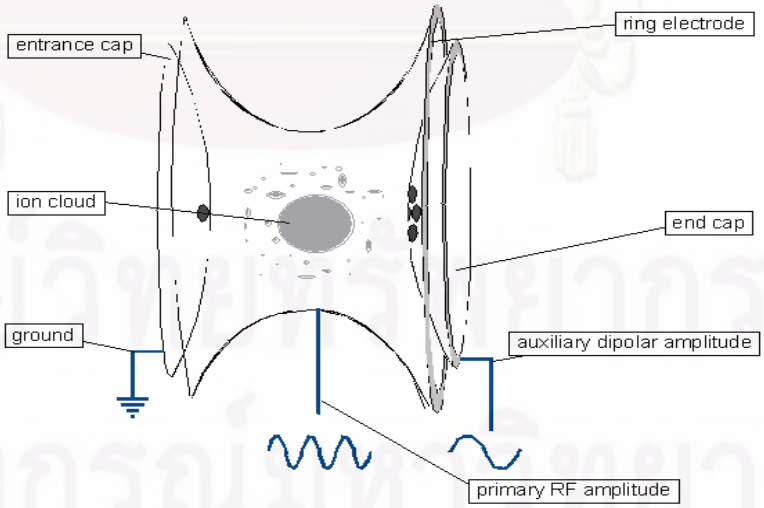


Figure 17 Ion Trap Mass Analyzer

(EsquireControl software version 5.2, Bruker Daltonik GmbH, Oct 2009)

2.7.2 Characterization of phospholipids molecular species by MS

Since membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl and headgroup compositions, the analysis of phospholipid requires multi-step procedures (Christie, 2003). Classical liquid-liquid extraction of cellular material readily results in heterogeneous mixtures of phospholipids, which can be further refined by the selective use of organic solvents. The next step is a class separation, generally relying on analysis by thin-layer chromatography, high-performance liquid chromatography (HPLC), and gas chromatography, any of which can necessitate prior derivatization as well as the availability of known standards (Wang and Gustafson, 1992; Abidi and Mounts, 1996; Genge et al, 2003). This conventional approach is time-consuming, requiring relatively large sample volumes for accurate separation and lacks the sensitivity required for quantifying minor components.

Characterization of phospholipids species by mass spectrometry (MS) has long been hindered by their properties to undergo fragmentation during analysis, but has recently become possible in large part because of dramatic technical advances. Mass spectrometry (MS) offers an attractive alternative for the analysis of phospholipids composition because of its high sensitivity, specificity, and simplicity. The introduction of “soft” ionization methods has completely opened new vista in this field. In particular, electrospray ionization-mass spectrometry (ESI-MS) has been shown to be a very promising technique. However, it is important to have a chromatographic system separate the different phospholipids classes to avoid possible mass overlap. Thus, in the analysis of lipids taken from a complex biological matrix, there is a need for class separation by LC followed by species identification by mass spectrometry. Therefore, HPLC/MS is a very useful tool for phospholipids analysis in any biological samples (Uran et al, 2001; Wang et al, 2004; Wang et al, 2005).

The characterization of individual phospholipids species from total lipid extract by ESI-MS is based on the ability of any given phospholipids class to acquire charge (positive or negative) in solution under the capillary source high energy. Lipids that do not possess inherently ionizable functions (e.g., diacylglycerols) can also be ionized under the conditions of ESI-MS, and as long as there is a sufficient dipole potential for interaction with small counter ions, these species can be analyzed. Thus, under suitable conditions of sample preparation, all molecular species that exist among cellular lipid classes can be detected in a single run of a total lipid extract (Ivanova et al, 2004). Typically, phospholipids classes are identified and characterized on the basis of their characteristic of fragment structures as shown in figure 18.

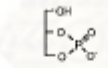
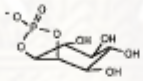
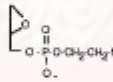
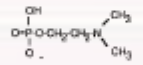
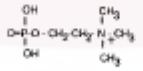
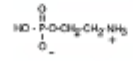
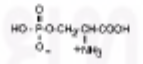
specificity	polarity	fragment structure	scan mode	fragment type	optimal collision offset
all $[M-H]^-$ ions of glycerophospho-lipids	neg.		precursor of 153	glycerol-phosphate $-H_2O$	+50 V
phosphatidyl-inositol	neg.		precursor of 241	head group $-H_2O$	+45 V
phosphatidyl-serine	neg.		neutral loss of 87	head group $-H_3PO_4$	+28 V
phosphatidyl-ethanolamine	neg.		precursor of 196	dilyso $-H_2O$	+50 V
sphingomyelin	neg.		sCID + precursor of 168	head group (demethyl-ated)	sCID +65 & +40 V
phosphatidyl-choline and sphingomyelin	pos.		precursor of 184	head group	-35 V
phosphatidyl-ethanolamine	pos.		neutral loss of 141	head group	-25 V
phosphatidyl-serine	pos.		neutral loss of 185	head group	-22 V

Figure 18 Summary of the phospholipid class-specific scan modes
available in positive and negative ion by ESI-Triple Quadrupole-MS (Brugger et al, 1997)

Triple quadrupole and ion trap mass spectrometers are the two common mass spectrometers used in combination with liquid chromatography (LC) for phospholipid analysis, particularly with soft ionization techniques like electrospray ionization. With the two MS systems, detection of molecular related ions appears very similarly. However, the physics behind mass separation and collision-induced dissociation (CID) processes are very difference. Both MS systems can be operated in full scan mode (FS), single ion monitoring mode (SIM), product ion scan mode (PIS) and multiple reaction monitoring (MRM). However, the ion trap mass spectrometer is unable to scan the phospholipid head group by using precursor and neutral loss scan modes like triple quadrupole mass spectrometer, thus the characterization and structural identification of phospholipid need to be achieved by collision-induced dissociation (CID) or fragmentation.

2.7.3 Collision-induced dissociation (CID) or fragmentation of phospholipid

In mass spectrometry, collision-induced dissociation (CID) is a mechanism by which to fragment molecular ions in the gas phase (Wells and McLuckey, 2005). The molecular ions are usually accelerated by some electrical potential to high kinetic energy in the vacuum of a mass spectrometer and then allowed to collide with neutral gas molecules (often helium, nitrogen or argon). In the collision some of the kinetic energy is converted into internal energy which results in bond breakage and the fragmentation of the molecular ion into smaller fragments. These fragment ions can then be analyzed by a mass spectrometer.

Fragmentation of phospholipid molecular ions in negative ionization generates ions derived from their constituent fatty acyl chains. Moreover, under appropriate experimental conditions, fatty acyl fragments from the sn-1 and sn-2 positions generate fragment ions of different abundance and this can be used to assign not only fatty acyl composition but also their regiospecificity. The plasmanyl and plasmenyl ether phospholipids are exceptions to this rule, as no fatty acid fragment is formed from the ether-linked component. Product ion scanning to determine the molecular species

components of individual ion peaks works well for acidic phospholipids, but is more problematic for PC. The intact PC molecule cannot acquire a negative charge due to the positively charged quaternary nitrogen in the choline headgroup. PC will ionize under negative conditions by loss of a methyl group $[M-15]^-$ or formation of a chloride adduct $[M+35]^-$, which in turn can be fragmented to generate the fatty acyl ions. This process also has problems, as it is often difficult to control the negative ionisation of PC and consequently multiple ions will be generated from a single molecular species resulting in overlapping fatty acyl fragments (Postle et al, 2007).

In ion trap mass spectrometer, collision-induced dissociation (CID) of parent molecular ions or fragmentation by an auxiliary pulsed voltage in the ion trap (MS^n) produces fragment ions corresponding to the fatty acid moieties required for assigning the particular mass peaks with phospholipids. Fragment ions corresponding to carboxylate anions of fatty acids are specifically obtained in the negative ionization mode of ESI after a collision-induced dissociation (CID) (Wolf and Quinn, 2008). In the negative ion mode the product ions were mainly sn-1 and sn-2 lyso-phospholipids with neutral loss of ketene in combination with neutral loss of the polar head group. Less abundant product ions were sn-1 and sn-2 carboxylate anions. As shown in figure 19 and 20, the charge-remote-type mechanism in ion trap mass analyzer is proposed for formation of the lyso-phospholipid product ions by loss of alpha-hydrogen on the fatty acid moiety, electron rearrangement and neutral loss of ketene. A second mechanism involves nucleophilic attack of the phosphate oxygen on the sn-1 and sn-2 glycerol backbone to form carboxylate anions with neutral loss of cyclo lysophospholipids. In addition, the ion-trap showed no loss in sensitivity in full scan compared to multiple reaction monitoring data acquisition. In combination with on-line liquid chromatography, it makes the ion-trap useful in the scanning modes for rapid screening of low concentrations of phospholipid species in biological samples (Larsen et al, 2001).

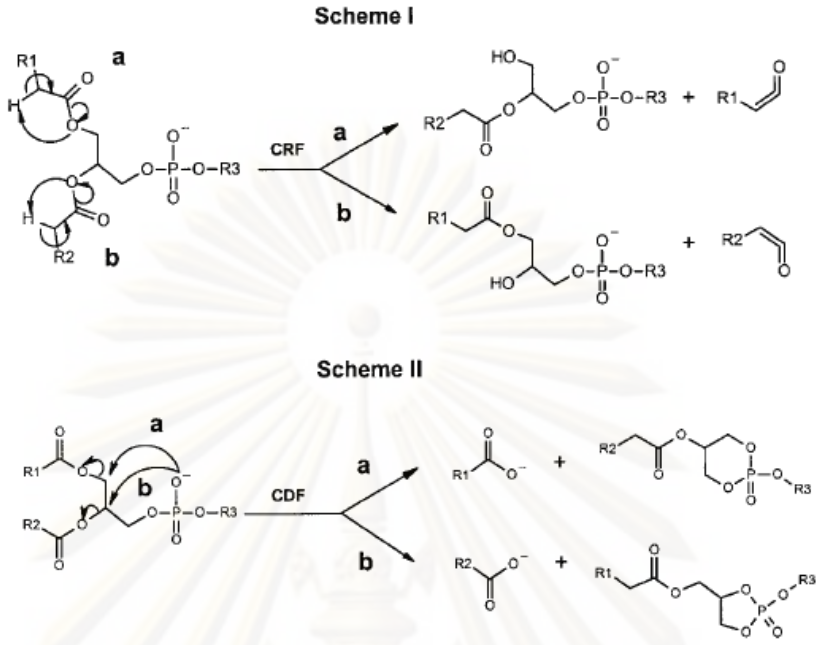


Figure 19 Proposed fragmentation mechanisms for CID of phospholipids using the ion-trap instrument. Both lyso-phospholipid anions (Scheme I) and carboxylate anions (Scheme II) were observed using the ion trap. CRF: charge-remote fragmentation, CDF: charge-driven fragmentation (Larsen et al, 2001)

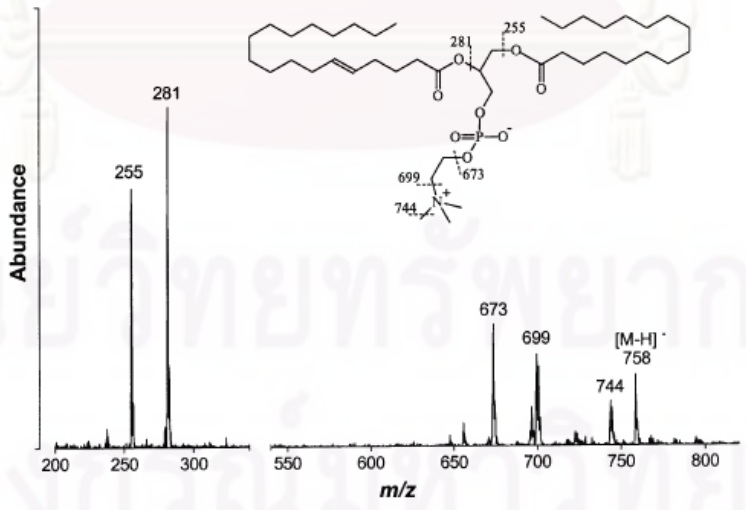


Figure 20 CID of PC 16:0/18:1 in negative mode MS (Brouwers et al, 1998)

2.7.4 Erythrocyte phospholipids analysis by MS

Over the last few years, phospholipids analysis by using mass spectrometer and liquid chromatography coupled with mass spectrometer have been dramatically increased and applied to not only erythrocytes but also to the other biological fluids such as whole blood, plasma, tissue extract and bronchoalveolar lavage fluid (Han and Gross, 1994; Heeley et al, 2000; Barroso and Bischoff, 2005; Gao et al, 2006; Bernhard et al, 2007). Early work from the laboratories of Gross and co-worker involved the analysis of the human erythrocyte plasma membrane, the starting point for many developments in lipid and membrane biochemistry, and where they first experienced the remarkable sensitivity of the technology for phospholipids analysis. This technique allowed structural determination and quantitative analysis of individual phospholipid molecular species from subpicomole amounts of human erythrocyte membrane. more than 50 human erythrocyte membrane phospholipld constituents were identified by direct infusion of chloroform extracts and it has been showed that the major phosphatidylethanolamine subclass in erythrocyte membranes was plasmenylethanolamine that was highly enriched in polyunsaturated fatty acids at the sn-2 position (Han and Gross, 1994). Since the sn-position of fatty acid in membrane lipids has an influence on the physiological function of cells, Beermann and colleagues have used reverse phase-HPLC coupled with on-line electrospray ionization ion trap MS for identification of fatty acids in phospholipids. They have demonstrated that saturated fatty acid were found to be located mainly in the sn-1 position of the glycerol backbones of erythrocyte phospholipids, whereas PUFA were found primarily in the sn-2 position (Beermann et al, 2005). Recently, ESI-MS was used for analysis of different circulating blood cells including erythrocyte of healthy human and it has been found that PC 34:1 is the major species beside PC 34:2 in erythrocyte and longer chain SM species (SM 24:1 and SM 24:0) were significantly elevated for erythrocyte as compared to other blood cells. In addition, they have observed that erythrocytes have a high fraction of species of PC, PE and PI with shorter chain length whereas an elevated proportion of long chain species was found in PS (Leidl et al, 2008).

2.8 Fourier Transform Infrared Spectroscopic analysis of erythrocyte contents

2.8.1 Overview of Fourier transform infrared (FT-IR) spectroscopy

Modern infrared spectrometers are usually Fourier transform infrared (FT-IR) spectrometers. Their name originates from the fact that the detector signal of these spectrometers is related by a Fourier transformation to the measured spectrum. The heart of an FT-IR spectrometer is an interferometer, like the Michelson interferometer shown in figure 21. It has a fixed and a movable mirror. The latter generates a variable optical path difference between two beams which gives a detector signal that contains the spectral information. Light emitted from the light source is split by a beam splitter: about half of it is reflected towards the fixed mirror and from there reflected back towards the beamsplitter where about 50% passes to reach the detector. The other half of the initial light intensity passes the beam splitter on its first encounter, is reflected by the movable mirror back to the beamsplitter where 50% of it is reflected towards the detector. When the two beams recombine, they interfere and there will be constructive or destructive interference depending on the optical path difference d. The instrument measures the light intensity relative to the position of the movable mirror and this is called an interferogram. It turns out that the interferogram is the Fourier transform of the spectrum. A second Fourier transform performed by a computer converts the measured data back into a spectrum. In total, a Fourier transform spectrometer performs two Fourier transformations: one by the interferometer, one by the computer. That the interferometer produces the Fourier transform of the spectrum is best seen when a monochromatic source is considered. Its spectrum $S(\lambda)$ is described by a delta function located at $\lambda 0$. Depending on the position of the movable mirror, one obtains constructive or destructive interference at the detector and the detector signal I(d) varies as a cosine function with the mirror position which determines the optical path difference d. This cosine function and the delta function describing the monochromatic spectrum are

related by a Fourier transformation. Thus, a second Fourier transformation performed by the computer generates the spectrum S(λ). The main advantage of Fourier transform spectrometers is the rapid data collection and high light intensity at the detector and in consequence the high signal to noise ratio. Therefore, a spectrum can be recorded in as few as 10 ms. Different types of detectors, light sources and other optical components are used in different regions of the infrared spectral range (Barth, 2007).

FT-IR spectroscopy is a technique that allows the molecular characterization of biological tissues and fluids. It utilizes the infrared region of light to obtain an IR spectrum which reflects the unique set of molecular vibrations for each molecular species in the sample examined. Therefore, the IR spectrum is like a ‘‘fingerprint’’ of the molecular species making up the sample. The intensities of IR spectra provide quantitative information while the absorption positions (wavelength, or the inverse ‘‘wavenumbers’’ (cm^{-1}) reveal qualitative characteristics about the nature of the chemical bonds, their structure and their molecular environment. In complex biological materials such as cells or biological fluids, the major IR absorption bands arise from N–H, C=O, C–H and P=O bonds found in proteins, lipids and nucleic acids of the studied materials (Liu et al, 2005).

In biological terms, the vibrations in the wavenumber region 2800–3050 cm^{-1}, for example, can be assigned to CH_2 and CH_3 stretching vibrations from fatty acids, whilst those in the wavenumber region 1500–1750 cm cm^{-1} (the amide I and II bands) are assigned to C=O, NH and C–N from proteins and peptides. In addition, FT-IR spectrometry has been recognized as a valuable tool for metabolic fingerprinting, owing to its ability to analyze carbohydrates, amino acids, fatty acids, lipids, proteins and polysaccharides simultaneously. However, one potential disadvantage of FT-IR in the mid-IR is that the absorption of water is very intense but this problem can be overcome in one of several ways such as dehydration of samples, subtraction of the water signal, or by application of attenuated total reflectance (ATR). Moreover, another perceived disadvantage is that as a holistic measurement is made with biochemical information

spread across the whole of the IR spectrum, validated and robust chemometrics must be used in order to turn data into information (Ellis and Goodacre, 2006).

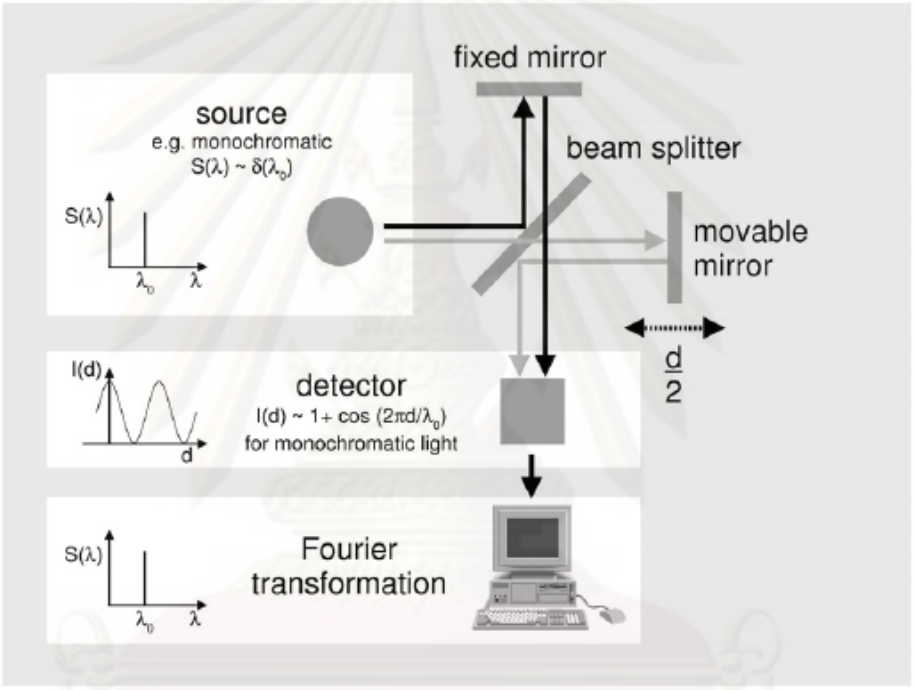


Figure 21 Scheme of a Fourier transform infrared spectrometer (Barth, 2007)

2.8.2 Studies of erythrocyte by FT-IR spectroscopy

Over the last few years, infrared spectroscopic studies have been undertaken in many biomedical applications such as disease diagnostics, cancer screening, metabolic disorders, etc. In general, the cell membrane of human erythrocytes is also widely recognized as an excellent model for the study of biological membranes since these membranes are relatively easy to obtain and purify. The frequency, intensity and width of particular vibrational IR spectral bands are extremely sensitive to the packing constraints, conformational changes and chemical variations in lipids and proteins present in erythrocyte membranes. In erythrocytes, phospholipids bilayer has been studied by FT-IR spectrometry (Moore et al, 1996). As shown in table 3 and figure 23, the main erythrocyte IR absorptivities should be easily interpreted: (1) fatty acyl moieties of erythrocytes stem exclusively from phospholipids; (2) hemoglobin accounts for about 80% of erythrocyte proteins, spectrin for about 10%, and there is no nucleus within this cell; (3) over membrane carbohydrate residues, sugars present within the cell are only lactate and possibly glucose for diabetics (Petibois and Deleris, 2008).

Recently, FT-IR spectroscopy has been widely used to study and apply to the elucidation of pathophysiological mechanisms in various erythrocyte disorders, such as erythrocyte peroxidation induced by free radicals (Sills et al, 1994), the alterations in membrane phospholipid domains and conformational orders of sickle cell anemia (Szostek et al, 1991), investigation of the secondary structure of hemoglobin (Gregoriou et al, 1995) and a screening tool for beta-thalassemia (Liu et al, 2003).

Table 3 Major organic content assignments for erythrocyte FT-IR spectra absorption bands (Petibois and Deleris, 2005)

Bands (cm^{-1})	Major assignments for erythrocyte contents
3020–3000	ν=(CH): (olefinic) unsaturated fatty acids, cholesterol esters
2990–2950	$\nu_{as}(CH_3)$: (methyl) phospholipids, cholesterol esters, fatty acids
2950–2880	$\nu_{as}(CH_2)$: (methylene) phospholipids, long chain fatty acids
2880–2860	$\nu_{s}(CH_3)$: (methyl) phospholipids, fatty acids
2870–2830	$\nu_{s}(CH_2)$: (methylene) phospholipids, long chain fatty acids
1739–1713	ν(C=O): phospholipids, cholesterol esters, glycerides
1713–1589	ν(C=O): (amide I) β-sheet: proteins, turns, coils
1589–1474	δ(N–H): (amide II) α-helix: proteins
1480–1430	$\delta_{as}(CH_3)$, $\delta_{as}(CH_2)$, $\delta_{s}(CH_3)$, $\delta_{s}(CH_2)$: phospholipids, fatty acids, glycerides
1420–1370	$\nu(COO^-)$: (carboxylate ions) amino-acids
1257–1201	ν(P=O): (phosphate) phospholipids
1138–1061	ν(C–O): polysaccharides, glucose, lactate

ν = stretching vibrations (s = symmetric, as = asymmetric), δ = bending (scissoring) vibrations.

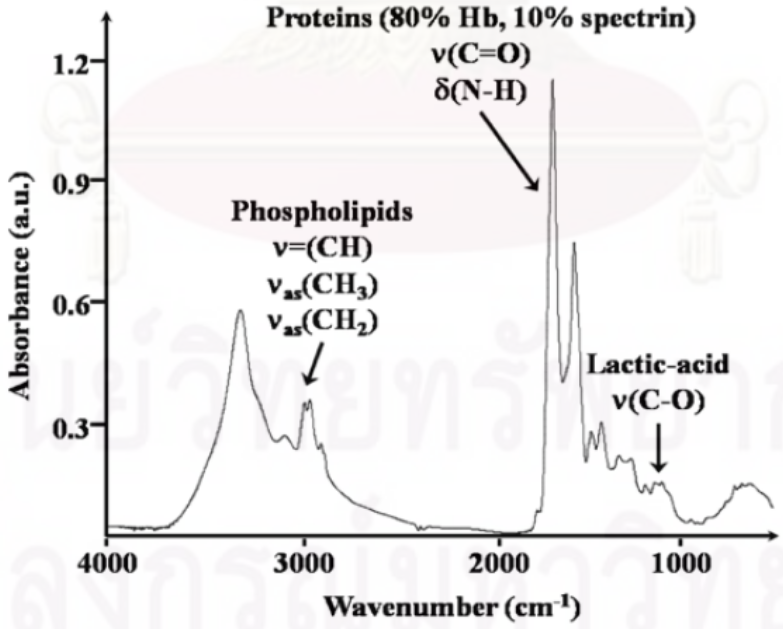


Figure 22 IR absorption bands assignation on erythrocyte FTIR spectrum (Petibois and Deleris, 2008)

CHAPTER III
MATERIALS AND METHODS

3.1 Part I: Study on the postprandial biochemical response after high fat meal period in control and type 2 diabetic group

3.1.1 Subjects

1. Design of experiments

After 12 hours overnight fasting, all subjects were given a single standard high fat meal containing 40 g fat/m^2 body surface area. The meal was ingested within 20 minutes. After the test meal, only water was allowed for drinking during the experiment. Blood samples were collected in EDTA-containing tubes before and four hours after the meal. After separation of plasma, erythrocyte was washed and divided into 2 parts. First part was used for FTIR-HTS-XT analysis for erythrocyte contents. The other one was extracted and analyzed for phospholipids molecular species by using LC-ESI-MS.

2. Subjects

Type 2 diabetic subjects

Twelve type 2 diabetic subjects were recruited from Theptarin general hospital, Bangkok, Thailand. The criteria of subjects were;

Inclusion criteria:

1. Diagnosed as type 2 diabetic patient at time of enrollment
2. Currently treated type 2 diabetic patients with oral anti-diabetic agents
3. Male or female, age between 21-60 years old
4. Available for fasting and 4 hours postprandial tests

Exclusion criteria:

1. Participation in another research projects
2. Having an evidence of acute infection, hepatic or renal disease
3. Pregnant or lactating
4. HbA1C > 10%
5. Fasting plasma triacylglycerol more than 500 mg/dL
6. Current insulin treatment
7. Taking lipid lowering drugs during the previous 3 weeks

Control subjects

11 healthy control subjects randomly selected from our laboratory staff. The control group was selected based on matching for age and sex. Subjects with hyperglycemia and dyslipidemia were excluded from this study.

The project and informed consents were reviewed and approved by ethical committee on researches involving human subjects, based on the Declaration of Helsinki, Theptarin General hospital, Bangkok, Thailand (Approved No. 1/2007).

Written inform consents were obtained from all subjects after explanation of the purpose, nature and potential risks of this study to the subjects.

3.1.2 Methodology

1. Anthropometric measurement

Anthropometric measurements comprised of height without shoes, weight with clothing, waist circumference (midway between the rib cage and the iliac crest), hip circumference (maximal circumference between the iliac crest and the thigh region) and blood pressure in the seating position. Body surface area was calculated from the Mosteller formula and body mass index was calculated as described in elsewhere.

2. Baseline biochemical test

After 12 hours overnight fasting, blood samples were collected to measure levels of glucose, glycated hemoglobin and lipid profiles. Serum glucose, cholesterol and triacylglycerol were measured with enzymatic techniques by using Vitalab Flexor clinical chemistry analyzer (Vital Scientific NV, Netherlands). The HDL cholesterol was measured after precipitation with phosphotungstate magnesium and the LDL cholesterol was calculated from the Friedewald equation (Friedewald et al, 1972) The glycated hemoglobin was measured by immunoturbidity assay. Serum insulin was measured by using ICMA assay (Immunochemiluminometric assay) with a Immutite 100 LKIN, DPC autoanalyzer. Homeostasis model assessment (HOMA), the insulin-resistant index, was estimated by homeostasis model assessment insulin resistance (HOMA-IR) index derived from fasting plasma insulin and glucose concentrations (insulin (μU/mL) x glucose (mg/dl)/405) (Matthews et al, 1985) ;

$$\text{HOMA-IR} = \frac{(\text{FIRI X FPG})}{405}$$

Where FIRI is fasting immunoreactive insulin (μU/mL) and

FPG is fasting plasma glucose level (mg/dl)

3. Study protocol

Study began at 8.00 am after 12 hours overnight fast. Fasting blood samples were drawn in EDTA-containing tubes from subjects for baseline biochemical parameters, erythrocyte contents and phospholipids composition analyzed by FTIR-HTS-XT and LC-ESI-MS, respectively. Subjects were given a single standard high fat meal in the form of milkshake with fresh daily cream and sliced butter bread as shown in figure 23. Subjects received 40 g fat/m^2 body surface area. The average of macronutrient energy distribution of the meal was 72% from fat, 24% carbohydrate and 4% protein. The fatty acid composition of the given high fat meal was shown in table 4. The meal was ingested within 20 minutes. Only water was allowed for drinking and subjects were not allowed to exercise or have other meals during 4 hours of experiment. Blood samples were collected in EDTA-containing tubes at four hours after the meal period for postprandial biochemical parameters. Single blood collection at 4 hours after the meal ingestion was used based on *in vitro* study in human erythrocyte, showing that the exchange or incorporation of fatty acid into lipid fraction of erythrocyte occurred at the first hour after incubation (Oliveira and Vaughan, 1964; Hodson et al, 2009) and the plasma triacylglycerol level reached to the maximum around 4-5 hours after the high fat meal administration (Guerci et al, 2001; Karamanos et al, 2001).

4. Study compliance

The compliance of all subjects on the test day was judged by observation of consumption. All subjects tolerated the test meal well.

Figure 23 Standard high fat meal used in this study

Table 4 Fatty acid composition of the given high fat meal

Fatty acid	Weight (%)
C8:0	0.48
C10:0	1.13
C12:0	5.27
C14:0	8.32
C16:0	39.89
C18:0	9.58
C19:0	0.07
C20:0	0.24
C22:0	0.06
C24:0	0.13
C16:1	0.91
C18:1	28.68
C22:1	0.13
C18:2	4.77
C20:2	0.08
C18:3	0.17
C20:4	0.10
MUFA/SFA Ratio	0.46
PUFA/SFA Ratio	0.08

3.2 Part II: FT-IR spectroscopic study of the biochemical changes of erythrocyte contents in type 2 diabetes after high fat meal period

3.2.1 Materials and methods

Materials

1. Blood samples

Blood samples from all subjects were collected in EDTA-containing tubes before and four hours after the meal period.

2. Chemicals and solvents

Deionized water was produced from Milli-Q synthesis system (Millipore, Milford, MA, USA)

3. Instruments

All experiments were carried out at the Lipid and Fat Science Research Center (LiFARC), Faculty of Allied Health Science and the Halal Science Center, Chulalongkorn University. The main instruments employed in this experiment were listed as below:

1. Fourier transform infrared spectrometer (Tensor 27) coupled with HTS-XT (Bruker Optics GmbH, Germany)
2. Deep freezer -80°C (ilShin Lab, Japan)
3. Refrigerated centrifuge CF 7D2 (Hitachi, Japan)
4. Vortex mixer (Labnet, USA)

3.2.2 Methodology

1. Erythrocyte preparation for FTIR-HTS-XT

After removing plasma, the erythrocyte was washed 3 times immediately with 3 volumes of ice-cold isotonic solution containing 0.15 M NaCl. The buffy coat was removed by aspiration after each wash. The packed erythrocyte was gently mixed and divided into small aliquots and kept under nitrogen at -80^{o}C until analysis.

2. FTIR spectrometer and sampling plate

A Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Germany) equipped with the high throughput extension (HTS-XT) accessory was used for the erythrocyte measurements. The HTS-XT is a micro-plate reader for automated infrared spectroscopic analysis of large sample numbers as presented in the standardized micro-plate format. A 96-well silicon plate (Bruker Optics GmbH) was used as a sampling plate for the erythrocyte measurements. The preparation of erythrocyte was used according to the method described by Petibois et al. with slightly modification (Petibois and Deleris, 2005). A 20 μl of washed packed erythrocyte was diluted with 180 ul of water and mixed for 60 seconds. Duplicated dry films were made for each sample by applying 5 μl of the hemolysate to 96 wells silicon microplate. The hemolysate films were left dry at 22^{o}C in desiccator under vacuum to prevent oxidation. After the films were dried, the microplate was mouthed in the Tensor 27 FTIR spectrometer coupled with the high throughput extension (HTS-XT) to enable acquisition of infrared spectra. The system was purged with dry nitrogen to reduce water vapor and CO_2. All samples were analyzed in the same period.

The infrared spectra were collected from 400 to 4000 cm^{-1} by a deuterium triglycine sulfate (DTGS) detector. For each acquisition, 512 interferogram scans and a spectral resolution of 4 cm^{-1} were used. A Blackman–Harris three term apodization and a zero filling factor of 2 were applied. Before recording the erythrocyte spectra, the spectra of a well of the blank silicon plate were collected and subtracted later from the spectra of the dried erythrocyte film on the silicon plate.

3. Spectral pre-processing and evaluation

The spectral pre-processing and evaluation was done using Bruker OPUS Software Version 6.0. Second derivative were performed to all spectra to resolve and enhance the weak bands and to remove variation in baselines. Vector normalization was then used in order to reduce the variation of film thickness (Kondepati et al, 2008).

The IR bands were the maximum in absorption spectra but it appeared as the minimum in second derivative spectra and of which intensity or integrated area arising from observed bands is directly proportional to the concentration. Therefore, it can be used for monitoring the concentration-sensitive changes (Toyran et al, 2006). The second derivative of the original spectra was used for identification the peak frequencies of characteristic components. The total peak areas were calculated from the second derivative spectra by using Optics User Software (OPUS), Version 6.0 (Bruker Optics, GmbH). Band assignments of major observed bands in IR spectra of erythrocyte were shown in table 5.

Table 5 Band assignments of major functional groups in IR spectra of erythrocyte observed in this study between 3020–1000 cm^{-1}

Frequency (cm^{-1})	Major assignments
Lipid region	
3020 - 3000	Olefinic=CH stretching: unsaturated lipids
2990 - 2945	CH_3asymmetric stretching: phospholipids, cholesterol esters, fatty acids
2945 - 2905	CH_2asymmetric stretching: phospholipids, long chain fatty acids
2885 - 2860	CH_3 symmetric stretching: phospholipids, fatty acids
2860 – 2840	CH_2 symmetric stretching: phospholipids, long chain fatty acids
Protein region	
1643 - 1611	Amide I: β-sheet structure
1672 - 1644	Amide I: α-Helix structure
1689 - 1673	Amide I: β-turn structure
1560 - 1527	Amide II: Protein N–H bending, C–N stretching
Sugar-phosphate region	
1260 - 1219	PO^{2-} asymmetric stretching: phospholipids
1139 - 1115	C–O stretching: Lactate
1064 – 1036	C–O stretching: Glucose

3.3 Part III: LC-MS study on the changes of erythrocyte phospholipids molecular species after high fat meal period in control and type 2 diabetic group

3.3.1 Materials and methods

Materials

1. Blood samples

Blood samples from all subjects were collected in EDTA-containing tubes before the meal and four hours after the meal period.

2. Chemicals and solvents

1. Deionized water was produced from Milli-Q synthesis system (Millipore, Milford, MA, USA)
2. Methanol HPLC grade (Merck KGaA, Darmstadt, Germany)
3. Dichloromethane HPLC grade (Merck KGaA, Darmstadt, Germany)
4. n-Hexane HPLC grade (Merck KGaA, Darmstadt, Germany)
5. Isopropanol HPLC grade (Merck KGaA, Darmstadt, Germany)
6. Standard Phospholipids , Avanti Polar Lipids, Inc.(Alabaster, AL, USA)
   - 1,2-Dimyristoyl-*sn*-Glycero-3-[phospho-L-serine]
   - 1,2-Dimyristoyl-*sn*-Glycero-3-phospho ethanolamine
   - 1-Heptadecanoyl-2-Hydroxy-*sn*-Glycero-3-phosphocholine
   - 1,2-Dimyristoyl-*sn*-Glycero-3-[phosphor-rac-(1-glycerol)]
   - 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine, 14:1 (Δ9-Cis) PC
7. EDTA (Ethylenediaminetetraacetic acid)
8. Formic acid (Merck KGaA, Darmstadt, Germany)
9. Ammonia Solution 25% (Merck KGaA, Darmstadt, Germany)

3. Instruments

All experiments were carried out at the Lipid and Fat Science Research Center (LiFARC), Faculty of Allied Health Science and the Halal Science Center, Chulalongkorn University. The main instruments employed in this experiment were listed as below:

1. HPLC system HP 1100 series, Agilent Technologies (Palo Alto, CA, USA) coupled on-line to Esquire HCT Ion trap mass spectrometer (Bruker Daltonics GmbH, Germany) with an Electrospray ionization source
2. Deep freezer -80°C (ilShin Lab, Japan)
3. Refrigerated centrifuge CF 7D2 (Hitachi, Japan)
4. Electronic balance with 4 digits CP2245, (Sartorius, Germany)
5. Dry block heater (Ratek, Australia)
6. Vortex mixer (Labnet, USA)

3.3.2 Methodology

1. Standard phospholipids preparation

The phospholipid standards were dissolved (approximately 1 mg/ml) in chloroform/methanol (2:1, v/v), and further diluted with hexane/isopropanol (4:1, v/v).

2. Erythrocyte preparation

Venous blood samples were taken from subjects and placed into Vacutainer tube pretreated with EDTA. Plasma was separated by low speed centrifugation at 3,000 rpm for 10 min at 4°C using a refrigerated centrifuge CF7D2 (Hitachi, Tokyo, Japan)

After removing plasma, the erythrocyte was washed 3 times with 3 volumes of ice-cold isotonic solution containing 0.15 M NaCl. The buffy coat was removed by aspiration after each wash. The packed erythrocyte was gently mixed and divided into small aliquots of 0.5 ml and kept under nitrogen at -80°C until analysis.

3. Erythrocyte phospholipid extraction

A 0.5 ml aliquot of the packed erythrocytes was taken for lipid extraction according to the method described by Blight and Dyer with modification (Blight and Dyer, 1959). Briefly, 500 ul of deionized water was added to 500 ul of the packed erythrocyte, then 3.75 ml of dichloromethane/methanol (1:2 v/v) and appropriate amounts of internal standards (50 μg) were added and the solution was mixed by vortex for 60 seconds. Then 1.25 ml of dichloromethane was added and vortex well. Finally, 1.25 ml of deionized water was added for giving phase separation before the solution was mixed for 60 seconds and centrifuged at 2,500 rpm for 10 min at 4°C using a refrigerated centrifuge CF7D2 (Hitachi, Tokyo, Japan). The lower chloroform phase was collected and dried by evaporation under nitrogen using a dry block heater

(Model.DBH20D, Ratek, Vic, Australia). The samples were stored dry at -20°C until analysis.

Prior to analysis, the extracted samples were reconstituted with 100 μl of chloroform/methanol (2:1, v/v) and further diluted with hexane/isopropanol (4:1, v/v), of which 5 μl was injected into LC-MS system.

4. Chromatographic conditions

An HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used. The lipids were separated on a diol column, Nucleosil 100-OH (Macherey-Nagel, Germany), (250 mm x 3.0 mm id. x 5 um particles size). The linear solvent gradient was used according to the method described by Wang C, et al with modification. Hexane/isopropanol (4:1, v/v) was used as mobile phase A, and mobile phase B was isopropanol/water/formic acid/ammonia (89.3:10:0.2:0.5, v/v/v/v). Separation was obtained by using a gradient elution starting at 30% B, increasing to 60% in 22 min, then maintained for another 2 min. After that, mobile phase B was increased to 80% over the following 11 min and maintained at this level for additional 28 min. Finally, the solvent B was quickly decreased to 30% in 2 min and the column was re-equilibrated for about 5 min before next injection. The flow rate was 0.45 mL/min and the column temperature was 35 °C. The volume of sample injection was 5 μl.

5. ESI-MS condition

The HPLC system was coupled on-line to Esquire HCT Ion trap mass spectrometer (Bruker Daltonics GmbH, Germany) with an electrospray Ionization source. The analytes eluted from HPLC column were directly entered the MS through a steel ES ionization needle set at 4.5 kV in the negative ion mode. The dry nitrogen gas flow rate was approximately 8.0 L/min at 300°C. All ion source and ion optic parameters were optimized with respect to the negative ion of the phospholipids standards. The MS data were collected under full scan mode (500-1,000 m/z at a rate of five spectra for

each time point). PE, pPE, PI and PS were all analyzed in negative mode as $[M–H]^-$. PC, SM and Lyso PC are zwitterionic molecules, they can be analyzed in both negative and positive mode. Thus, in this experiment, these phospholipids molecular species were well detected as the formate adduct, $[M+45]^-$.

Since there was the combination of phospholipids molecular species that have the same m/z (Isobaric species), thus, in the present study the phospholipids molecular species were displayed as the total number of carbon atoms and double bonds in the fatty acid moiety for lipid classes containing two fatty acids esterified to the glycerol-backbone (PE, PI, PS and PC). For example, a PC 34:1 was represented different combination of fatty acids such as 18:0/16:1, 16:0/18:1, etc. Lysophospholipids (LPC) and sphingolipids (SM) contain one fatty acid denominated by the species nomenclature such as LPC16:0. (Leidl et al, 2008).

6. Data analysis

All chromatograms and spectra of phospholipids were analyzed by using Data analysis™ software version 3.2 (Bruker Daltonik GmbH, Germany). In this study, absolute phospholipid concentrations can not be shown due to the differences in the ionization efficiency and instrument response of the different head groups and also of the unsaturated degree of acyl chains in phospholipid structure (Koivusalo et al, 2001). Thus, all phospholipids molecular species obtained after high fat meal period were calculated as percentage relative to its total concentration in fasting state (Before giving a meal) as such calculation has been used for monitoring the changes of phospholipids molecular species in the study regarding the chemical-induced modification in phospholipids (Davis et al, 2008).

3.4 Statistical analysis

All variables were normal distributions tested by One Sample-Kolmogorov-Smirnov Test. Values were expressed as mean ± SEM. The paired and 2-sample t tests were used for comparison between fasting state and 4 hours postprandial period, and between control and type 2 diabetic group, respectively. Pearson correlation analysis was used to find the relationships between HOMA (Insulin resistance index) and biochemical parameters including phospholipids molecular species. Statistical significance means significant difference at p value < 0.05. All calculations were performed using SPSS software version 11.5 (SPSS Inc, Illinois, US).

CHAPTER IV

RESULTS

4.1 Part I: Study on the postprandial biochemical response after high fat meal period in control and type 2 diabetic group

4.1.1 Clinical and metabolic characteristics of subjects

Twelve type 2 diabetic and eleven healthy control subjects were enrolled in this study. After screening of fasting biochemical parameters, 1 of 12 in type 2 diabetic group was excluded due to abnormal liver function and 1 of 11 in control group was excluded due to abnormal fasting triacylglycerol.

Therefore, the results in the present study were then based on the remaining of 21 subjects. The clinical and metabolic characteristics of subjects were shown in Table 6. As expected, all type 2 diabetic subjects had fasting glucose, HbA_1C and HOMA index (Insulin resistance index) that were all significant difference from control group. Fasting insulin in type 2 diabetic group was higher than that in control group, however, it didn't reach statistically significant difference. For fasting lipid profile, it has been found that no significant difference was observed in type 2 diabetic group as compared to control group.

Table 6 Clinical and metabolic characteristics of all type 2 diabetic and control group*

Parameters	Control (n=10)	Type 2 diabetes (n=11)
Age (years)	46.0 ± 1.7	48.36 ± 2.2
Sex (M/F)	4/6	4/7
Duration of diabetes (years)	-	6.5 ± 1.0
Systolic blood pressure (mmHg)	121.0 ± 4.8	123.8 ± 6.8
Diastolic blood pressure (mmHg)	68.1 ± 1.8	77.9 ± 3.6 [a]
Height (cm)	158.4 ± 2.1	162.6 ± 2.5
Weight (kg)	59.3 ± 2.1	68.9 ± 5.0
Body surface area (m^2)	1.61 ± 0.03	1.74 ± 0.07
BMI (kg/m^2)	23.64 ± 0.72	25.89 ± 1.56
Waist (cm)	80.30 ± 2.10	86.14 ± 3.28
Hip (cm)	96.80 ± 1.45	100.27 ± 2.29
WHR	0.83 ± 0.02	0.86 ± 0.02
Fasting Glucose (mg/dl)	89.0 ± 2.7	130.1 ± 9.1 [a]
Fasting Insulin (μU/ml)	5.20 ± 0.80	7.55 ± 1.52
HbA_1C (%)	5.7± 0.2	7.3 ± 0.4 [a]
Total cholesterol (mg/dl)	203.5 ± 13.6	188.7 ± 7.0
Triacylglycerol (mg/dl)	99.6 ± 11.9	110.2 ± 14.8
HDL cholesterol (mg/dl)	51.7 ± 3.6	46.0 ± 2.8
LDL cholesterol (mg/dl)	131.8 ± 12.6	120.7 ± 7.2
HOMA-IR	1.17 ± 0.20	2.37 ± 0.46 [a]

* Values were expressed as mean ± SEM

[a] $p < 0.05$ vs. control group

4.1.2 Correlation between Insulin resistance index (HOMA) and some baseline biochemical parameters

Since insulin resistance is accepted as a risk factor of many diseases, as well as causing hypertension, dyslipidemia and atherosclerotic vascular disease. In order to find the relationship of some clinical parameters associated with insulin resistance, the correlations between HOMA (Insulin resistance index) and baseline biochemical parameters were performed.

The results have revealed that some baseline parameters were significantly correlated with HOMA. Insulin resistant index measured by HOMA was strongly significant correlated with weight (r = 0.774, p<0.001), Body Mass Index (BMI)(r = 0.654, p = 0.001), Waist to hip ratio (WHR) (r = 0.547, p<0.01), fasting triacylglycerol (r = 0.679, p = 0.001) but inversely correlated with HDL Cholesterol (r = -0.542, p = 0.011)

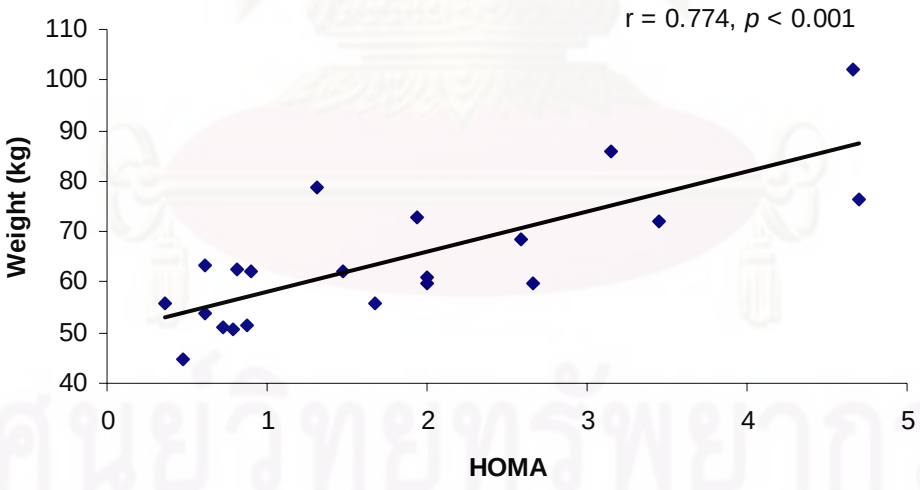


Figure 24 Relationship between HOMA (Insulin resistance index) and weight

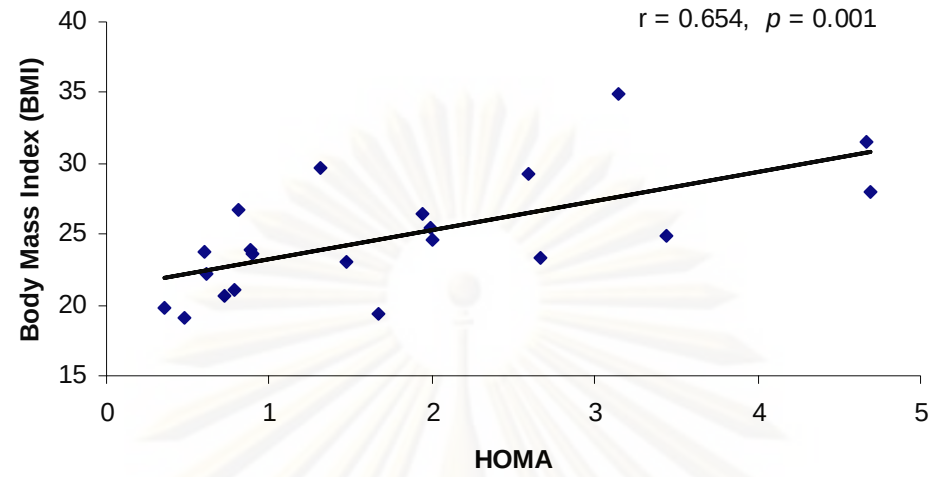


Figure 25 Relationship between HOMA (Insulin resistance index) and body mass index (BMI)

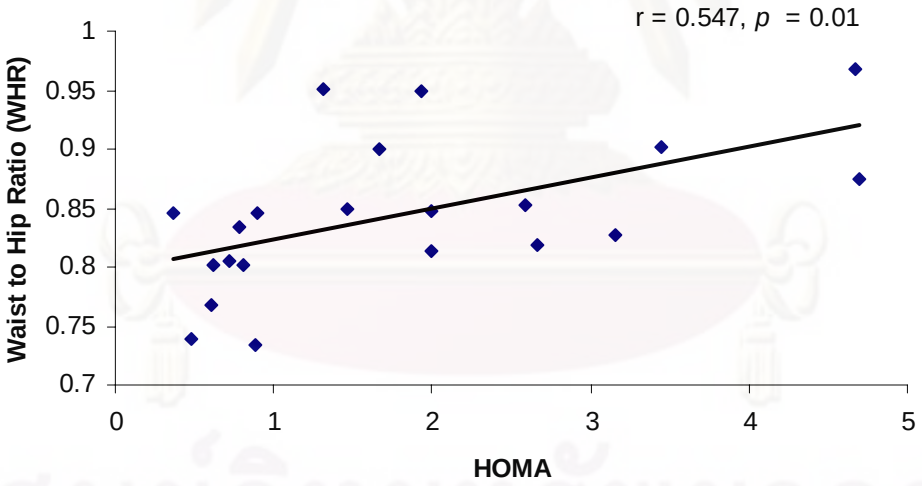


Figure 26 Relationship between HOMA (Insulin resistance index) and waist to hip ratio (WHR)

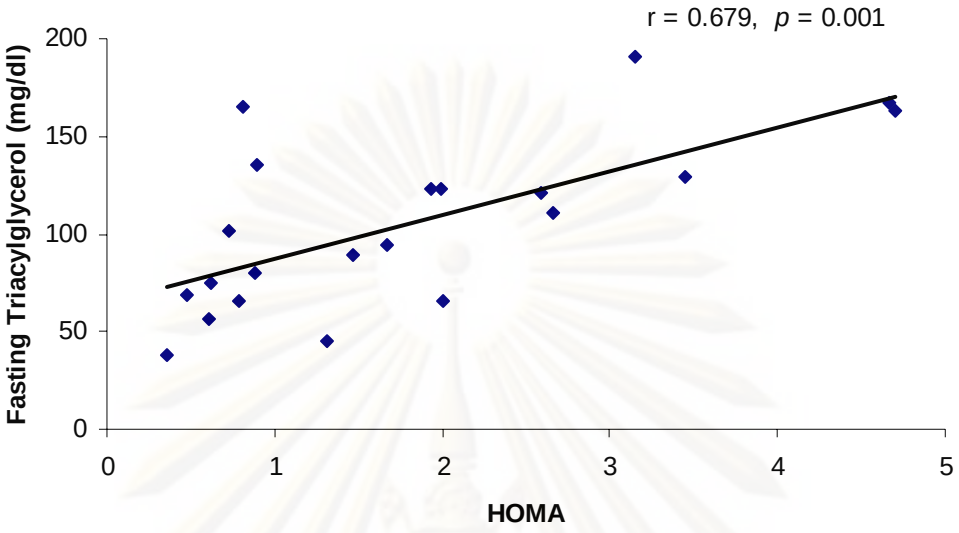


Figure 27 Relationship between HOMA (Insulin resistance index) and fasting triacylglycerol

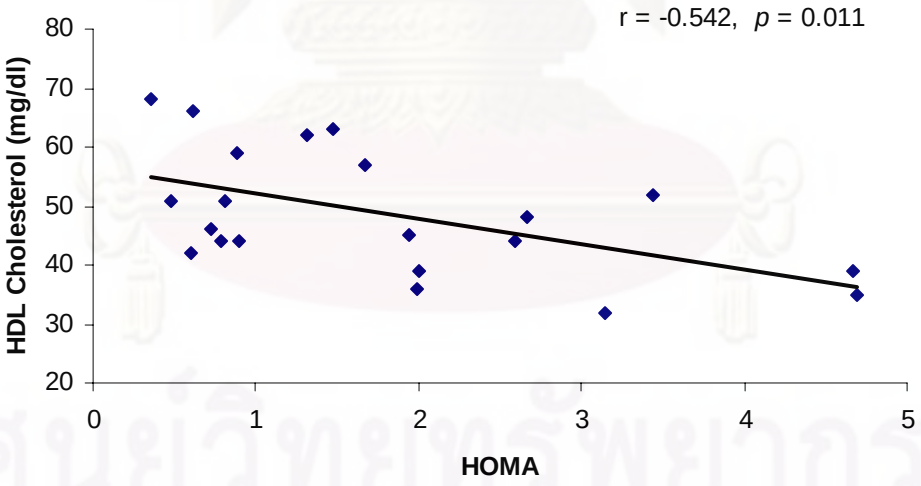


Figure 28 Relationship between HOMA (Insulin resistance index) and HDL-Cholesterol

4.1.3 Postprandially biochemical response after high fat meal period

After the meal period, both groups showed lipemic plasma compared to that in fasting state (Figure 29). Postprandially biochemical parameters were presented in table 7. Postprandial glucose in both groups tend to decrease to the baseline level, however, postprandial glucose in type 2 diabetic group was still higher than control group. In addition, postprandial glucose in control group was found to be significantly higher than fasting state.

Four hours postprandial insulin in both groups was significantly increased as compared to fasting state. Postprandial insulin in type 2 diabetic group was significantly higher than that in control group. The change in insulin concentrations (Δ insulin) was shown in Figure 30. The differences of insulin between fasting and four hours postprandial period in control group and type 2 diabetic group were 4.20 ± 1.70 μU/ml and 10.91 ± 2.50 or 96% and 180% as compared with fasting level, respectively.

For four hours postprandial lipid profile, the significant increase of postprandial cholesterol compared with fasting state was observed only in type 2 diabetic group. Postprandial triacylglycerol in both groups was significantly increased from fasting state. As shown in figure 31, the changes of postprandial triacylglycerol (ΔTG) in control group and type 2 diabetic group as compared with fasting state were 139.6 ± 28.3 mg/dl and 159.9 ± 25.5 mg/dl, respectively. In addition, cholesterol was not found to be significant difference in both groups compared to fasting state.

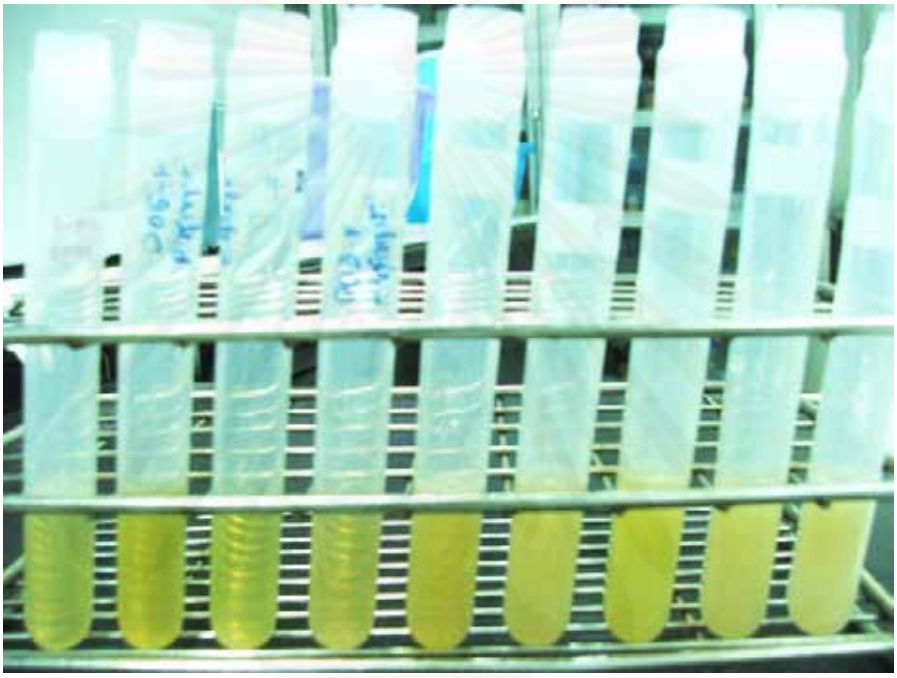


Figure 29 Fasting and four hours postprandial plasma obtained from subjects

Table 7 Four hours postprandial biochemical parameters after high fat meal period*

4 PP parameters	Control (n=10)		Type 2 diabetes (n=11)	
	Fasting	4 PP	Fasting	4 PP
Glucose (mg/dl)	89.0 ± 2.7	99.5 ± 3.9 [a]	130.1 ± 9.1 [b]	132.6 ± 10.2 [b]
Insulin (μU/ml)	5.20 ± 0.80	9.40 ± 1.8 [a]	7.55 ± 1.52	18.45 ± 3.59 [a, b]
Lipid profiles				
- Total cholesterol (mg/dl)	203.5 ± 13.6	204.1 ± 14.7	188.7 ± 7.0	198.6 ± 8.4 [a]
- Triacylglycerol (mg/dl)	99.6 ± 11.9	239.2 ± 32.6 [a]	110.2 ± 14.8	270.1 ± 37.5 [a]
- HDL cholesterol (mg/dl)	51.7 ± 3.6	51.7 ± 3.5	46.0 ± 2.8	45.8 ± 2.4

* Values were expressed as mean ± SEM, 4 PP = 4 hours postprandial period

[a] $p < 0.05$ fasting state vs. 4 hours postprandial period in the same group

[b] $p < 0.05$ vs. control group in the same period

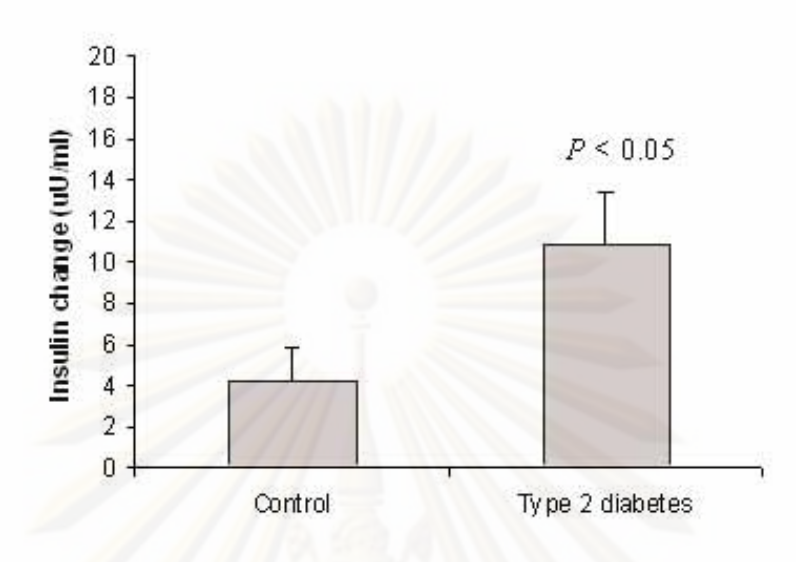


Figure 30 Change in insulin concentrations between fasting and four hours postprandial state. Values were shown as mean change compared to fasting state

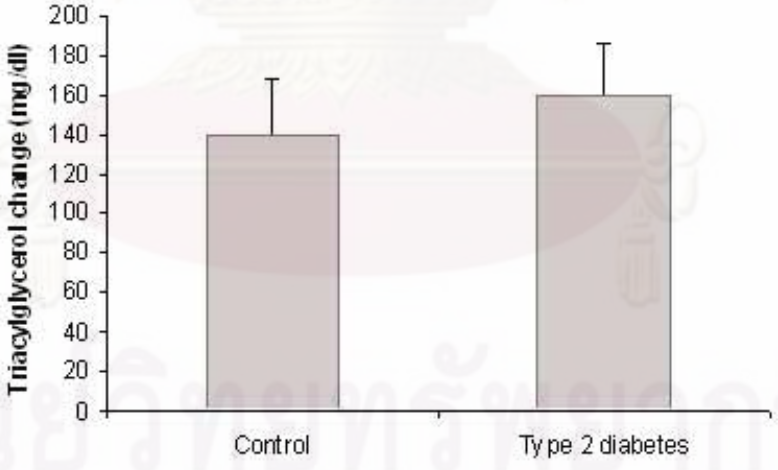


Figure 31 Change in TG concentrations between fasting and four hours postprandial state. Values were shown as mean change compared to fasting state

4.2 Part II: FT-IR spectroscopic study of the biochemical changes of erythrocyte contents in type 2 diabetes after high fat meal period

4.2.1 FT-IR erythrocyte spectra

The dried film of hemolysate was shown in figure 32. The microplate was then mouthed into the FTIR spectrometer coupled with the HTS-XT (Figure 33) for acquisition of the spectra. Typical FT-IR absorption and second derivative spectra obtained from all subjects were shown in Figure 34. After spectra have been preprocessed, the FT-IR absorption bands were transformed to second derivative spectra in order to enhance the intensity of the weak bands, and in which observed bands were integrated in accordance with the band assignments described in Materials and Methods section.

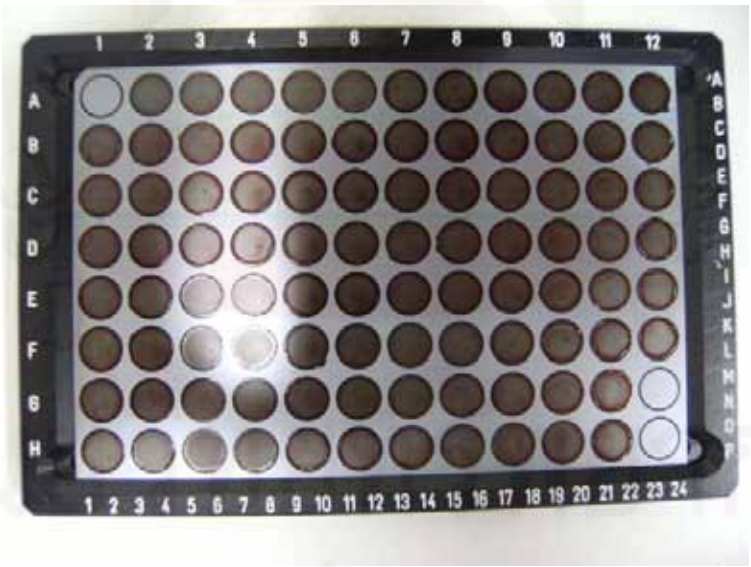


Figure 32 Dried film of erythrocyte on silicon plate of FTIR-HTS-XT

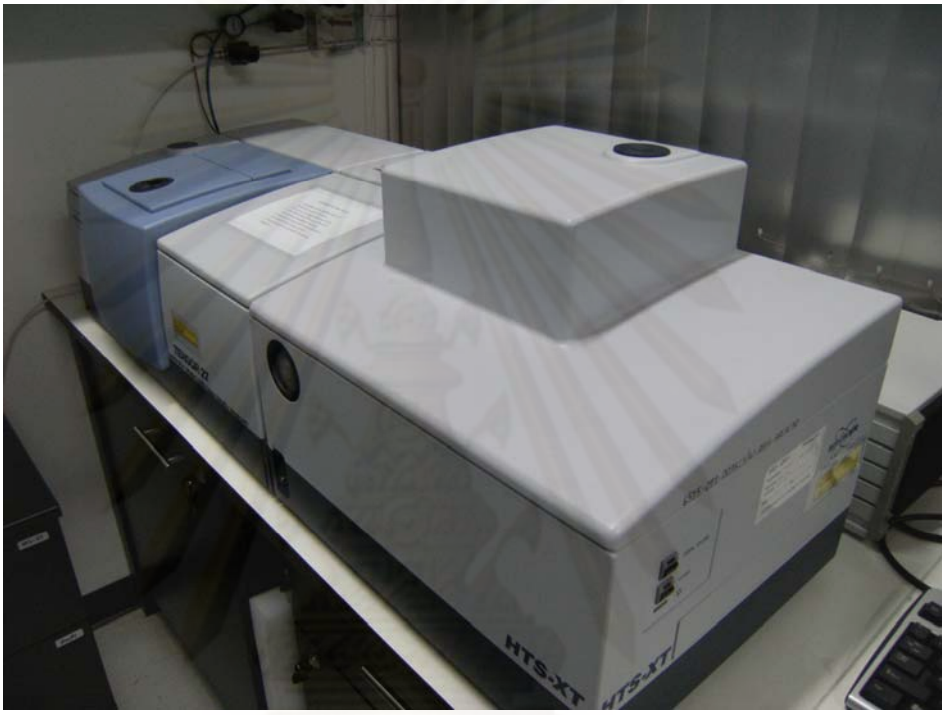


Figure 33 FTR-HTS-XT (Bruker Optics, Germany) used in this study

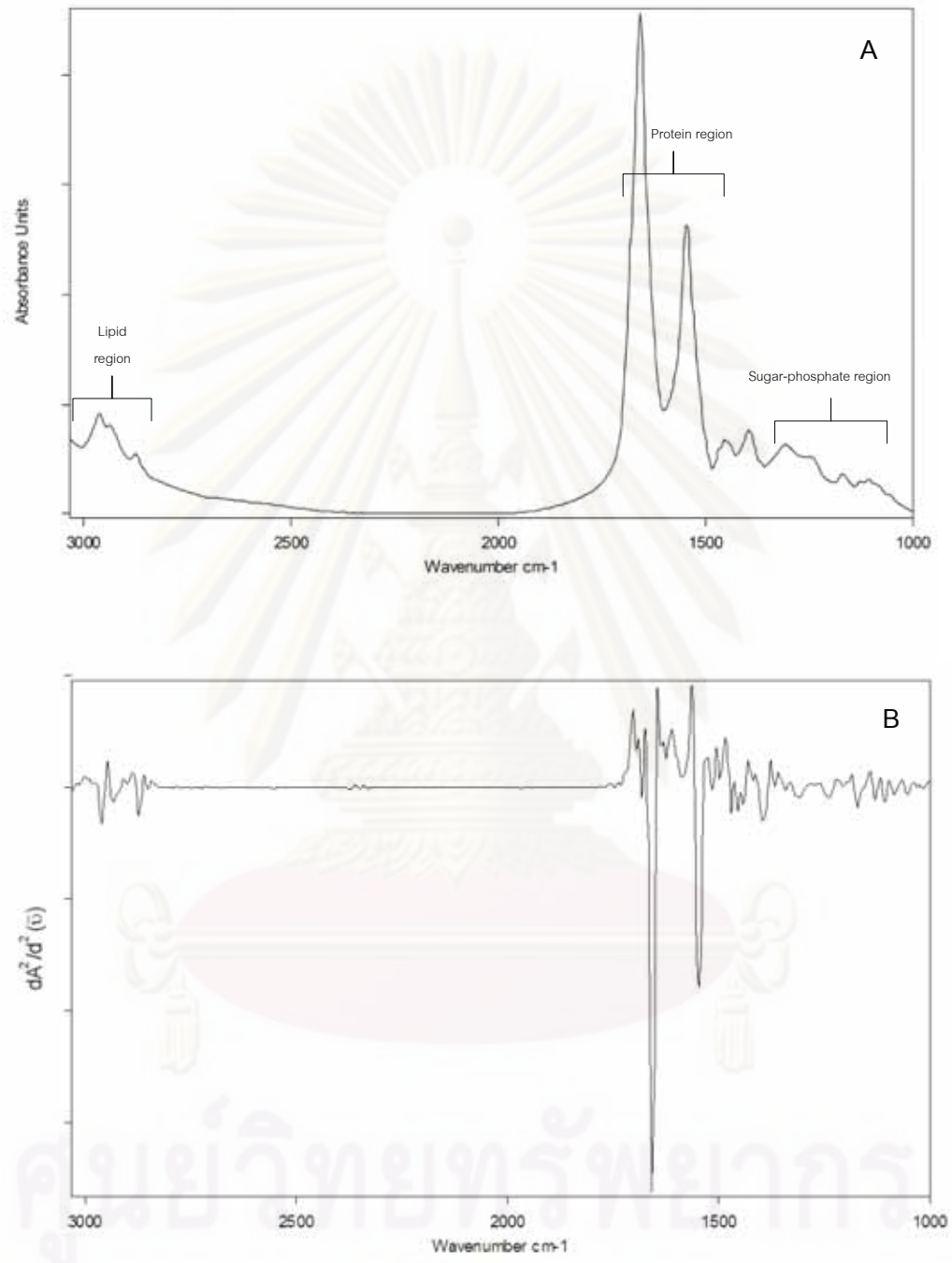


Figure 34 Typical FT-IR absorption (A) and second derivative (B) spectra of erythrocyte observed in this study

4.2.2 The changes in FTIR integrated areas of the major functional groups of erythrocytes obtained from fasting and four hours after the meal period in control and type 2 diabetic group

1. Diabetes-induced alterations in erythrocytes contents observed in fasting state

In order to investigate diabetes-induced alterations in erythrocytes contents, the erythrocyte from fasting state in both groups was analyzed by FT-IR and used for finding the differences as compared to control group. Table 8 was shown the changes in integrated areas of lipid region in erythrocytes obtained from fasting state in both groups, all integrated areas rising from erythrocyte lipids in type 2 diabetic group seemed to be higher than those in control group, especially a significant increase in $\nu_s(CH_3)$. However, the integrated area of olefinic band (ν=(CH)) in type 2 diabetic group was found to be lower than that in control group with strongly significant difference ($p<0.001$).

For protein region (table 9), the integrated area of β-turn conformation in type 2 diabetic group was only found to be significantly lower than that in control group. Moreover, the integrated area of Amide II was also significantly lower in type 2 diabetic group compared to control group.

Table 10 was presented the integrated areas of sugar-phosphate region in erythrocytes obtained from fasting state in both groups, PO^{2-} asymmetric stretching vibration (ν(P=O)) which was exclusive from phospholipids was found to be higher in type 2 diabetic group but not reach statistically significant difference. In addition, the integrated areas of C-O stretching from glucose and lactate in type 2 diabetic group were significantly higher than those in control group.

Table 8 Changes in integrated areas of lipid region in erythrocytes obtained from fasting state in type 2 diabetic compared to control group

Functional groups	Control (n=10)	Type 2 diabetes (n=11)	*p* values
Lipid region			
Olefinic=CH stretching, ν=(CH)	-0.034 ± 0.002*	-0.025 ± 0.001	<0.001
CH_3 asym. Stretching, ν_{as} (CH_3)	-1.019 ± 0.006	-1.032 ± 0.010	0.281
CH_2 asym. stretching, ν_{as} (CH_2)	-0.720 ± 0.007	-0.738 ± 0.012	0.183
CH_3 sym. stretching, ν_s (CH_3)	-0.480 ± 0.002	-0.492 ± 0.004	0.012
CH_2 sym. stretching, ν_s (CH_2)	-0.118 ± 0.003	-0.128 ± 0.005	0.097

* All values were expressed as mean ± SEM

Table 9 Changes in integrated areas of protein region in erythrocytes obtained from fasting state in type 2 diabetic compared to control group

Functional groups	Control (n=10)	Type 2 diabetes (n=11)	*p* values
Protein region			
Amide I : β-turn structure	-0.766 ± 0.008*	-0.703 ± 0.002	<0.001
Amide I : α-helix structure	-5.582 ± 0.008	-5.568 ± 0.017	0.465
Amide I : β-sheet structure	-0.931 ± 0.004	-0.930 ± 0.006	0.822
Amide II : N–H bending, C–N stretching	-4.033 ± 0.017	-3.937 ± 0.010	<0.001

* All values were expressed as mean ± SEM

Table 10 Changes in integrated areas of sugar-phosphate region in erythrocytes obtained from fasting state in type 2 diabetic compared to control group

Functional groups	Control (n=10)	Type 2 diabetes (n=11)	*p* values
Sugar-phosphate region			
PO^{2-} asym. stretching, ν(P=O)	-0.337 ± 0.005*	-0.347 ± 0.005	0.173
C-O stretching (Lactate)	-0.271 ± 0.004	-0.289 ± 0.005	0.004
C-O stretching (Glucose)	-0.165 ± 0.002	-0.173 ± 0.003	0.047

* All values were expressed as mean ± SEM

4.2.3 Changes of erythrocytes contents after high fat meal period as compared to fasting state in both groups

High fat meal was given to all subjects in order to study the effect of transient hypertriacylglycerolemia on the changes of erythrocyte contents analyzed by FT-IR. Table 11 and 14 were shown changes in integrated areas of lipid region in erythrocytes obtained from fasting and 4 hours after the meal period in control and type 2 diabetic group, respectively. In type 2 diabetic group, the integrated area of ν=(CH) still remained unchanged after the meal period whereas $\nu_{as}(CH_3)$, $\nu_{as}(CH_2)$, $\nu_{s}(CH_3)$ and $\nu_{s}(CH_2)$ seemed to be decrease when compared to those in the fasting state. In contrast to diabetic group, all integrated areas of lipid stretching vibrations in control group seemed to be increased as compared to those in fasting state, especially a strongly significant increase of ν=(CH) (p<0.001).

The conformations of protein at four hours after the meal period were also changed. Table 12 and 15 were presented the changes in integrated area of protein region in control and type 2 diabetic group, respectively. All integrated areas of protein conformations from Amide I and Amide II in control group were significantly different from those in fasting state. However, only the integrated area of β-turn conformation in type 2 diabetic group was found to be significantly increased as compared to fasting state.

As shown in table 13 and 16, in type 2 diabetic group, the integrated area of ν(P=O), which is mainly from phosphate moiety in phospholipid structure, was found to be lower than that in fasting state but not reach significant difference (p=0.121). This was in contrast with control group that the increase in integrated area of ν(P=O) was found to be significantly increased when compared to fasting state. Additionally, the significant decreases in integrated areas of C-O stretching from glucose and lactate were observed in type 2 diabetic group after the meal period whereas control group had apparently significant increases in integrated areas of those when compared to fasting state.

Control group

Table 11 Changes in integrated areas of lipid region in erythrocytes obtained from fasting and 4 hours after the meal period in control group

Functional groups	Fasting	4 hours PP	*p* values
Lipid region			
1. Olefinic=CH stretching, ν=(CH)	-0.034 ± 0.002*	-0.058 ± 0.002	<0.001
2. CH_3 asym. Stretching, ν_{as} (CH_3)	-1.019 ± 0.006	-1.053 ± 0.006	<0.001
3. CH_2 asym. stretching, ν_{as} (CH_2)	-0.720 ± 0.007	-0.732 ± 0.005	0.076
4. CH_3 sym. stretching, ν_s (CH_3)	-0.480 ± 0.002	-0.482 ± 0.003	0.544
5. CH_2 sym. stretching, ν_s (CH_2)	-0.118 ± 0.003	-0.116 ± 0.002	0.489

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

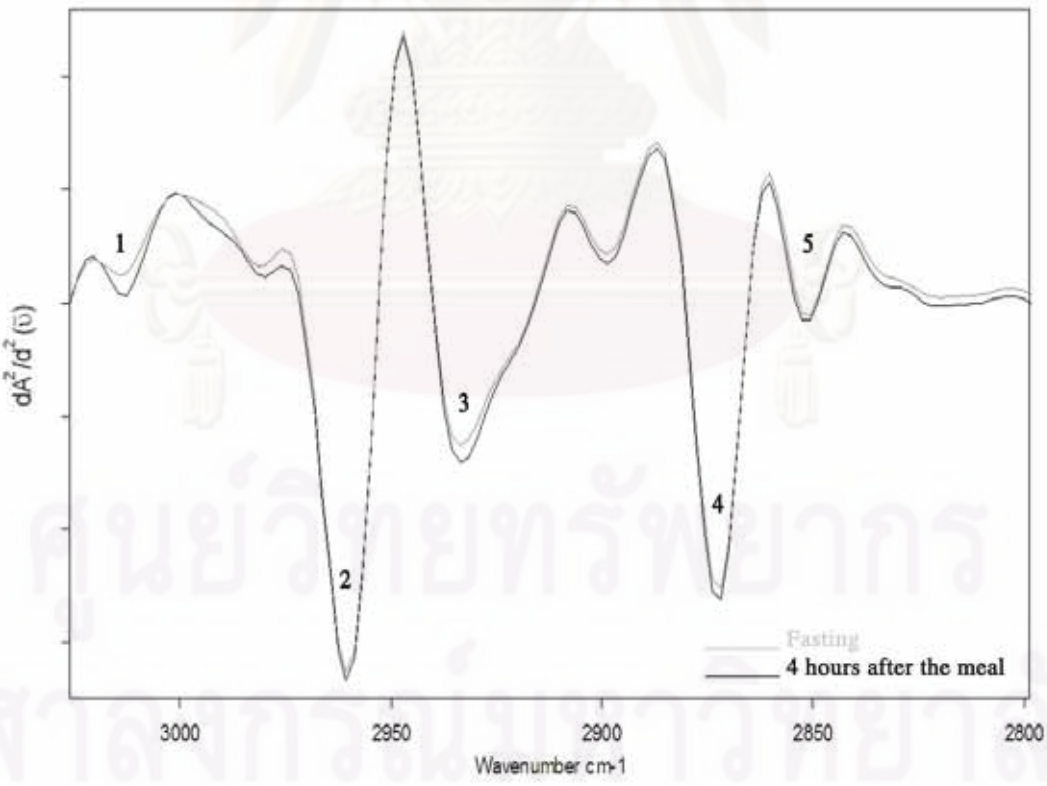


Figure 35 Second derivative average spectra in lipid region of erythrocytes obtained from fasting and 4 hours after the meal period in control group

Table 12 Changes in integrated areas of protein region in erythrocytes obtained from fasting and 4 hours after the meal period in control group

Functional groups	Fasting	4 hours PP	*p* values
Protein region			
6. Amide I : β-turn structure	-0.766 ± 0.008*	-0.846 ± 0.005	<0.001
7. Amide I : α-helix structure	-5.582 ± 0.008	-5.446 ± 0.026	<0.001
8. Amide I : β-sheet structure	-0.931 ± 0.004	-0.954 ± 0.007	0.011
9. Amide II : N–H bending, C–N stretching	-4.033 ± 0.017	-4.198 ± 0.016	<0.001

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

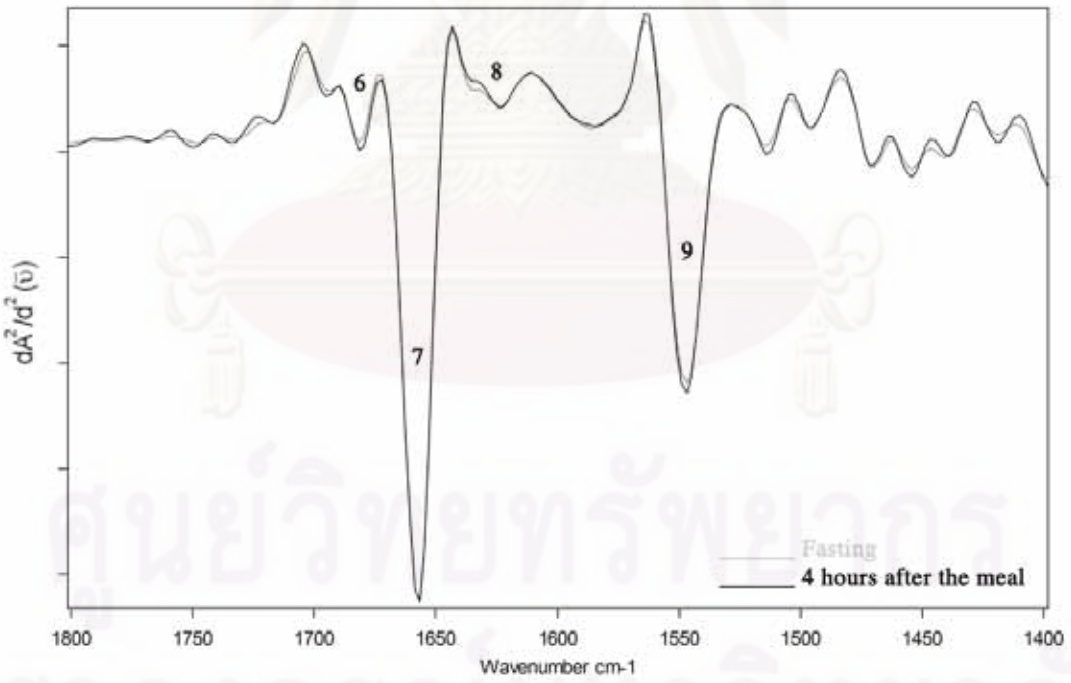


Figure 36 Second derivative average spectra in protein region of erythrocytes obtained from fasting and 4 hours after the meal period in control group

Table 13 Changes in integrated areas of sugar-phosphate region in erythrocytes obtained from fasting and 4 hours after the meal period in control group

Functional groups	Fasting	4 hours PP	*p* values
Sugar-phosphate region			
10. PO^{2-} asym. stretching, ν (P=O)	-0.337 ± 0.005*	-0.376 ± 0.006	<0.001
11. C-O stretching (Lactate)	-0.271 ± 0.004	-0.295 ± 0.005	0.001
12. C-O stretching (Glucose)	-0.165 ± 0.002	-0.173 ± 0.002	0.017

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

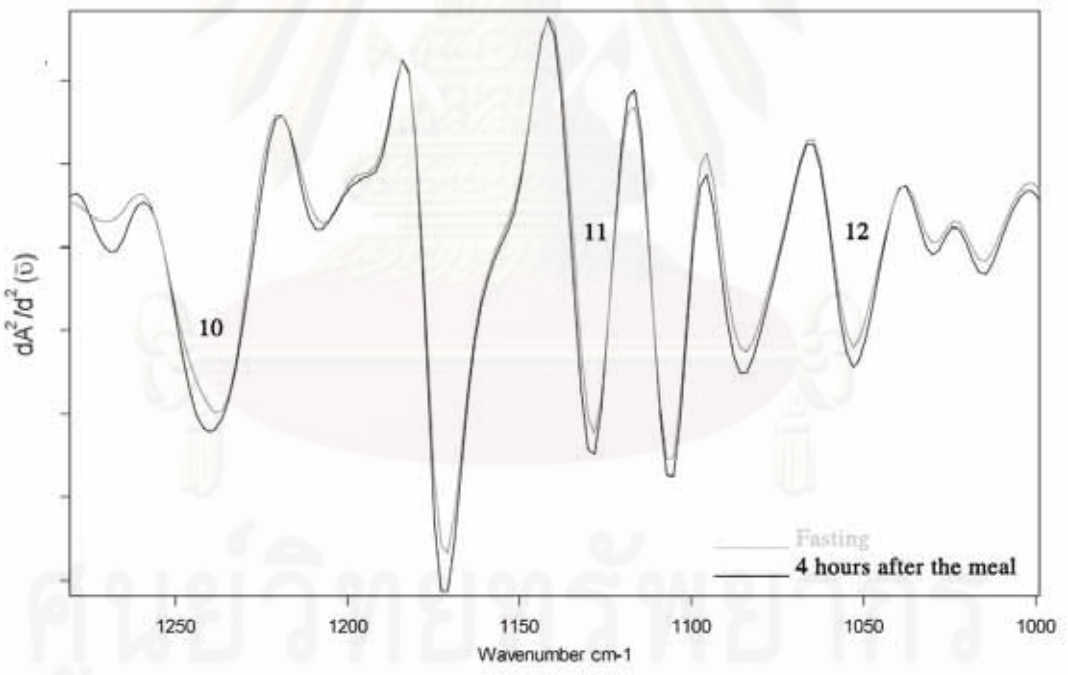


Figure 37 Second derivative average spectra in sugar-phosphate region of erythrocytes obtained from fasting and 4 hours after the meal period in control group

Type 2 diabetic group

Table 14 Changes in integrated areas of lipid region in erythrocytes obtained from fasting and 4 hours after the meal period in type 2 diabetic group

Functional groups	Fasting	4 hours PP	*p* values
Lipid region			
1. Olefinic=CH stretching, ν=(CH)	-0.025 ± 0.001*	-0.023 ± 0.001	0.209
2. CH_3 asym. Stretching, ν_{as} (CH_3)	-1.032 ± 0.010	-1.006 ± 0.006	0.028
3. CH_2 asym. stretching, ν_{as} (CH_2)	-0.738 ± 0.012	-0.705 ± 0.006	0.006
4. CH_3 sym. stretching, ν_s (CH_3)	-0.492 ± 0.004	-0.483 ± 0.003	0.080
5. CH_2 sym. stretching, ν_s (CH_2)	-0.128 ± 0.005	-0.113 ± 0.002	0.001

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

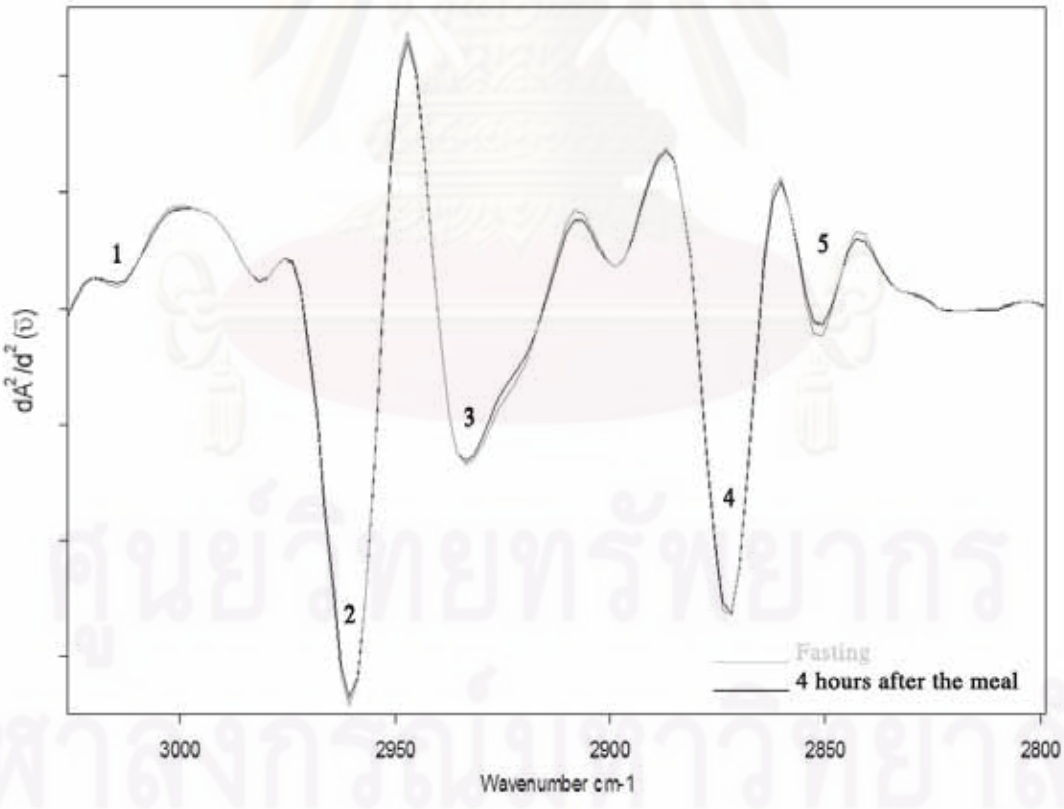


Figure 38 Second derivative average spectra in lipid region of erythrocytes obtained from fasting and 4 hours after the meal period in type 2 diabetic group

Table 15 Changes in integrated areas of protein region in erythrocytes obtained from fasting and 4 hours after the meal period in type 2 diabetic group

Functional groups	Fasting	4 hours PP	*p* values
Protein region			
6. Amide I : β-turn structure	-0.703 ± 0.002*	-0.722 ± 0.003	<0.001
7. Amide I : α-helix structure	-5.568 ± 0.017	-5.603 ± 0.009	0.114
8. Amide I : β-sheet structure	-0.930 ± 0.006	-0.935 ± 0.003	0.359
9. Amide II : N–H bending, C–N stretching	-3.937 ± 0.010	-3.954 ± 0.007	0.242

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

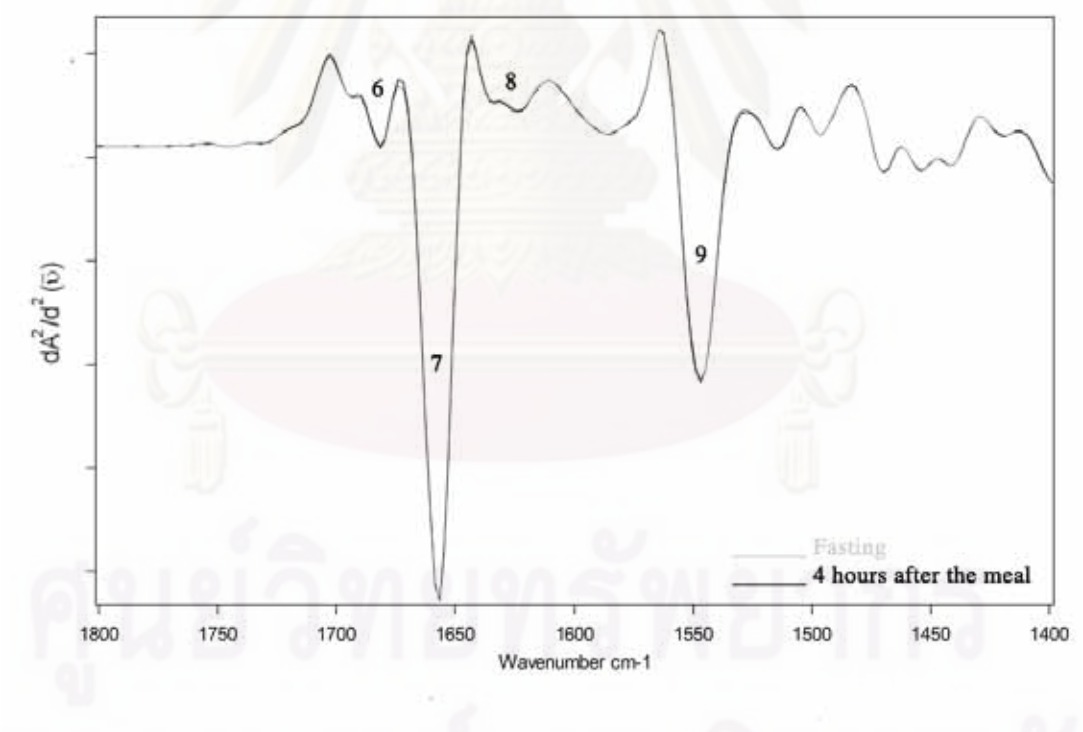


Figure 39 Second derivative average spectra in protein region of erythrocytes obtained from fasting and 4 hours after the meal period in type 2 diabetic group

Table 16 Changes in integrated areas of sugar-phosphate region in erythrocytes obtained from fasting and 4 hours after the meal period in type 2 diabetic group

Functional groups	Fasting	4 hours PP	*p* values
Sugar-phosphate region			
PO^{2-} asym. stretching, ν (P=O)	-0.347 ± 0.005*	-0.338 ± 0.003	0.121
C-O stretching (Lactate)	-0.289 ± 0.005	-0.277 ± 0.004	0.017
C-O stretching (glucose)	-0.173 ± 0.003	-0.162 ± 0.003	0.003

* All values were expressed as mean ± SEM, 4 hours PP = 4 hours postprandial period

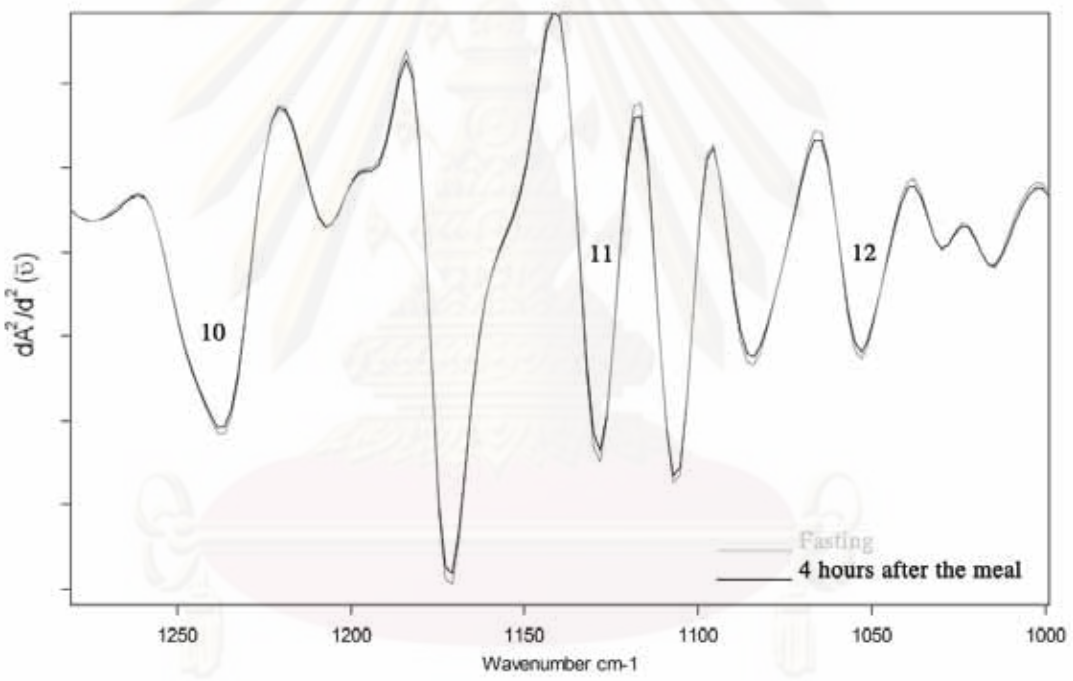


Figure 40 Second derivative average spectra in sugar-phosphate region of erythrocytes obtained from fasting and 4 hours after the meal period in type diabetic group

4.3 Part III: LC-MS study on the changes of erythrocyte phospholipids molecular species after high fat meal period in control and type 2 diabetic group

4.3.1 Separation and characterization of phospholipids classes and molecular species by LC-ESI-MS

The chromatographic system was firstly used to separate phospholipids classes and followed by MS for identification of molecular species. The typical base peak chromatogram was shown in figure 41. Six major classes of erythrocyte phospholipids were measured and it can be seen that PE/pPE was firstly eluted and followed by PI/PS, PC, SM and LPC. Individual phospholipids class was identified by comparing the retention time and the mass spectra with the standards.

Phospholipids molecular species were detected by full-scan ESI-MS analysis in negative mode. Many classes of phospholipids possess net negative charge at neutral pH. Accordingly, negative-ion ESI-mass spectra of these phospholipids can be effectively obtained with $[M–H]^-$ as the molecular ions, such as PE, pPE, PI and PS. However, PC, SM and LPC are zwitterionic molecules, they can also be analyzed by negative mass spectra. Thus, in this experiment, these phospholipids classes were well detected as the formate adduct, $[M+45]^-$. In order to determine the phospholipids molecular species, tandem mass spectrometry were conducted with the ion-trap mass analyzer. Negative ion fragments on MS^2 and MS^3 spectra were formed after collision induced dissociation (CID) and used to determine the structure of molecular species. The mass spectra of phospholipids molecular species of each class were shown in figure 42.

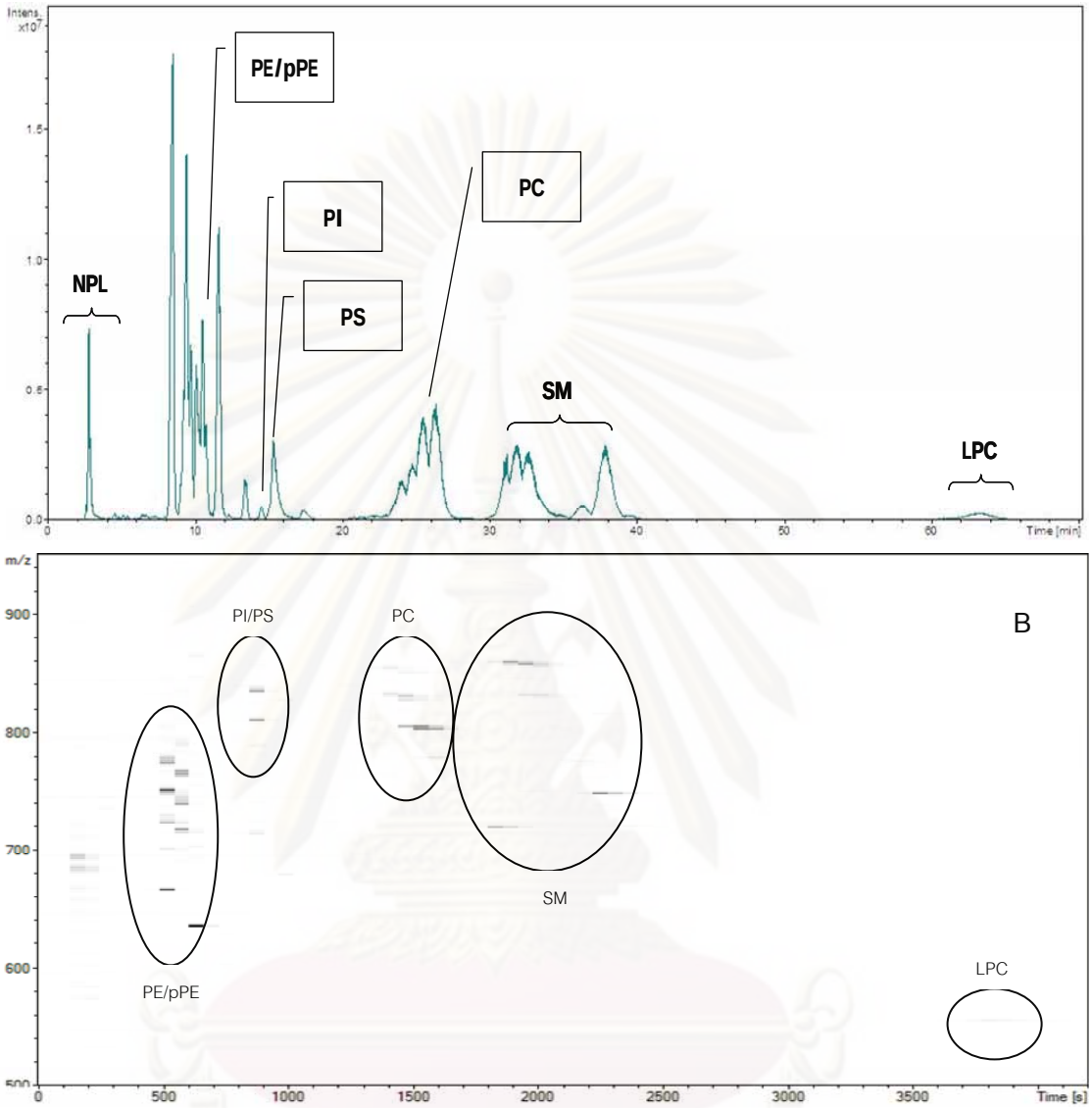


Figure 41 Two dimensional (2D) map of phospholipids (PLs) from erythrocyte obtained by LC-ESI-MS using the normal-phase column in the negative ion mode. The 2D map has the m/z value of $[M–H]^-$ or $[M +45]^-$: (A) Base Peak Chromatogram and (B) 2D map.

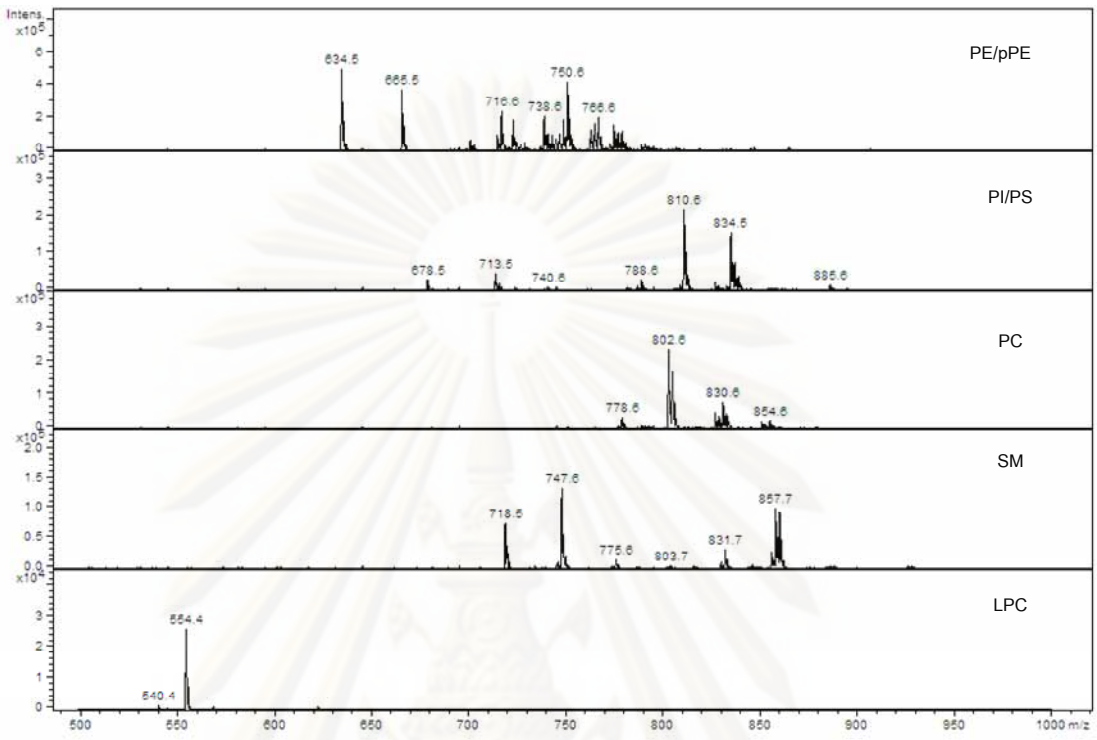


Figure 42 Phospholipids molecular species of each class obtained from erythrocyte. PE: Phosphatidylethanolamine, pPE: Plasmalogen phosphatidylethanolamine, PI: Phosphatidylinositol, PS: Phosphatidylserine, PC: Phosphatidylcholine, SM: Sphingomyelin and LPC: Lysophosphatidylcholine

4.3.2 The overall of the changes in erythrocyte phospholipids molecular species obtained from fasting and postprandial period in both groups

The erythrocyte phospholipids molecular species obtained from fasting and four hours postprandial period in both groups were categorized by unsaturated degree of fatty acyl chain into saturated (SFA), monounsaturated (MUFA) fatty acid-containing species, polyunsaturated fatty acid (PUFA)-containing species (double bond = 2) and highly unsaturated fatty acid (HUFA)-containing species (double bond >2) (Table 17).

In control group, 4 hours after the meal period, the total highly unsaturated fatty acid-containing species of PE and PS was significant decreased relative to fasting state. In contrast with PI and PC, the overall of PI molecular species seemed to be increased from fasting state, especially of SFA, MUFA-containing species whereas the significant increase of total SFA and MUFA-containing PC was found as compared to fasting state. In addition, no significant changes of SM and LPC molecular species were found, however, the total LPC molecular species seemed to be decreased but not reach statistic significance.

In contrast to the control group, type 2 diabetic group seemed to have small changes of phospholipids molecular species after the high fat meal period as compared to fasting state. However, it has been found that some phospholipids classes in type 2 diabetic group were significantly different from control group when compared in the same period.

Table 17 The overall of erythrocyte phospholipids molecular species categorized by unsaturated degree of fatty acyl chain [#]

	Control (n=10)		Type 2 diabetes (n=11)	
	Fasting	4 PP	Fasting	4 PP
Phosphatidylethanolamine (PE)				
SFA and MUFA species	13.71 ± 0.63	14.30 ± 0.56	13.48 ± 0.67	12.84 ± 0.62
PUFA species	6.39 ± 0.35	6.54 ± 0.32	7.73 ± 0.51 [b]	7.40 ± 0.45
HUFA species	29.94 ± 0.65	24.90 ± 1.61 [a]	27.33 ± 0.53 [b]	29.05 ± 1.51
Plasmalogen PE	49.96 ± 0.61	44.05 ± 2.90	51.46 ± 0.85	54.28 ± 2.36 [b]
Phosphatidylserine (PS)				
SFA and MUFA species	6.84 ± 0.52	6.78 ± 0.51	7.48 ± 0.50	7.26 ± 0.47
PUFA species	2.23 ± 0.31	2.43 ± 0.31	2.97 ± 0.55	2.82 ± 0.51
HUFA species	90.93 ± 0.55	68.96 ± 5.24 [a]	89.55 ± 0.77	95.97 ± 5.63 [b]
Phosphatidylinositol (PI)				
SFA and MUFA species	6.08 ± 0.76	17.57 ± 3.89 [a]	6.84 ± 1.08	5.96 ± 0.77 [b]
PUFA species	9.85 ± 0.92	21.14 ± 4.13 [a]	11.93 ± 1.46	10.86 ± 0.80 [b]
HUFA species	84.07 ± 1.64	86.84 ± 7.63	81.23 ± 2.50	87.59 ± 4.49
Phosphatidylcholine (PC)				
SFA and MUFA species	38.05 ± 0.98	42.95 ± 1.36 [a]	38.13 ± 1.04	39.79 ± 1.53
PUFA species	38.78 ± 1.13	39.85 ± 1.82	39.99 ± 1.21	41.94 ± 1.83
HUFA species	22.09 ± 0.90	18.87 ± 1.41	20.41 ± 1.06	22.96 ± 1.55
Sphingomyelin (SM)				
SFA and MUFA species	99.72 ± 0.01	103.78 ± 2.00	99.58 ± 0.01 [b]	105.14 ± 3.10
PUFA species	0.28 ± 0.01	0.30 ± 0.01	0.42 ± 0.01 [b]	0.43 ± 0.03 [b]
Lysophosphatidylcholine (LPC)				
SFA and MUFA species	83.13 ± 0.53	76.61 ± 4.92	77.85 ± 0.53 [b]	72.98 ± 2.22
PUFA species	8.38 ± 0.40	8.16 ± 0.23	11.75 ± 0.36 [b]	11.41 ± 0.38 [b]
HUFA species	8.49 ± 0.29	8.34 ± 0.33	10.40 ± 0.31 [b]	9.74 ± 0.46 [b]

[#] The percentages in 4 PP (4 hour postprandial period) were calculated relative to the total concentration in fasting state and all were expressed as the means ± SEM.

[a] Significant difference from fasting state within group, [b] Significant difference from control group in the same period

4.3.3 Diabetes-induced alterations of erythrocyte phospholipids molecular species in type 2 diabetes compared with control group

Since the analysis of phospholipids of erythrocyte obtained from fasting state can reflect the disease-induced abnormal phospholipids composition, thus, erythrocyte obtained from fasting state in control and type 2 diabetic group were analyzed for phospholipids molecular species which were then used to find whether there were any alterations of phospholipids molecular species in type 2 diabetes compared to control subjects.

PE molecular species profiles were shown in figure 43. In both groups, the predominant molecular species of pPE and PE were pPE38:4 and PE34:1, respectively. As compared to control group, type 2 diabetic group had significantly low percentage of some PE molecular species which were PE36:4, PE38:6, PE38:5 and PE38:1 but had high percentage of some plasmenyl PE molecular species, pPE34:2, pPE34:1 and pPE36:2.

As shown in figure 44 and 45, two species of PS, PS38:5 and PS40:6 were significantly lower in type 2 diabetic group whereas no PI molecular species have been found to be different when compared to control group.

For choline-containing phospholipid, as shown in figure 46-48, PC36:2, PC38:3, SM14:0 and SM22:2 in type 2 diabetic group were significantly higher whereas PC30:0, PC34:0, PC36:0, PC38:2, PC38:1 and SM16:1 were found to be significantly lower than those in control group. In addition, LPC16:0, LPC18:2, LPC18:1 and LPC22:3 were significantly higher in type 2 diabetic group while LPC20:1 and LPC22:1 were lower than those in control group.

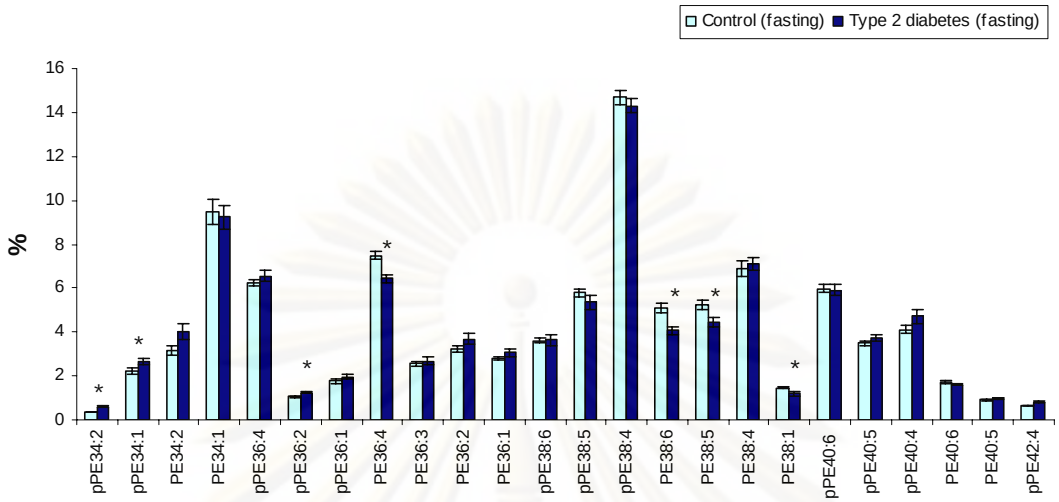


Figure 43 PE and pPE molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

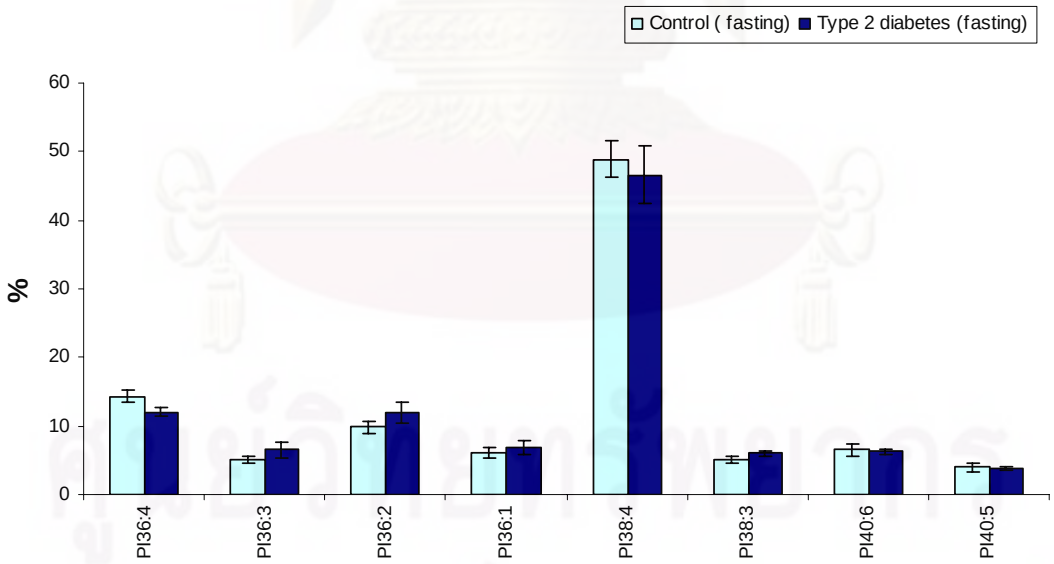


Figure 44 PI molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

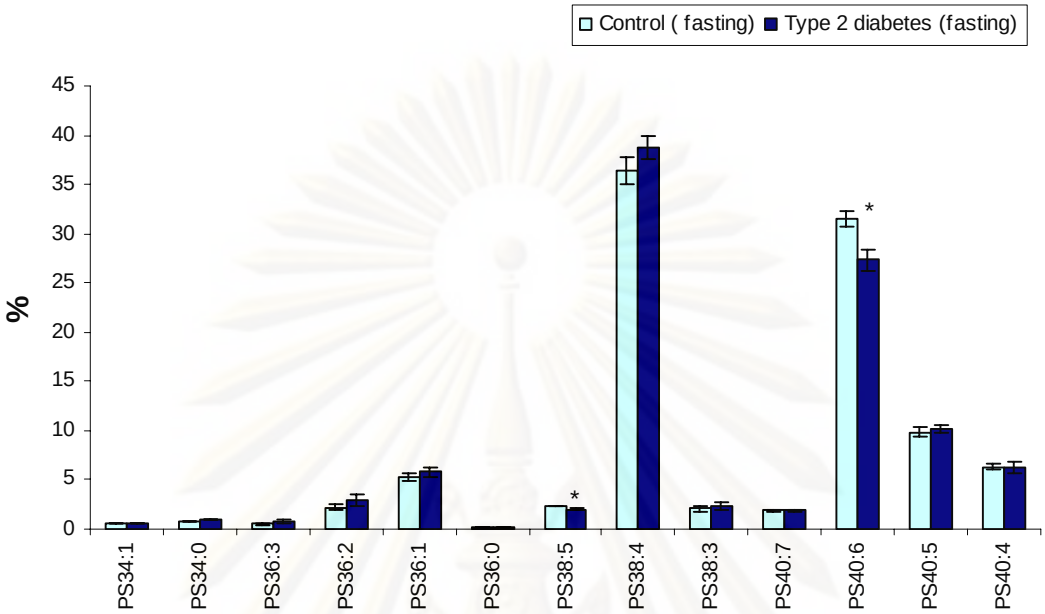


Figure 45 PS molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

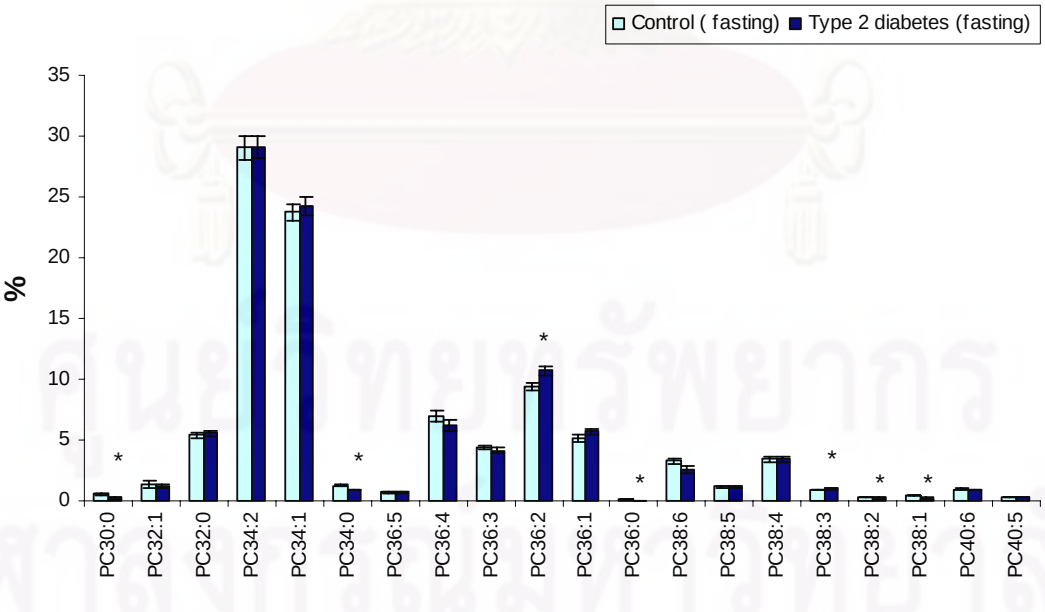


Figure 46 PC molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

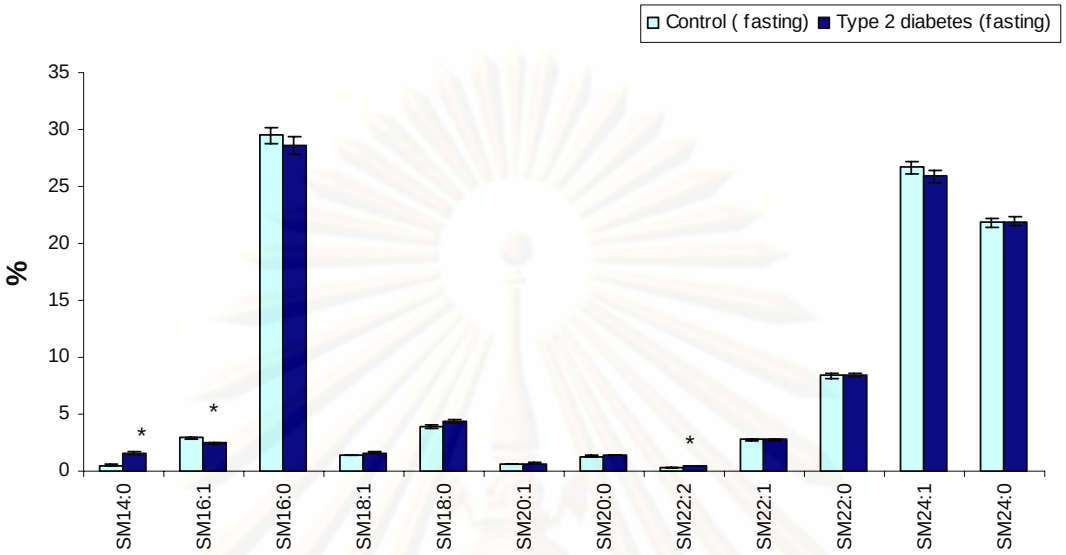


Figure 47 SM molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

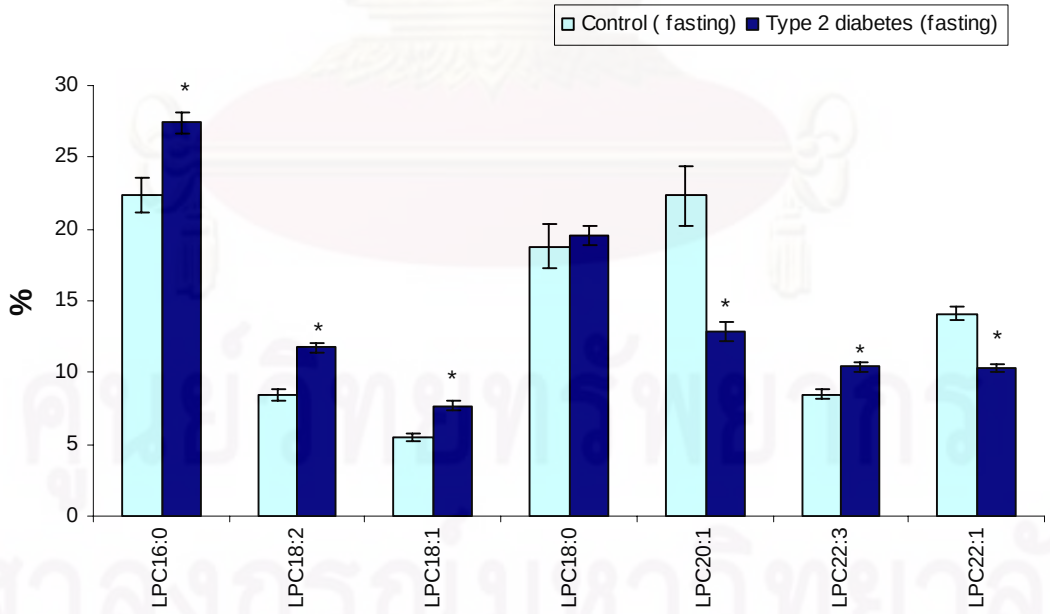


Figure 48 LPC molecular species of erythrocytes obtained from fasting state.

* Significant difference from control group.

4.3.4 The changes of erythrocyte phospholipids molecular species after high fat meal period in control and type 2 diabetic group

PE and pPE molecular species

Four hours after administration of high fat meal to all subjects, the changes of PE and pPE molecular species were observed and shown in figure 49. In type 2 diabetic group, pPE36:1 was found only to be slightly decrease but significant difference as compared to fasting state. In contrast to type 2 diabetic group, most of PE and pPE molecular species in control group were significantly decreased as compared to those in fasting state and there were preferential decreases in HUFA-containing PE and pPE species. After the meal period, total PE and pPE in type 2 diabetic group did not markedly change from those in fasting state. However, there was approximately 10% decrease of total PE and pPE in control group when compared to fasting state.

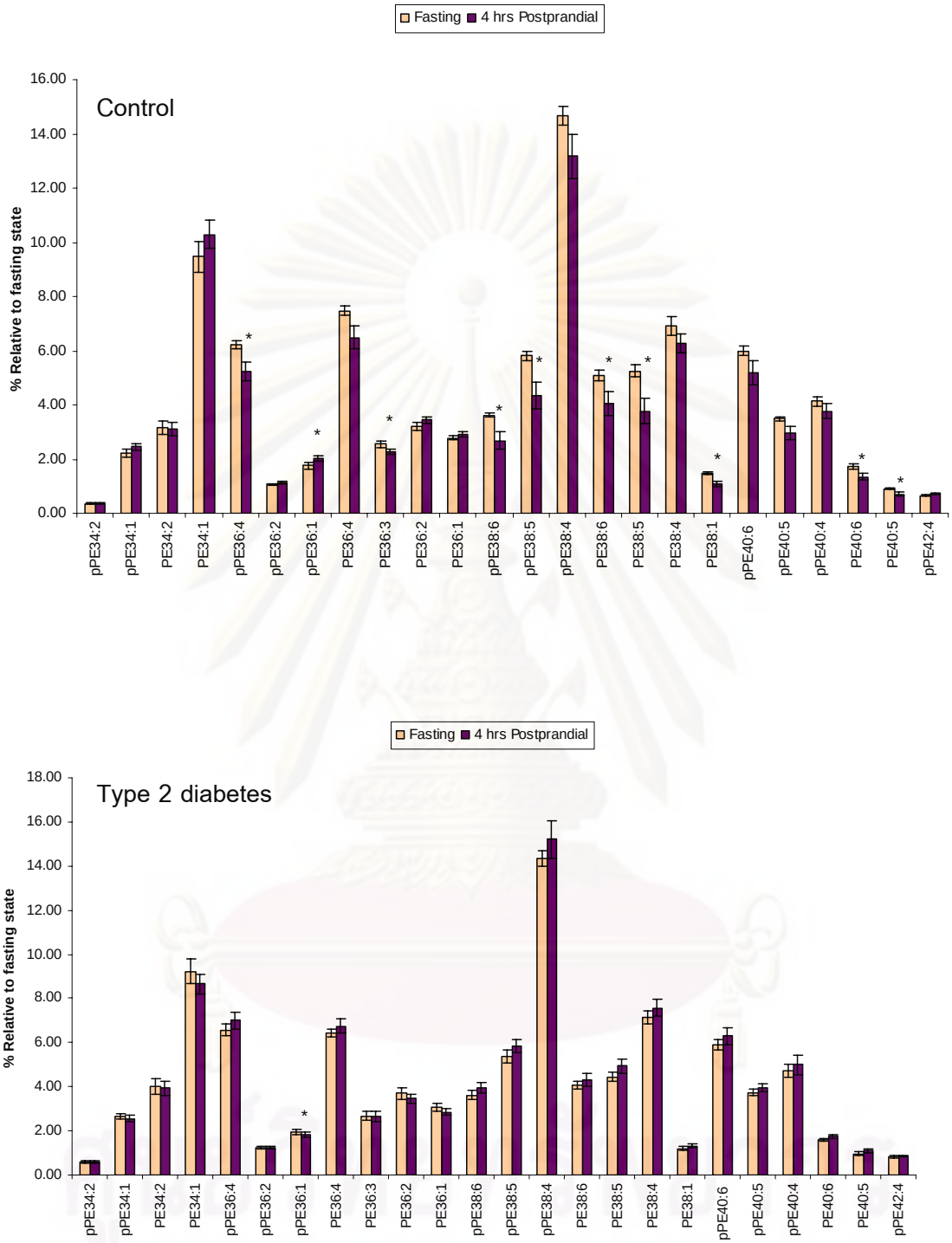


Figure 49 PE and pPE molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

PI molecular species

As shown in figure 50, PI which has been reported to be low concentration in erythrocyte, had a predominant species of PI38:4. No change of PI molecular species was found in type 2 diabetic group with respect to fasting state whereas some PI molecular species in control group were significantly increased. In addition, the increase of total PI was approximately 25% and 4% in control and type 2 diabetic group, respectively.

PS molecular species

PS molecular species of both groups were shown in figure 51. The predominant PS molecular species of erythrocytes were PS38:4. After the meal period, no significant difference of PS molecular species was observed in type 2 diabetic group, however, some PS molecular species were apparently decreased in control group when compared to fasting state. In control group, PS38:4 and PS40:6 were approximately 10% reduction from those in fasting state. The total PS after high fat meal period in type 2 diabetic group was approximately 6% increase from fasting state, however, 21% reduction was found in control group.

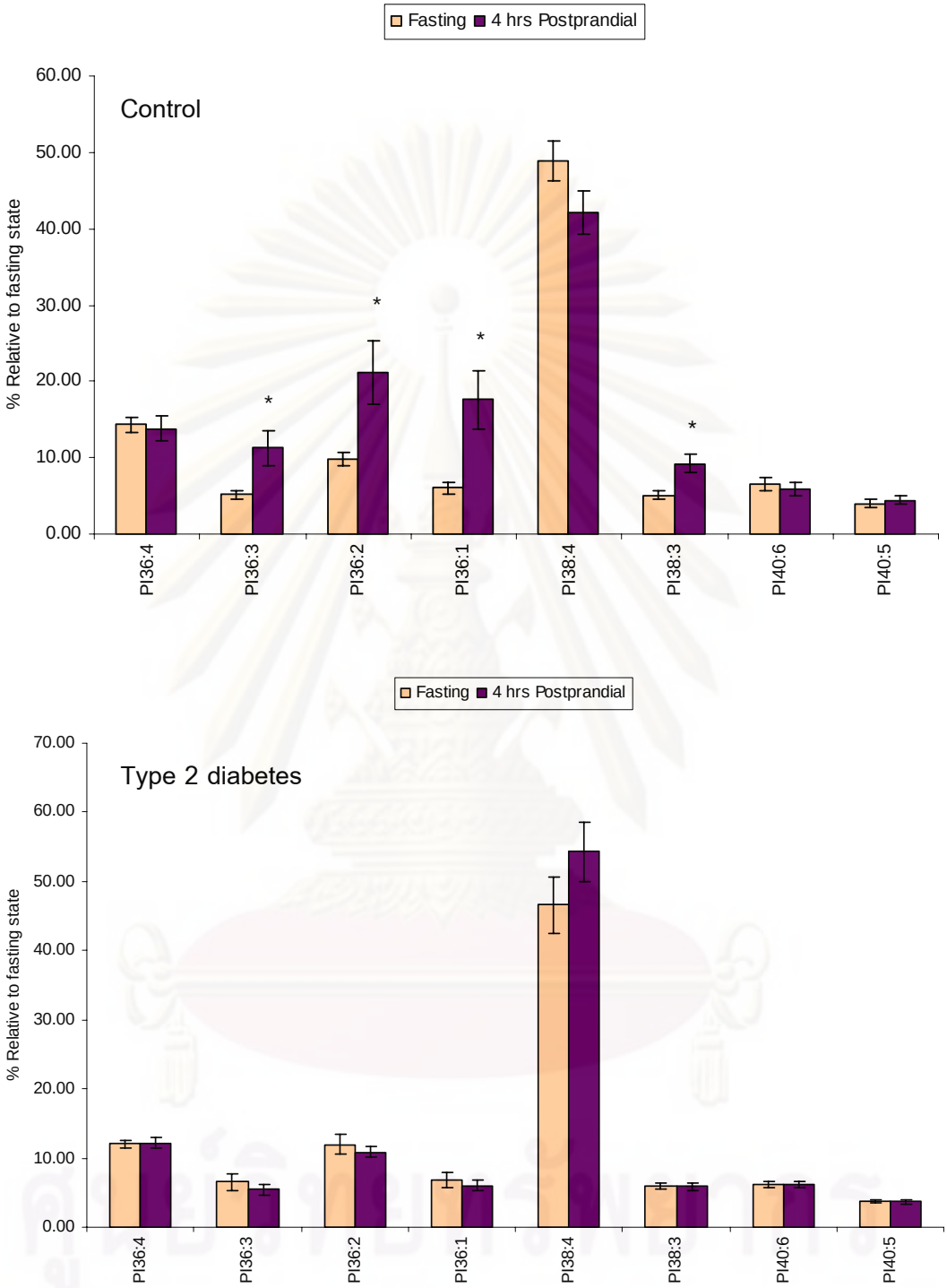


Figure 50 PI molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

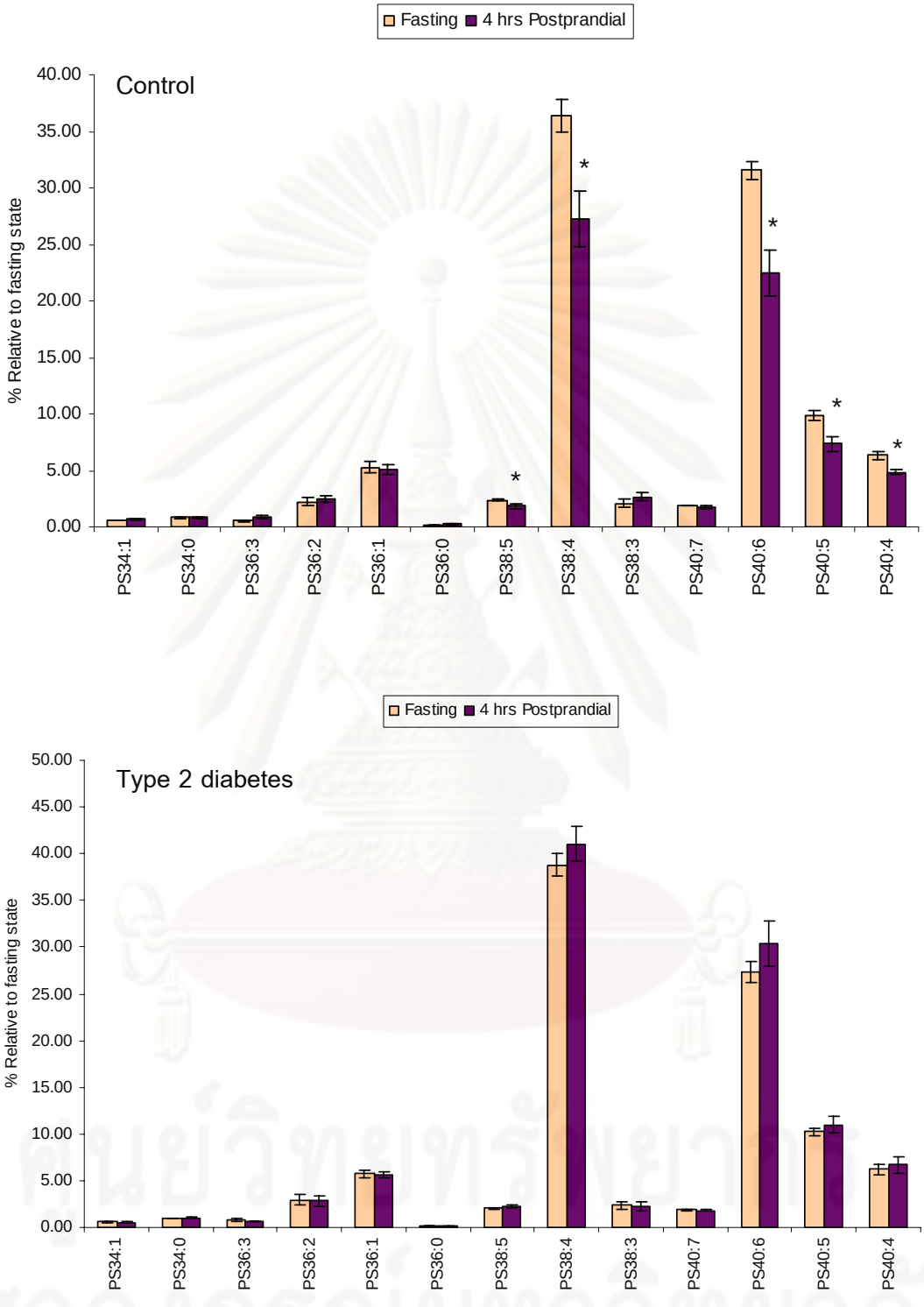


Figure 51 PS molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

PC molecular species

Figure 52 was shown the changes of PC molecular species in both groups after the meal period. The predominant PC molecular species of erythrocyte were PC34:2, PC34:1, which were accounted for approximately 28% and 24% of total PC molecular species, respectively.

Both groups tend to have the increase of PC molecular species after the meal period. In type 2 diabetic group, four PC molecular species, PC30:0, PC36:3, PC38:4 and PC38:1, were found to be significant changes from fasting state. However, the apparent changes of PC molecular species were found in control rather than those in type 2 diabetic group. In control group, there were significant increases of PC30:0, PC32:1, PC32:0, PC34:1, PC36:2, PC36:1, PC38:2 and PC38:1 as compared to fasting state.

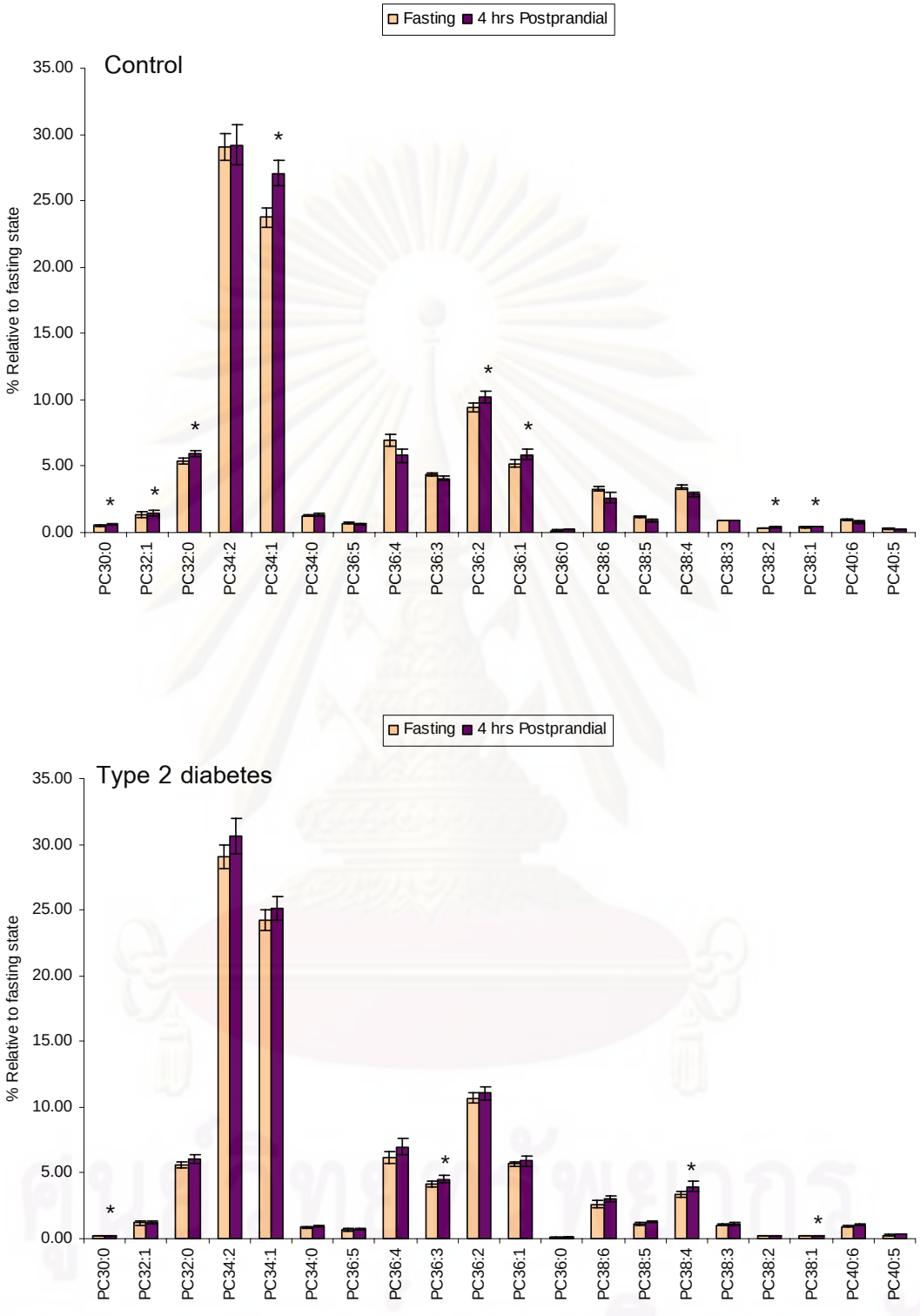


Figure 52 PC molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

SM molecular species

SM molecular species were shown in figure 53. 16:0, 24:0, 24:1 fatty acid-containing SM were major molecular species of erythrocyte in both groups. Four hours after the meal, most of SM molecular species tend to be increased with respect to those in fasting state. There were no changes in SM molecular species observed in type 2 diabetic group, however, only SM 24:0 in control group was found to be significant increased from fasting state.

LPC molecular species

Figure 54 was shown molecular species of LPC of erythrocyte. No significant change of LPC was observed in type 2 diabetic group. LPC molecular species in control group have changed into two directions. LPC16:0 was significant increased whereas there was significant decrease in LPC20:1 when compared to fasting state. However, the total LPC in both groups tend to decrease approximately 6% from total LPC in fasting state.

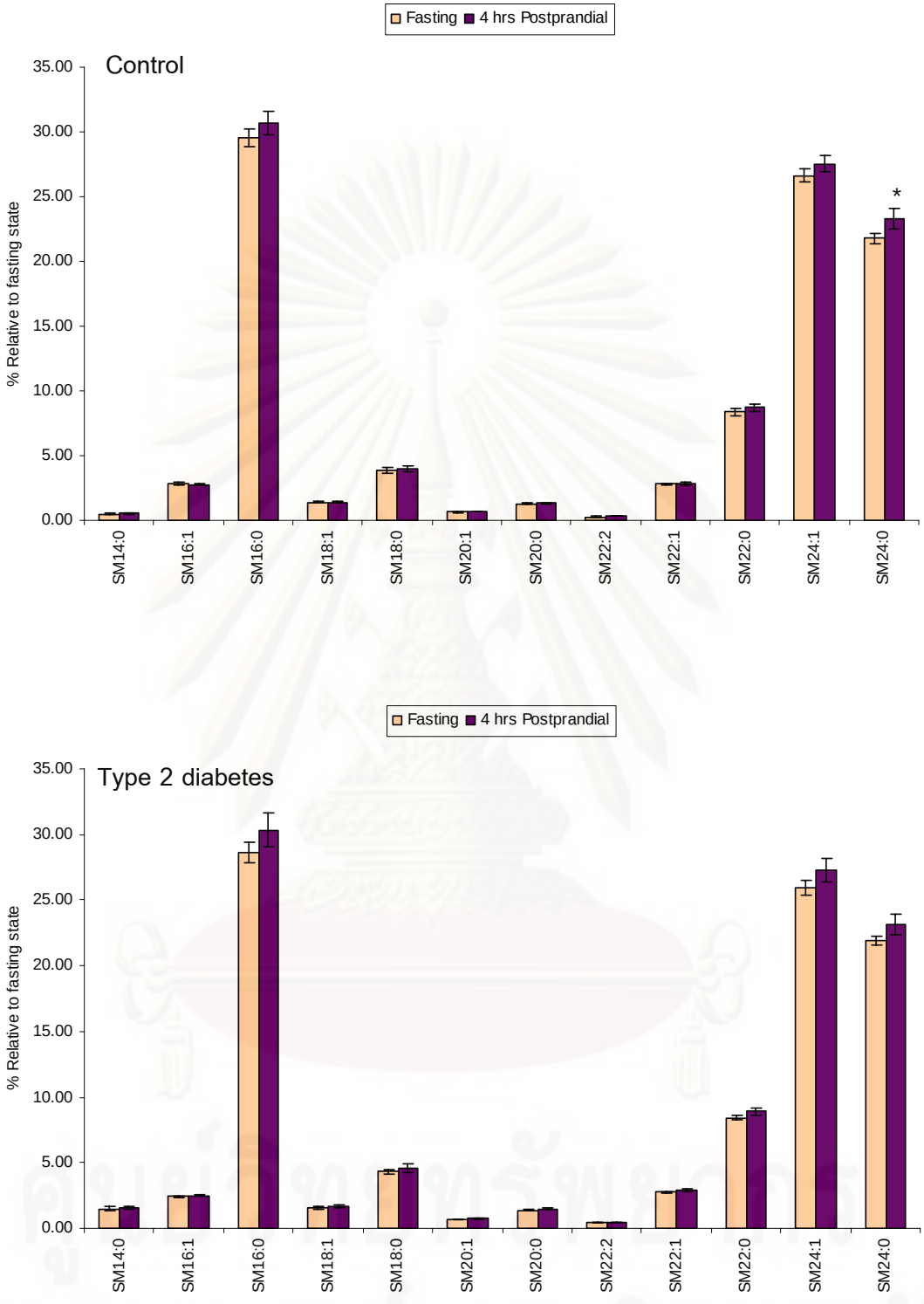


Figure 53 SM molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

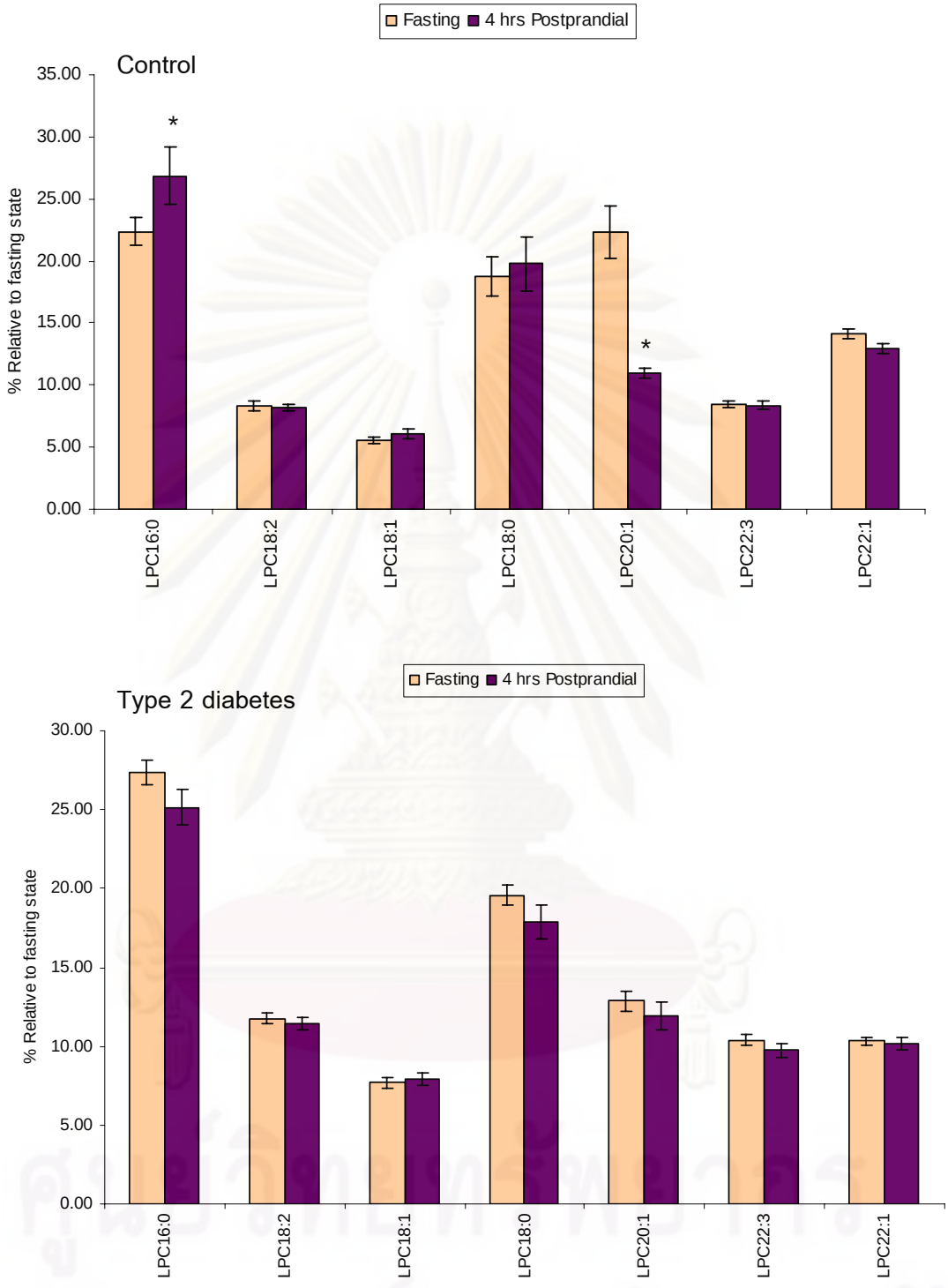


Figure 54 LPC molecular species of erythrocytes in fasting and postprandial state.

* Significant difference from fasting state

4.3.5 The correlation between insulin resistance index (HOMA) and some erythrocyte phospholipids molecular species

Since insulin resistance is a major risk factor of many diseases including type 2 diabetes and phospholipids plays an important role in cell structure and many biological processes, the relationships between some erythrocyte phospholipids molecular species in fasting state and insulin resistance measured by HOMA were determined. As shown in figure 55, it has been found that HOMA was significantly correlated with the total HUFA-containing PE species, (r = -0.468, p = 0.032).

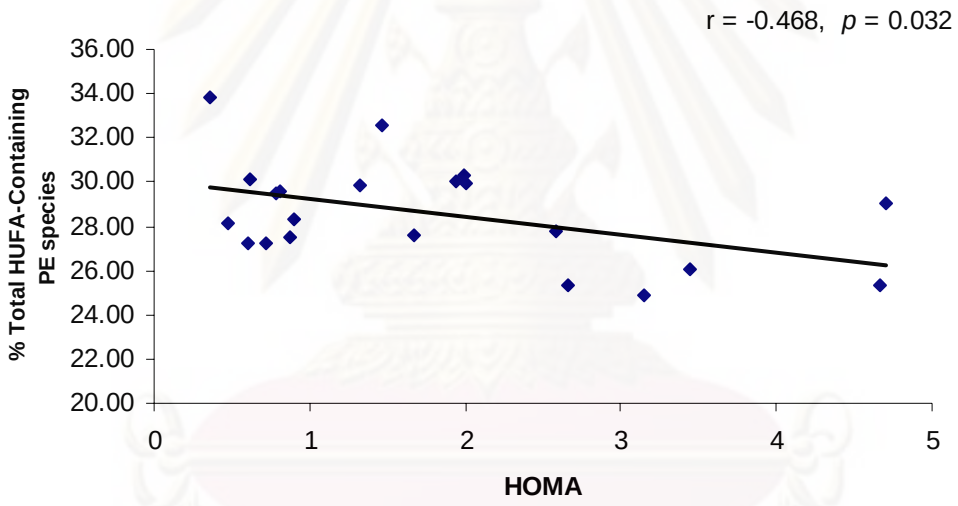


Figure 55 Correlation between insulin resistance index (HOMA) and total HUFA-containing PE species

In addition, the correlations between insulin resistance index (HOMA) with some erythrocyte phospholipids molecular species were shown in figure 56 and 57. PE36:4 was inversely correlated with HOMA (r=-0.465, p<0.05). Moreover, it has been found that SM16:0 was also inversely correlated with HOMA (r=-0.637, p<0.01) with strong significance.

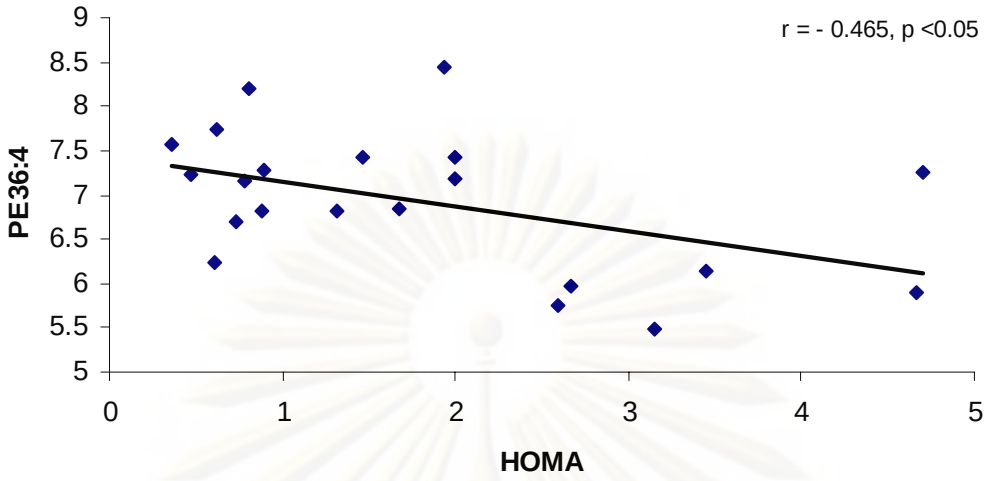


Figure 56 Correlation between insulin resistance index (HOMA) and PE36:4

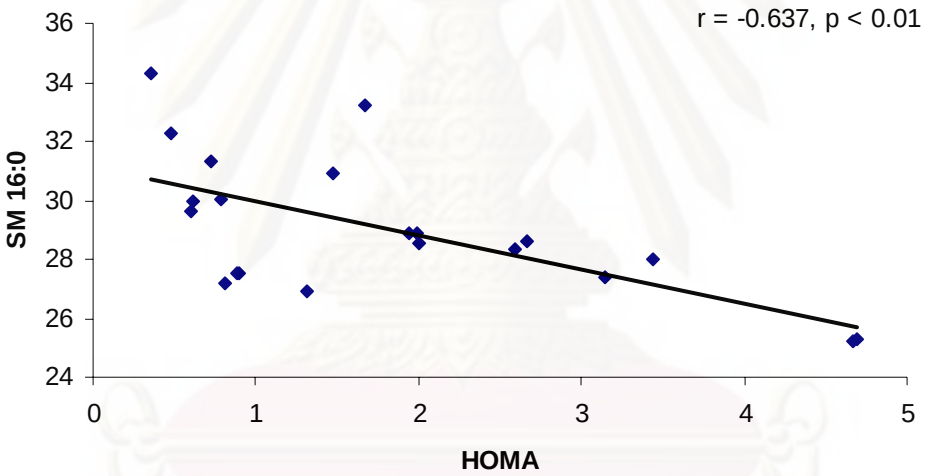


Figure 57 Correlation between insulin resistance index (HOMA) and SM 16:0

In addition, since aminophospholipids (PE, PS) and PI are situated on the inside of the erythrocyte membrane and have interactions with the membrane proteins, thus, the correlations between the changes in PE, PS and PI after the meal priod and the changes in protein conformations analyzed by FTIR spectroscopy were evaluated. The change in α-helix conformation was only found to be significantly correlated with the change in total HUFA-containing PS (r=0.507, p<0.019).

CHAPTER V
DISCUSSION

5.1 Part I: Study on the postprandial biochemical response after high fat meal period in control and type 2 diabetic group

Most of type 2 diabetic patients have a high level of plasma cholesterol, triacylglycerol, LDL and low level of HDL that are the characteristic of diabetic dyslipidemia. Since lipid-lowering drugs affected erythrocyte lipids (Levy et al, 1992) and to avoid this effect, all subjects including type 2 diabetes in this study were recruited based on free of use of such drugs. After screening, eleven type 2 diabetic and ten control subjects were finally enrolled and performed the high fat meal loading test.

As expected, fasting blood glucose and glycated hemoglobin in type 2 diabetic were higher than those in control group. The level of glycated hemoglobin in type 2 diabetes was not found to be high. This may be due to the dietary control and use of anti-hyperglycaemic drug of diabetic subjects.

Fasting hyperinsulinemia has been widely used as a surrogate measurement of insulin resistance and can predict the type 2 diabetic condition (Weyer et al, 2000). In present study, fasting insulin concentration in type 2 diabetic group seemed to be higher than the control but do not reach statistically significant difference. The fasting level of insulin variation depends on many factors, for instance, diet, racial difference, etc. However, our results of fasting insulin in type 2 diabetic subjects are consistent with other results from Thai diabetic subjects (Davis et al, 1995). Homeostasis model assessment (HOMA) which was the simple method to access insulin-resistant index was calculated and compared between both groups. Although fasting insulin in type 2 diabetic group was not found to be significantly different from that in control group, the calculated HOMA-IR values in type 2 diabetic group was significant higher as compared to control group. It can be suggested that the insulin concentration found in type 2

diabetic group was not enough to control blood glucose into normal level whereas nearly the same insulin concentration in control group can do.

Since type 2 diabetic subjects in this study have a good control of their diet and lifestyle, thus, no significant differences of fasting lipid profile were observed as compared to control group. HDL chlolesterol, the liproprotein that plays important role in reverse cholesterol transport (RCT), has been reported to be low level in insulin resistant state and type 2 diabetes (Rashid et al, 2002; Rashid et al, 2003). Many mechanisms were involved with lowering of HDL cholesterol. The enhance of CETP-mediated exchange of triglycerides and cholesteryl ester between HDL and triacylglycerol-rich lipoproteins together with the action of hepatic lipase (HL), are the causes of reduction in plasma HDL cholesterol and apoA-I plasma concentrations. In agreement with others, our results have shown that the level of HDL cholesterol in type 2 diabetic group seemed to be lower than that in control group, however, it can not show the statistically significant difference (p = 0.222).

5.1.1 Correlation between Insulin resistance index (HOMA) and baseline biochemical parameters

The relationships of some fasting baseline parameters and insulin resistance index measured by HOMA were performed in order to find the factors that may be associated with development of insulin resistance. Insulin resistance index (HOMA) was mainly correlated with the body composition parameters (Hettihewa et al, 2007). All type 2 diabetes seemed to have body mass index, weight, waist circumference, hip circumference and waist to hip ratio higher than those in control group. Our results have shown that HOMA had strongly significant correlation with weight, Body Mass Index (BMI) and waist to hip ratio (WHR). The mechanisms underlie these correlations were that the increasing of intra abdominal or visceral adipose tissue resulting in a higher rate of flux of adipose tissue-derived free fatty acids to the liver. In the liver, fatty acid produces an increased production of glucose, triacylglycerol and lipoproteins secretion.

Moreover, fatty acid also reduces insulin sensitivity in muscle by inhibiting insulin-mediated glucose uptake. These results of high circulating glucose and fatty acid can increase pancreatic insulin secretion that lead to hyperinsulinemia (Eckel et al, 2005) and finally caused insulin resistance.

Moreover, our results have demonstrated that insulin resistance index was found to be significantly correlated with fasting triacylglycerol and inversely correlated with HDL cholesterol. Insulin resistance can increase triacylglycerol by two mechanisms, the first is the increasing of free fatty acid flux to the liver and the second one is hepatic insulin resistance. These finally result to an overproduction of VLDL seen in insulin resistant state and/or type 2 diabetic patients. Adiels and co-workers have studied in type 2 diabetes regarding the overproduction of VLDL and have proposed that hyperglycemia was the driving force that enhanced overproduction of VLDL1 particles under conditions of insulin resistance, moreover, the significant correlation between plasma triacylglycerol and HOMA value was also observed in that study (Adiels et al, 2005).

5.1.2 The oral high fat meal loading

The high fat meal given to all subjects in this study was calculated based on their body surface area (BSA) since the body surface area is a better indicator of metabolic mass than body weight because it is less affected by abnormal adipose mass. In the present study, the Mosteller equation was used to estimated BSA as it has been reported that this equation was a reliable method for estimating BSA and was also more convenient to apply in daily clinical practice than a formula requiring the use of logarithms (Vu, 2002).

Because of the absence of a standardized meal for postprandial studies, many studies have used a variety of different test meals consisting of “everyday” foods such as dairy products, breads or liquid formulations. The amounts of fat is varied around 33–66%; up to 110 g , carbohydrate 14–43%; up to 75 g and protein 16–20%; up to 46 g

(Mohanlal and Holman, 2004). In this study, 40 grams of fat/m^2 BSA in the form of chocolate milkshake and a piece of butter bread were given and all subjects tolerated well to the meal since an intake of 40–50 g of fat in a meal results in significant lipemia in healthy adults (Sanders, 2003).

Most studies on postprandial lipemia cover at least an 6-8 hours period and the area under the curve (AUC) was the index most frequently used to evaluate postprandial lipemia (Lemieux et al, 2000; Pruneta-Deloche et al, 2005). In the present study, the single time point of 4 hours after the high fat meal test was used to study the effect transient hypertriacylglycerolemia induced by the high fat meal on the biochemical changes of erythrocyte contents and membrane phospholipids molecular species. This single time point was selected base on the *in vitro* and *in vivo* studies in erythrocyte that have shown that the exchange or incorporation of fatty acid into lipid fraction of erythrocyte occurred at the first hour after incubation (Oliveira and Vaughan, 1964; Hodson et al, 2009) and the concentration of triacylglycerol in human reached the peak around 4-5 hours after high fat meal administration (Dallongeville and Fruchart, 1998).

5.1.3 Four hours postprandial biochemical response after meal period

Four hours after the meal period, postprandial glucose was decreased to fasting level in both groups, however, postprandial glucose in type 2 diabetic group was still higher than control group. In addition, postprandial insulin concentration in type 2 diabetic group was significantly higher as compared to control group. Our results agree with others that have shown that type 2 diabetic patient or their relatives had higher postprandial insulin concentration in 3 and 4 hours after the meal as compared with healthy control subjects (Axelsen et al, 1999). Moreover, Greenfield and colleagues have studied insulin sensitive compared with insulin resistant subjects. Two types of meal, high and low carbohydrate meal were given to their subject and measure plasma glucose and insulin over 6 hours period. Postprandial increasing of glucose and insulin were greater following the high-carbohydrate in both insulin-sensitive and insulin-

resistant subjects (Greenfield et al, 2007). Although the high fat meal given to all subjects in this study contained small amount of carbohydrate (Approximately 30 grams), however, it can stimulate insulin response seen in both groups. Approximately 180% (Twofold as compared to control) increase of insulin concentration relative to fasting state was found in type 2 diabetic group. The markedly increased response may be due to their high glycaemic index of the given carbohydrate (Rasmussen et al, 1992; Galgani et al, 2006) together with the increase of insulin response in diabetes in order to stimulate glucose uptake from the blood into the cells and insulin sensitive tissues.

Lipid profiles were observed at four hours after the meal period and our results have shown that total cholesterol was significantly increased only in type 2 diabetic group whereas no change of HDL cholesterol was observed in both groups. Our results of postprandial cholesterol in type 2 diabetic group were in contrast with the others. Annuzzi and colleagues (Annuzzi et al, 2004) have studied the association of insulin resistance with postprandial alterations of TRL in type 2 diabetes mellitus. After the meal, no significant difference was observed in cholesterol concentrations between groups and this was in agreement with the study in normal healthy subjects (Ferreira et al, 2004). Cholesterol metabolism in diabetes has been reported to abnormal. Gylling and Miettinen have found that cholesterol turnover and removal of LDL apo B were high in NIDDM subjects as compared with healthy control subjects whereas cholesterol absorption efficiency was abnormally low (Gylling and Miettinen, 1997). However, our results can not show the efficiency of cholesterol absorption in type 2 diabetes.

In the past few years, several studies have shown that postprandial hypertriacylglycerolemia has been associated with coronary heart and/or carotid artery disease in nondiabetic and diabetic subjects (Groot et al, 1991; Teno et al, 2000; Hyson et al, 2003). In the present study, all subjects have exhibited an increased postprandial triacylglycerol response to the given meal. It can be shown that the given meal can stimulate the production of chylomicron since dietary fat was composed principally of triacylglycerol that can induce the production of chylomicron (Summers et al, 1998).

Postprandial triacylglycerol induced by the given meal was significantly

increased in both groups relative to baseline fasting level. Although, our study can not show the significant difference of postprandial triacylglycerol between control and type 2 diabetic group (p= 0.545), however, it seemed that postprandial triacylglycerol of type 2 diabetes was higher than that in control group, even both had no difference in fasting triacylglycerol and the amount of fat intake.

Since the present study used the single time point for investigating the influence of postprandial hypertriacylglycerolemia on erythrocyte contents and phospholipids compostition rather than measurement of the total triacylglycerol concentration during the high fat meal period, thus, our results can not show whether postprandial triacylglycerol was still high and prolonged in type 2 diabetic or not. To support this hypothesis, Iovine and colleagues have evaluated the daily triacylglycerol profile of type 2 diabetic patients during their everyday life and found that most of type 2 diabetic patients have postprandial triacylglycerol above optimal concentration for several hours after meals (Iovine et al, 2004). It can be indicated that diabetic patients have worse handling of the fat after the meal.

Additionally, the results of fasting and postprandial triacylglycerol which were correlated with insulin resistance index (HOMA), were consistent with the others. Eriksson and co-workers have studied postprandial blood lipids and adipose tissue lipoprotein lipase in type 2 diabetic patients by following 3.5 hours after lipid-enriched meal, have found that HOMA was significantly associated with both fasting and postprandial serum triacylglycerol concentrations. Moreover, they have shown that insulin resistance was inversely correlated with post-heparin plasma LPL activity (Eriksson et al, 2003). Plasma LPL activity which plays an important role in the clearance of chylomicrons derived from dietary fat, is a key factor of postprandial lipid metabolism. It has been well known that type 2 diabetes have strong reduction of TRL-bound LPL activity in the postprandial period. This has contributed to postprandial hypertriacylglycerolemia (Pruneta-Deloche et al, 2004). Recently, Annuzzi and colleagues have demonstrated that in insulin-resistant conditions of obesity with and without diabetes, the large VLDL was increased after lipid-enriched meal and diabetic

patients have an increased postprandial chylomicron response and a reduced adipose tissue LPL activity (Annuzzi et al, 2008). In addition, LPL in the circulation has been reported to be reduced after both hyperglycemia and combined hyperinsulinemia and hyperglycemia induced by clamp techniques (Kovar et al, 2004). Moreover, Ruge and colleagues have demonstrated that LPL activity in postprandial period of human subjects was lower than that in the fasting state (Ruge et al, 2001). Therefore, taken together, if the increase in both glucose and insulin can suppress the LPL activity and the low LPL activity was found in postprandial period, it could be expected that LPL activity may be markedly reduced in the postprandial period of type 2 diabetes due to the low level of LPL activity typically found in type 2 diabetes (Nikkila et al, 1977; Saheki et al, 1996) and it may be from the possible effects of the highly postprandial glucose and insulin to suppress plasma LPL activity.

5.2 Part II: FT-IR spectroscopic study of the biochemical changes of erythrocyte contents in type 2 diabetes after high fat meal period

5.2.1 Characteristics of erythrocytes contents studied by FT-IR

Recently, FT-IR spectroscopy has become a routine technique for many clinical applications because it can provide global biochemical information on any biological fluids or cells. With a simple preparation of sample and easy to run method, the whole biochemical contents of any samples can be identified at sub-molecular level simultaneously. In the present study, erythrocyte from all subjects in fasting state was analyzed in order to investigate the diabetes-induced alterations of erythrocyte contents in type 2 diabetes compared to healthy control subjects.

In fasting state, some lipid stretching bands, especially $\nu_s(CH_3)$ in type 2 diabetic group seemed to be higher than those in control group. It can be implied that the relative lipid contents in the diabetic erythrocyte may be higher than those in control subjects. This was consistent with the study of Liu and colleagues that have used infrared spectroscopy to determine the biochemical and physical profiles in diabetic platelets. They have found an increasing of $\nu_s(CH_2)$ and the shift of $\nu_{as}(CH_2)$ band in diabetic platelets (Liu et al, 2002).

The integrated area of olefinic band can be used as an index of relative concentration of double bonds in the lipid structure from unsaturated fatty acyl chains and our results have shown that the integrated area of such band in fasting state in type 2 diabetic group was significantly lower than control group. The unsaturated degree of membrane is involved with the fluidity of the cell membrane and some studies have demonstrated that the increasing the ratio of polyunsaturated to saturated fatty acid can increase the fluidity of cell membranes and the number binding of insulin receptors (Ginsberg et al, 1982). Our results agree with the others that studied erythrocyte fatty acid in diabetic patients. Bakan and colleagues have studied fatty acid of type 2 diabetic using gas chromatographic method, have revealed that type 2 diabetic patients

showed a significantly higher proportion of C16:0 fatty acid not only in erythrocyte but also in leukocyte membranes and plasma samples (Bakan et al, 2006). Recently, Allen and colleague have studied fatty acid in black and white type 2 diabetes and also found significantly higher of S/P ratios in diabetic subjects relative to control group (Allen et al, 2006). The unsaturation of the cell membrane was influenced by several factors such as diet, hepatic FA synthesis, desaturase enzymes, hormones and other chemicals.

Moreover, the other possible mechanisms that can be explained for the loss or low level of olefinic band found in type 2 diabetes was lipid peroxidation by free radicals. As an increase in reactive oxygen species has been reported in diabetes and it was mainly from autooxidation of glucose and protein glycosylation (Wolff and Dean, 1987). Our results were in accordance with the results of Sills and colleagues that have studied erythrocyte peroxidation by using FT-IR spectroscopy. They have found that the olefinic band has diminished in intensity during membrane peroxidation as a result of loss of unsaturation in the phospholipid acyl chains. Moreover, they have confirmed that the primary site of peroxidation occurred at phospholipid containing arachidonate acyl chains (Sills et al, 1994). However, the relationship between olefinic band and lipid peroxidation is controversial. Severcan et al. have studied lipid peroxidation of diabetic rat microsomal membrane and found significant increase of such band in diabetic samples compared to normal control subjects (Severcan et al, 2005). They have suggested that an increase of this band was due to the accumulation of end products of lipid peroxidation and the variation of the olefinic band area/intensity as a result of lipid peroxidation may be due to the structural difference of cell in the samples.

Infrared (IR) spectroscopy is one of the oldest and well established experimental technique for the analysis of secondary structure of polypeptides and proteins. Proteins are frequently referred to as having a certain fraction of structural components (α-helix, β-sheet, etc.). The secondary structural composition is some of the most important information for a structure-unknown protein. Therefore, the estimation of protein secondary structure is one of the major applications of the FTIR technique. Study of protein conformation by using FTIR has been reported to be well match with X-ray

crystallography (Jand and Yu, 2007). As erythrocyte membrane proteins has been shown to be altered or increased in diabetic patient (Kolosova et al, 2000; Petropoulos et al, 2007), our results have shown that type 2 diabetic erythrocyte had low β-turn conformation and Amide II compared to control group. It can be suggested that the differences in secondary protein structure in type 2 diabetes compared to healthy control subjects reflected an alteration of erythrocyte protein conformations. This may be due to the chronic hyperglycemia caused oxidation and non-enzymatic glycosylation (Maillard reaction) of erythrocyte membrane proteins (Miller et al, 1980) since the FT-IR study has shown that the incubation of glucose with protein can induce the alteration of protein conformations, especially Amide I. (Rondeau et al, 2007). In the present study, however, the results from FT-IR can not show which proteins have been modified or altered in erythrocyte of type 2 diabetes, therefore it can only provide the information regarding the overall of the diabetic-induced alterations in erythrocyte protein conformations.

In the fingerprint region of FTIR spectrum, there are a large number of different vibrations that occur here. These include single bond stretches and a wide variety of bending vibrations. In the present study, the vibrations of phosphate, C-O of glucose and lactate were mainly observed. The band of ν (P=O) which mainly rises from phospholipid moieties in erythrocyte membrane was found to be higher in type 2 diabetes compared to control subjects. It can be implied that there may be high phospholipid content in erythrocyte of type 2 diabetes. In addition, lactate is the main substrate for erythrocyte metabolism and the monocarboxylate transporter of erythrocyte membrane widely accepts lactate from plasma. Normally 6-8 μmol of glucose/g hemoglobin/hour is broken down to lactate via the Embden-Meyerhof pathway. Our FTIR results have shown that the integrated area of ν(C-O) of lactate in type 2 diabetic group was significantly higher when compared to control group. As high plasma lactate has been reported in type 2 diabetes (Chen et al, 1993), the increase of lactate band area seen in erythrocyte of type 2 diabetes may be due to the uptake of plasma lactate by erythrocyte (Skelton et al, 1998). As expected, the integrated area of ν(C-O) of glucose

in type 2 diabetic group was also significantly higher than that in control group. It may increase from glucose that reacted non-enzymatically with erythrocyte protein particularly hemoglobin, which can be measured as glycated hemoglobin (HbA_1C) that was found to be high level in type 2 diabetic group observed in this study.

5.2.2 Changes in erythrocytes contents after the meal period as compared to fasting state in both groups

Recently, postprandial period has become an important condition to study cell adaptation or modification together with the several reports of metabolic abnormalities occurred in postprandial period. Thus, erythrocyte from fasting and four hours after the high fat meal period were obtained from all subjects for investigating the changes of its contents by FT-IR spectroscopy. After the meal period, almost of integrated areas of CH stretching vibrations from lipid in type 2 diabetic group tend to be decreased in parallel with the decrease in integrated area of phosphate asymmetric stretching. Moreover, the olefinic band area still remained unchanged when compared to fasting state. Since phospholipid of erythrocyte is the predominant lipid that could produce the olefinic band (ν=(CH) stretching of unsaturated lipids), $\nu_{as}(CH_3)$, $\nu_{as}(CH_2)$, $\nu_s(CH_3)$, $\nu_s(CH_2)$ and ν(P=O) absorption bands found on FT-IR spectra, therefore, the changes of these bands were mainly involved with the modification or alteration of erythrocyte phospholipids. The decreases in CH stretching vibrations from lipid and ν(P=O) have been reported to be a result from phospholipid peroxidation (Petibois and Deleris, 2005).

The increasing of lipid peroxidation and oxidative stress in postprandial hypertriacylglycerolemic state has been reported in type 2 diabetic patients (Saxenaa et al, 2005; Anderson et al, 2006). Together with the decrease in integrated areas of CH stretching vibrations from lipid and phosphate stretching vibration seen in our FTIR results, therefore, it can be proposed that erythrocyte of type 2 diabetic patients has undergone oxidative damage by free radicals generated during postprandial hypertriacylglycerolemia. This may lead to the deterioration of erythrocyte membrane.

The other possible explanation for the changes of lipid-related stretching vibrations may involve the exchange or incorporation of ingested fatty acid to the lipid fraction of erythrocyte after the meal period. In contrast to type 2 diabetic group, the olefinic band, CH stretching vibrations from lipid and phosphate stretching vibration in control group tend to be all increased relative to fasting state. Since erythrocyte is the major blood cells the circulation and its membrane phospholipid is continuously renewed during the circulation of the cell, it can be indicated that during postprandial state, there may be an exchange or incorporation of ingested fatty acid/phospholipid among erythrocyte, plasma and lipoproteins by the action of some enzymes. The enzymes that were involved with the exchange or promoting the efflux of phospholipid, cholesterol and reverse cholesterol transport have been reported to be defect in type 2 diabetes. This may result in no apparent changes or increases in erythrocyte lipid contents of type 2 diabetes, especially unsaturated lipids which has been recently reported to be rapidly incorporated into lipid fraction of erythrocyte (Hodson et al, 2009).

The conformations of protein after the meal period in both groups were also changed when compared to fasting state. In erythrocyte, hemoglobin normally accounts for about 90% and spectrin for about 10%. Thus, significant changes in FTIR bands of protein conformations would likely be due to the hemoglobin denaturation and/or possibly to spectrin described by Petibois and Deleris (Petibois and Deleris, 2005). However, the other possible explanation for such changes of protein conformations after postprandial period may be due to the change of glucose transporter conformation when glucose was bound and released from its binding site. The effects of sugar on glucose transporter have been shown the alternation of conformation model for transport studied by FT-IR (Alvarez et al, 1987). In addition, shape alteration of erythrocyte may be involved with the changes of protein conformation by the uptake or loss of phospholipids of erythrocyte as lipids take part in structural arrangements of the bilayer and preserve conformational changes of membrane enzymes or proteins (Kolosova et al, 2000).

Four hours after the meal period, our FTIR results have shown that the integrated areas of glucose and lactate stretching bands tend to be decreased in type 2 diabetic group, however, the increases of such were found in control group. Regarding to the metabolism of erythrocyte, glucose is the energy source of the erythrocytes. Under normal physiological circumstances, 90% of glucose is catabolized anaerobically to pyruvate or lactate by the Embden-Meyerhof Pathway or glycolysis (Van Wijk and Van Solinge, 2005) and, in addition, insulin can increase the rate of glycolysis in human erythrocytes that result in increase lactate production (Zancan and Sola-Penna, 2005). From our results after meal period, it can be indicated that erythrocyte in control group can uptake and utilize glucose better than those in type 2 diabetic group. This may be due to the defect of insulin-bingind receptor of erythrocyte in type 2 diabetes and the alterations of many enzymes in erythrocyte that play important role in the modulation of glycolytic rates through the control of insulin receptor phosphorylation (Marques et al, 2000).

5.3 Part III: LC-MS study on the changes of erythrocyte phospholipids molecular species after high fat meal period in control and type 2 diabetic group

5.3.1 Separation and characterization of phospholipids classes and species from erythrocytes

Unlike the triple quadrupole mass spectrometer, the ion trap and other mass analyzers are unable to perform the direct analysis of crude lipid extracts by using selective detection modes, i.e., classs specific precursor ion or neutral loss scans (Brugger et al, 1997; Wakelam et al, 2007). Pre-separation of the phospholipids classes is necessary with most samples to allow reliable quantification. Therefore, in this study, liquid chromatography was firstly used for class separation and followed by mass spectrometer for species characterization (Kim et al, 1994).

In normal phase separation mode, phospholipids are separated by class on the basis of that head-group polarity. Typically, neutral lipids elute firstly, followed closely by PE, PI, PS and choline-containing phospholipids elute later. In the present study, hexane/isopropyl alcohol-based mobile phase was used and give the eluted pattern that in accordance with the others (Wang et al, 2004; Gao et al, 2006). PE was firstly eluted followed by PI, PS, PC, SM and LPC. Our results have shown that erythrocytes have a small amount of PI and LPC whereas the major phospholipids compositions were PE, PC and SM. Furthermore, the phospholipids molecular species composition from our results were consistent with the others that have shown the major phosphatidylethanolamine subclass in erythrocyte was plasmenylethanolamine which was highly enriched in polyunsaturated fatty acids at the sn-2 position and the predominance of polyunsaturated fatty acids in phosphatidylserine and phosphatidylinositol. (Myher et al, 1989; Han and Gross, 1994; Leidl et al, 2008).

5.3.2 The alteration of erythrocyte phospholipids molecular species of type 2 diabetes compared with control subjects

The fatty acid composition of erythrocyte phospholipid is affected by fat intake and might reflect that of membranes function which may be altered by disease state. This could affect the fluidity of the membrane and therefore influence the function of cell surface receptors (including the interaction between insulin and its receptor) and glucose transporters (Stubbs and Smith, 1984). Alterations in phospholipids classes, subclasses and individual molecular species have previously been shown to modulate the physical properties of cell membranes (Brenner, 1984), the enzymatic activity of transmembrane proteins and the production of lipid second messengers of signal transduction (Muderhwa and Brockman, 1992).

The alteration of erythrocyte membrane phospholipids has been reported in type 2 diabetic patients. In 1996, Labrouche and colleagues have studied the changes in phospholipids composition of blood cell membranes in different types of diabetes and found that PE in erythrocyte was increased as compared to healthy control. (Labrouche et al, 1996) and this was consistent with the results of white diabetics but not of black diabetics described by Allen and co-workers. (Allen et al, 2006). Our results have found that only total PUFA containing-PE species in type 2 diabetic group was higher as compared with control group. This phenomenon may be due to the abnormal rate of conversion of PE to PC in erythrocyte.

Some erythrocyte phospholipids molecular species were also altered in type 2 diabetic patients relative to control subjects. Some species of PE, PS and PC of type 2 diabetic erythrocyte tend to be lower than those in control group. This can be indicated that the reduction of such species may result from oxidative damage since it was well recognized that free radical production was increased in diabetes together with some phospholipids classes in human erythrocytes were enriched with high amount of polyunsaturated fatty acids that were susceptible to peroxidation (Clemens and Waller, 1987). To support this, Mawatari and colleagues have studied peroxidation of normal

erythrocytes with tBHP and AAPH and found the extent decrease especially in PE, PS and their polyunsaturated fatty acids after incubation (Mawatari et al, 2004). Moreover, our result has found that PE36:4, of which predominant species was PE16:0/20:4, was inversely correlated with insulin resistant index (HOMA) and also found to be significant lower in type 2 diabetic group. This was consistent with the results of Rodriguez and colleagues that have found the negative correlation between insulin resistance and with 20:4 ω6 fatty acid (r =0.40, $p < 0.01$) of erythrocyte in type 2 diabetes (Rodriguez and Christophe, 2005). Therefore, it can be implied that such species may be a main target for peroxidation by free radical in insulin resistant state and/or type 2 diabetes. However, Han and colleagues have studied phospholipids molecular species in rat diabetic myocardium and found specific alterations of lipid molecular species after induction of the diabetic state by streptozotocin treatment. They have found the decrease of PE 18:0/20:4 in rat myocardium and proposed that the decrease in this species could be due either to a decreased synthetic capacity for the production of this molecular species or may result from the persistent stimulation of one or more phospholipases (Han et al, 2000). Since erythrocyte is unable to *de novo* synthesis its lipid composition, thus, the latter suggestion may be the other possible cause that affected such species.

ω3-fatty acid has been reported to be increased in membrane PE more than PC (Popp-Snijders et al, 1986). Our results have found that the total HUFA-containing PE and PS species in type 2 diabetic group, of which predominant species were PE16:0/22:6, PE 18:0/20:5, PS18:0/20:5 and PS18:0/22:6 were significantly lower than those in control group. This was consistent with the results of the others which have reported the low or absence of such fatty acids in erythrocyte membrane of diabetic patients as compared with healthy control subjects (Bakan et al, 2006; Krachler et al, 2008). The possible mechanisms which can be explained for the ω3-fatty acid in diabetes may be involved the low intake, abnormal incorporation and the potential for increased lipid peroxidation of long chain ω3-fatty acid as high oxidative stress and free radical production have been well known in diabetes. In addition, increased oxidative stress in diabetic patients may be related to an increase in inflammation and can be

determined by the increase of proinflammatory cytokines (Pradhan et al, 2001). Thus, it can be indicated that ω3-fatty acid may be rapidly metabolized in diabetic state due to its anti-inflammatory action since ω3-fatty acid has been reported to have anti-inflammatory effects (Calder, 2006).

The total SFA with MUFA and PUFA-containing PC in type 2 diabetic group were slightly higher whereas HUFA-containing PC was lower than those in control group, however, all were not statistically significant difference. It has been found that the apparently significant difference was PC36:2. Generally, type 2 diabetic patients tend to have high fasting and daylong plasma triacylglycerol (van Wijk et al, 2003) and this can increase the incubation time of PC between lipoprotein and cells since the major phospholipid composition of plasma and plasma lipoprotein was PC (Ravand and Kuksis, 2006). In type 2 diabetic group, MUFA-containing LPC was lower whereas SFA and PUFA-containing LPC were higher than those in control group, however, the overall of LPC seemed to be apparently increased as compared to control subjects. Phospholipase A_2 (PLA_2) is a major enzyme that removes oxidized acyl chains from membranes in order to maintain cellular integrity. Any oxidative modification of membrane phospholipid, whether enzymatic or nonenzymatic, is a deleterious process. The best way for such repair is the selective cleavage of the peroxidized fatty acid residues and their subsequent replacement by native fatty acid. Since in membrane phospholipid, the PUFA are preferentially located in the sn-2 position so that this position is especially prone to lipoxygenation or lipid peroxidation, PLA_2 preferentially hydrolyzes peroxidized fatty acids from membranes, and there is an absolute requirement for PLA_2 to allow glutathione peroxidase (GSH-PX) to reduce fatty acid hydroperoxides in membrane (Chakraborti, 2003). Under some pathological conditions such as type 2 diabetes which have high oxidative stress, PLA_2 play important role in this repair process which can increase a production of LPC found in type 2 diabetes including erythrocytes.

Some SM molecular species in type 2 diabetic group were found to be different with respect to control group. Moreover, the positive correlation between SM16:0 with

insulin resistance index (HOMA) was observed in the present study. In the best of our knowledge, no data has been reported the relationship between insulin resistance index (HOMA) with some specific SM molecular species. Erythrocyte membrane sphingomyelin has been reported to be associated with fasting plasma insulin and insulin resistance. Study in skeleton muscle has shown that insulin sensitivity was associated with ceramides consisting of palmitic and linoleic acid since ceramides were the products of sphingomyelin hydrolysis and might interfere insulin signaling pathway (Straczkowski et al, 2004). Ceramide might interfere with the insulin signaling pathway through maintaining protein kinase B (PKB)/Akt in an inactive dephosphorylated state (Teruel et al, 2001). A further consequence of ceramide action is the reduction of GLUT4 translocation to plasma membrane and a decrease in insulin-stimulated glucose uptake (Han and Gross, 1994; Summers et al, 1998). Moreover, sphingomyelin/cholesterol ratio has been reported to be an important determinant of glucose transport mediated by GLUT-1 in preadipocytes (Al-Makdissy et al, 2003).

5.3.3 The changes of erythrocyte phospholipids molecular species after the high fat meal period in both groups

Since erythrocyte was considered as a poor surrogate of insulin sensitive cells unlike adipocyte, hepatocyte, or skeletal muscle cell. However, some studies have shown that changes in erythrocyte membrane parallel with those in adipocyte membrane in obese subjects were correlated with the degree of insulin resistance (Zeghari et al, 2000). Therefore, it may be implied that the changes in erythrocyte phospholipids can reflect those occurred in an insulin sensitive cells.

Phospholipids molecular species of all classes in both groups were analyzed and compared in order to investigate and compare the changes of erythrocyte phospholipids molecular species obtained from type 2 diabetes and healthy control subjects after the high fat meal period. Transient postprandial hypertriacylglycerolemia was induced by giving high fat meal to all subjects. Our results have revealed that

erythrocyte phospholipids molecular species in control group were shown apparent changes after meal period rather than type 2 diabetic group. HUFA-containing PE and PS were found to be significantly decreased in control group whereas no differences of those were observed in type 2 diabetic group. PI seemed to be increased especially in control group after the meal period. Significant increase of SFA and MUFA-containing PC was only found in control group whereas no change of SM was found in both groups. LPC in all groups tend to be decrease as compared with fasting state but apparent changes of its species were observed in control group.

The changes of PE and PS molecular species after the meal period

The mechanisms of the changes of erythrocyte phospholipids after the high fat meal period could be explained by the exchange, uptake or incorporation of fatty acid and phospholipid between erythrocyte and plasma or lipoprotein. As phospholipids and cholesterol are the major lipid found in erythrocyte, these lipids are capable to exchange and efflux into plasma by the action of some enzymes, such as LCAT. However, PE and PS in erythrocyte membrane are not involved in this exchange due to the concept that these phospholipids are situated on the inside of the erythrocyte membrane. From our results of control subjects, there were marked losses of HUFA-containing PE and PS molecular species observed after high fat meal period whereas small changes of such were found in type 2 diabetic group.

In normal physiologic condition, erythrocyte has a potential to loss its cholesterol to plasma in order to be esterified with fatty acid by LCAT. Cholesterol associated with phospholipid (0.2–55% of total haemoglobin) in erythrocyte has been reported to be firmly bound to Hb yielding an Hb-lipid adduct (termed Hb-Ch) with a binding of approximately two molecules of each cholesterol and phospholipids per single Hb tetramer (Nikolic et al, 2004). Moreover, Nikolic and colleagues have recently studied the efflux of lipids from normal human erythrocyte into fasting plasma. Plasma was incubated with a two-fold excess of autologous erythrocyte, the plasma cholesterol and

phospholipid level were increased due to the loss of cholesterol and phospholipid from the erythrocyte membrane. They have proposed that the possible mechanisms for exchange of Hb-lipids between erythrocyte and plasma lipoproteins would most likely include a two-step process. Firstly, the influx of cholesterol is associated with phospholipids from plasma via the erythrocyte membrane, followed by their binding to Hb forming the stable Hb-Ch adduct. In addition, they have strongly suggested that a diet enriched in both cholesterol and phospholipids (particularly PS) could be a primary factor that influences the quantity of the Hb-lipids adduct. Secondly, the efflux of cholesterol (in association with phospholipids) from the Hb-Ch fraction back into the circulation with its preferential distribution into HDL particles appears to be an attractive and unique characteristic of Hb-Ch. Therefore, it can be speculated that the Hb-Ch adduct may play an important role in RCT in vivo, as the transfer of Hb-lipid-derived cholesterol into plasma would lead to its removal by the liver (Nikolic et al, 2007). Additionally, Tomasevic and colleagues have demonstrated that in the presence of insulin, PS, PE and cholesterol were found to be bound to Hb (Tomasevic, 2003). Taken together, the possible explanation that can support our results of the reduction of PE and PS after high fat meal period may be involved the loss of cholesterol associated with phospholipid from erythrocyte into plasma.

During the high fat meal period, the presence of active LCAT and CETP are very effective in promoting efflux of cholesterol from erythrocyte and the level of cholesterol released from erythrocytes into plasma can be increased by supplementing it with chylomicrons (Chung et al, 1998). Thus, it may be indicated that cholesterol in erythrocyte from control subjects may be lost into plasma together with PE and PS. Moreover, our findings can speculate that HUFA-containing PE and PS species were major species participating in this process. In contrast with control group, there were no changes of PE and PS after the meal period observed in type 2 diabetic group. It can be suggested that cholesterol may be not firmly bound with phospholipid to form Hb-lipid complex or there may be the internalzation of this lipid complex in erythrocyte since the

supraphysiological concentrations of insulin in vitro can result in the internalization of such lipid complex. (Tomasevic et al, 2003)

As the supraphysiological concentrations of insulin has influence on the internalization of Hb-lipid complex together with a marked increase of postprandial insulin found in type 2 diabetic group, thus, it can be indicated that this can affect the capacity of erythrocyte to lose cholesterol into plasma during postprandial period. The movement of cholesterol from peripheral tissues or any cells through the plasma was termed reverse cholesterol transport (RCT). As mentioned, Hb-Ch was recently postulated as a new form of cholesterol in circulation that contributed to the permanent removal of excess of non-esterified cholesterol from the circulation. The impairment of the loss of such lipids or lipid complexes may affect the RCT mechanism that can increase risks of atherosclerosis and/or coronary heart disease, the major complications found in type 2 diabetes.

The changes of PI molecular species after the meal period

After the meal period, PI in control group seemed to be increased as compared to fasting state whereas type 2 diabetic group did not. The increase of PI in erythrocyte may be due to the rapid exchange of PI from plasma by the contribution of phosphatidylinositol transfer protein (PITP). The PITP family is one of the two families of phosphoinositide transfer proteins that can bind and exchange one molecule of either phosphatidylinositol (PI) or phosphatidylcholine (PC) and facilitate the transfer of these lipids between different membranes (Hsuan and Cockcroft, 2001). Human erythrocytes are highly specialized cells whose function is oxygen transport. These cells' sole metabolic source of energy is the fermentation of glucose via glycolysis. They contain an active insulin receptor and respond to insulin by increasing phosphorylation of tyrosine residues in several proteins. In postprandial period, the increased insulin level can stimulate glucose uptake in erythrocyte via insulin signaling pathway. A part of such pathway, polyphosphoinositides are diverted out of the kinase/phosphatase substrate cycles , causing a local depletion of PI in the membrane. The PITP then catalyzes a net

transfer of PI to the membrane from plasma or other membranes (Currie et al, 1997). Since the high concentration of PI was found in plasma, therefore, such transfer may cause the increase of some PI species in erythrocyte observed after the meal period. However, this phenomenon hasn't been observed in type 2 diabetic group. The possible explanation may be due to the alteration of erythrocyte insulin receptor, causing insulin resistance that can alter the insulin signaling pathway. In addition, the abnormal activities of phospholipid transfer protein, especially PITP activity in type 2 diabetes may be also the other mechanism to support this.

The changes of PC, SM and LPC molecular species after high fat meal period

PC is a major phospholipid in erythrocyte membrane and of which molecular species are primarily involved in its renewed process. As mentioned before, this renewal occurs without the *de novo* synthesis of fatty acids. Two major pathways for this renewal were a passive exchange of intact phospholipid between serum lipoproteins/plasma and erythrocyte cell membranes, and an energy-dependent acylation within the membrane of either endogenous erythrocyte or plasma LPC by free fatty acids derived from plasma.

Our results have found that the total of SFA and MUFA-containing PC species was significantly increased relative to those in fasting state and this was only found in control group. SM seemed to be unchanged whereas LPC tend to be decreased as compared to fasting state in both groups.

There were apparently significant increases of PC34:1, PC36:2 and PC36:1 observed in control group rather than in type 2 diabetic group after the meal period. This indicated the rapid turnover of phospholipids, particularly PC in of erythrocyte. Since plasma PC have been reported to be increased after high fat diet (Lindman et al, 2003) and erythrocyte has a passive exchange with plasma PC until equilibrium, it can be speculated that the increases of such PC species may be from the exchange of intact PC from plasma lipoproteins. However, the acylation of LPC is also another process that was involved with the fatty acid remodeling of PC in erythrocyte. As the predominant

fatty acid composition of the high fat meal in this study was palmitic acid (16:0) and oleic acid (18:1), it can be assumed that the increases of some PC species may be from an ATP-dependent acylation of such fatty acids with either endogenous erythrocyte or plasma LPC. The mechanism of this process was that plasma-derived fatty acids are esterified to acyl-CoA by acyl-CoA synthetases and transferred to lysophosphatidylcholine acyl-CoA transferases (LAT) to form PC molecules (Soupene et al, 2008) which finally incorporated into phospholipids fraction of erythrocyte. The extents of the incorporation of various fatty acids into erythrocyte were reported. Oliveira and Vaughan have studied the incorporation of C^{14}-labeled fatty acids into phospholipids of human erythrocyte and found that a labeled palmitic acid from 1-C^{14}-palmitoyl CoA was incorporated in the absence of added cofactors, the extent of incorporation of linoleic acid was greater than that of oleic, palmitic, or stearic acids. Moreover, of the radioactive fatty acid taken up into phospholipids 80-90% was found in the lecithin fraction (Oliveira and Vaughan, 1964). This results were consistent with the recent study that has reported the markedly rapid incorporation of linoleate from a single meal into phospholipids fractions of erythrocyte (Hodson et al, 2009).

Although the overall of LPC seemed to be decreased in both groups, however, some LPC molecular species in control were apparently increased as compared to fasting state, particularly LPC16:0 whereas no significant changes were observed in type 2 diabetic group. Generally, there were some possible mechanisms that can be explained for this phenomenon. First, the oxygen-carrying function of the erythrocyte leads to free radical damage of unsaturated acyl chains that need to be removed and oxidized acyl chains are rapidly removed from membranes by phospholipase A_2 action which generated LPC (van den Berg et al, 1993). Second, there may be a passive exchange of LPC from plasma in postprandial state since the rapid exchange of LPC has been reported to be equilibrium with that in plasma (Renooij and Van Golde, 1976) and the increasing of LPC may be formed via LCAT (Glomset, 1968) which have been known to be very active in postprandial period. Third, it may be involved the direct transfer of fatty acid from PC to PE before its final release to serum. Shohet have done

such experiment and supported the concept of the independent transfer of fatty acid on the 2-position of PC to PE in erythrocyte incubated in fresh serum (Figure 58). However, they were unable to show an increase of LPC (Shohet, 1971). In control group of present study, PUFA containing-PC and PE seemed to be decreased in parallel with increasing of some LPC species, it can be implied that there may be a possible transfer of fatty acid from PC to PE in erythrocyte during postprandial period and such fatty acids may finally release to serum for further metabolism, however, the lysoPE species have not been investigated in this study (Figure 58).

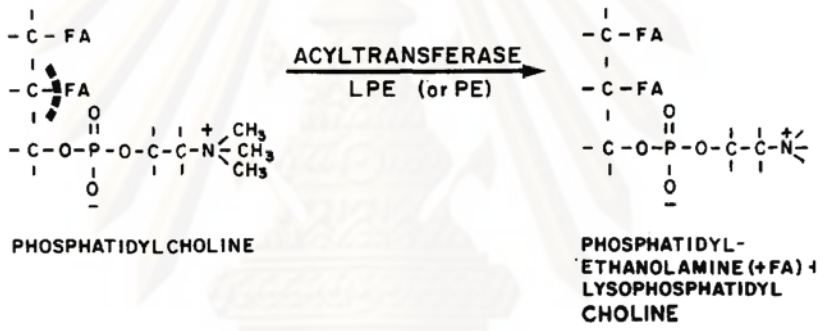


Figure 58 Possible pathways for the apparent conversion of PC to PE (Shohet, 1971)

As PC is a predominant phospholipid in plasma and triacylglycerol-rich lipoprotein and is involved in the exchange between cell membranes including erythrocyte. However, our finding has shown that the mild alterations of PC molecular species of type 2 diabetic group after meal period. It is possibly due to the abnormal activities of some enzymes involved the exchange or renewed processes. The principal enzymes that were involved in such processes were phosphatidylcholine-transfer protein (PC-TP), acyl-CoA synthetases and lysophosphatidylcholine acyl-CoA transferases (LAT). The PC-TP is a major protein that catalyzed PC one for one exchange, as well as net transfer of PC between the membranes (Roderick et al, 2002) and its activity has been found in human erythrocyte hemolysate (Kuroda and Ohnishi,

1983). The two latter enzymes have a major role in acylation of ingested fatty acid to LPC. In type 2 diabetic human, there were no evidences that have been shown whether these enzymes was defective in activities. In animal experiments, some evidences were shown the alteration of the activity of key enzymes of the membrane phospholipid fatty acid turnover, LAT and phospholipase A_2 in streptozotocin(STZ)-induced diabetic rats (Dang et al, 1984) and the decreased ability of diabetic rat erythrocytes to reacylate membrane PC by LAT. In addition, these abnormalities can be restored to control values by ATP and insulin treatment (Le Petit-Thevenin et al, 1988). However, such experiments were done in animal model of type I diabetes which was defined as insulin deficiency. In the present study, high plasma insulin was found among type 2 diabetic patients, especially after the meal period when compared to control subjects. Consequently, it can be speculated that the activity of principal enzymes of the membrane phospholipid fatty acid turnover may be suppressed by the high insulin concentration in type 2 diabetes, especially during postprandial period since the high concentration and prolongation of insulin had influence on the activity of some lipid transfer proteins (Dullaart and van Tol, 2001; Kaser et al, 2001). Further studies need to study the influence of high insulin concentration during postprandial period on the activity of key enzymes of the membrane phospholipid fatty acid turnover in erythrocyte of insulin resistant type 2 diabetes.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The present study were summarized herewith

1. Type 2 diabetes group has a significantly higher fasting glucose, HbA_1C and insulin resistant index (HOMA-IR) than those in healthy control group. Moreover, the HOMA-IR was significantly associated with some biochemical parameters e.g. weight ($r = 0.774$, $p<0.001$), Body Mass Index (BMI)($r = 0.654$, $p=0.01$), Waist to hip ratio (WHR) ($r = 0.547$, $p =0.01$), fasting triacylglycerol ($r = 0.679$, $p =0.01$) and HDL Cholesterol ($r = -0.542$, $p =0.011$).

2. Four hours after ingestion of high fat meal, plasma triacylglycerol in both groups was markedly increased as compared with fasting state. This has indicated that all studied subjects have well response to the given meal. Insulin was also increased in both groups, however, the postprandial insulin and percentage change in insulin in type 2 diabetic group was significantly higher than that in control group.

3. FTIR analysis of erythrocyte contents revealed that the integrated areas of lipid and phosphate stretching vibrations seemed to decrease after the meal period, especially the olefinic band, which indicates double bonds in lipid structure, still remained unchanged relative to fasting state. In contrast to diabetic group, our results showed an increasing of such band in control group. The conformations of erythrocyte protein after the meal period in both groups were also changed and different in some conformations. Glucose and lactate region seemed to decrease in type 2 diabetic group, however, there were significant increases of those found in control group.

4. Insulin resistant index (HOMA-IR) was inversely correlated with some erythrocyte phospholipids molecular species, PE36:4 and SM16:0.

5. After the meal period, most of erythrocyte phospholipids molecular species in type 2 diabetes still remained unchanged, however, the apparent changes were found in control group. Some amino-containing phospholipids molecular species were decreased whereas there were increases of some choline-containing phospholipids molecular species observed in control group, particularly the significant increase of total SFA and MUFA-containing PC ($p<0.05$).

In conclusion, in contrast with healthy control, the results of the present study indicated that there were the abnormalities of carbohydrate, protein and lipid metabolism in erythrocyte of T2DM patients, especially of the remodeling of phospholipid fatty acid during the meal period which was an important process for phospholipid turnover in erythrocyte. The impairment of such, particularly the incorporation or uptake of unsaturated fatty acid, directly affected the erythrocyte membrane fluidity, leading to the abnormal membrane functions and properties. In addition, the impaired alterations occurred in biochemical structure of erythrocytes of T2DM probably explain the less flexibility regularly observed in blood cells of diabetic patients. The underlying mechanisms still remained unclear, however, it can be speculated that the high insulin concentration in postprandial period together with the insulin resistance found in type 2 diabetic patients may have the influence on the activities of many enzymes involved the exchange and incorporation of ingested lipids between erythrocyte and plasma and/or lipoproteins. Consequently, these findings could be the potential information for further researches in nutritional and pharmaceutical fields in order to find the new therapeutic treatments to correct or restore these impairments that would decrease the risks of microvascular complications in type 2 diabetic patients.

REFERENCES

Abidi, S. L. and Mounts, T. L. Reversed-phase high-performance liquid chromatography of molecular species of phospholipid derivatives. J. Chromatogr. A 741 (1996): 213-222.

Adiels, M. ; Borén, J. ; Caslake, M. J., et al. Overproduction of VLDL1 driven by hyperglycemia is a dominant feature of diabetic dyslipidemia. Arterioscler. Thromb. Vasc. Biol. 25 (2005): 1697-1703.

Aekplakorn, W. ; Stolk, R. P. ; Neal, B., et al. The prevalence and management of diabetes in Thai adults: the International Collaborative Study of Cardiovascular Disease in Asia. Diabetes Care 26 (2003): 2758-2763.

Ahmad, J. ; Hameed, B. ; Das, G. ; Siddiqui, M. A. and Ahmad, I. Postprandial hypertriglyceridemia and carotid intima-media thickness in north Indian type 2 diabetic subjects. Diabetes Res. Clin. Pract. 69 (2005): 142-150.

Al-Makdissy, N. ; Younsi, M. ; Pierre, S. ; Ziegler, O. and Donner, M. Sphingomyelin/cholesterol ratio: an important determinant of glucose transport mediated by GLUT-1 in 3T3-L1 preadipocytes. Cell. Signal. 15 (2003): 1019-1030.

Allen, H. G. ; Allen, J. C. ; Boyd, L. C. ; Alston-Mills, B. P. and Fenner, G. P. Determination of membrane lipid differences in insulin resistant diabetes mellitus type 2 in whites and blacks. Nutrition 22 (2006): 1096-1102.

Alvarez, J. ; Lee, D. C. ; Baldwin, S. A. and Chapman, D. Fourier transform infrared spectroscopic study of the structure and conformational changes of the human erythrocyte glucose transporter. J. Biol. Chem. 262 (1987): 3502-3509.

Anderson, R. A. ; Evans, L. M. ; Ellis, G. R., et al. Prolonged deterioration of endothelial dysfunction in response to postprandial lipaemia is attenuated by vitamin C in Type 2 diabetes. Diabet. Med. 23 (2006): 258-264.

Annuzzi, G. ; De Natale, C. ; Iovine, C., et al. Insulin resistance is independently associated with postprandial alterations of triglyceride-rich lipoproteins in type 2 diabetes mellitus. Arterioscler. Thromb. Vasc. Biol. 24 (2004): 2397-2402.

Annuzzi, G. ; Giacco, R. ; Patti, L., et al. Postprandial chylomicrons and adipose tissue lipoprotein lipase are altered in type 2 diabetes independently of obesity and whole-body insulin resistance. Nutr. Metab. Cardiovasc. Dis.18 (2008): 531-538.

Avramoglu, R. K. ; Basciano, H. and Adeli, K. Lipid and lipoprotein dysregulation in insulin resistant states. Clin. Chim. Acta 368 (2006): 1-19

Avramoglu, R. K. ; Basciano, H. and Adeli, K. Lipoprotein Metabolism in Insulin-Resistant States.In S. K. Cheema, Biochemistry of Atherosclerosis, 205-246. New York: Springer 2006.

Axelsen, M. ; Smith, U. ; Eriksson, J. W. ; Taskinen, M. R. and Jansson, P. A. Postprandial Hypertriglyceridemia and Insulin Resistance in Normoglycemic First-Degree Relatives of Patients with Type Diabetes. Ann. Intern. Med. 131 (1999): 27-31.

Bae, J. ; Schwemmer, M. ; Lee, I., et al. Postprandial hypertriglyceridemia-induced endothelial dysfunction in healthy subjects is independent of lipid oxidation. Int. J. Cardiol. 87 (2003): 259-267.

Bae, J. H. ; Bassenge, E. ; Lee, H. J., et al. Impact of postprandial hypertriglyceridemia on vascular responses in patients with coronary artery disease: effects of ACE inhibitors and fibrates. Atherosclerosis 158 (2001): 165-171.

Bagdade, J. D. ; Lane, J. T. ; Subbaiah, P. V. ; Otto, M. E. and C, R. M. Accelerated cholesteryl ester transfer in noninsulin-dependent diabetes mellitus. Atherosclerosis 104 (1993): 69-77.

Bakan, E. ; Yildirim, A. ; Kurtul, N., et al. Effects of type 2 diabetes mellitus on plasma fatty acid composition and cholesterol content of erythrocyte and leukocyte membranes. Acta Diabetol. 43 (2006): 109-113.

Bansal, S. ; Buring, J. E. ; Rifai, N., et al. Fasting Compared With Nonfasting Triglycerides and Risk of Cardiovascular Events in Women. JAMA 298 (2007): 309-316.

Barroso, B. and Bischoff, R. LC-MS analysis of phospholipids and lysophospholipids in human bronchoalveolar lavage fluid. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 814 (2005): 21-28.

Barth, A. Review : Infrared spectroscopy of proteins. Biochim. Biophys. Acta 1767 (2007): 1073-1101.

Beermann, C. ; Mobius, M. ; Winterling, N. ; Schmitt, J. J. and Boehm, G. sn-position determination of phospholipid-linked fatty acids derived from erythrocytes by liquid chromatography electrospray ionization ion-trap mass spectrometry. Lipids 40 (2005): 211-218.

Bernhard, W. ; Schmiedl, A. ; Koster, G., et al. Developmental changes in rat surfactant lipidomics in the context of species variability. Pediatr. Pulmonol. 42 (2007): 794-804.

Blight, E. G. and Dyer, W. J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (1959): 911-917.

Bloomgarden, Z. T. Insulin Resistance:Current Concepts. Clin. Ther. 20 (1998): 216-231.

Brenner, R. R. Effect of unsaturated acids on membrane structure and enzyme kinetics. Prog. Lipid Res. 23 (1984): 69-96.

Brouwers, J. F. ; Versluis, C. ; van Golde, L. M. and Tielens, A. G. 5-Octadecenoic acid: evidence for a novel type of fatty acid modification in schistosomes. Biochem. J. 334 (1998): 315-359.

Brugger, B. ; Erben, G. ; Sandhoff, R. ; Wieland, F. T. and Lehmann, W. D. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 94 (1997): 2339-2344.

Calder, P. C. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am. J. Clin. Nutr. 83 (2006): 1505S-1519S.

Chakraborti, S. Phospholipase A(2) isoforms: a perspective. Cell. Signal. 15 (2003): 637-665.

Chen, Y. D. ; Varasteh, B. B. and Reaven, G. M. Plasma lactate concentration in obesity and type 2 diabetes. Diabetes Metab. 19 (1993): 348-354.

Christie, W. W. Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids. England: Oily Press, Bridgewater, 2003.

Christie, W. W. Sphingomyelin and related lipids [Online]. 2009. Available from http://www.lipidlibrary.co.uk/Lipids/sph/index.html [2009, October, 23]

Chung, B. H. ; Franklin, F. ; Cho, B. H. S., et al. Potencies of lipoproteins in fasting and postprandial plasma to accept additional cholesterol molecules released from cell membranes. Arterioscler. Thromb. Vasc. Biol. 18 (1998): 1217-1230.

Clemens, M. R. and Waller, H. D. Lipid peroxidation in erythrocytes. Chem. Phys. Lipids 45 (1987): 251-268.

Currie, R. A. ; MacLeod, B. M. and Downes, C. P. The lipid transfer activity of phosphatidylinositol transfer protein is sufficient to account for enhanced phospholipase C activity in turkey erythrocyte ghosts. Curr. Biol. 7 (1997): 184-190.

Dahlan, W. Intravenous infusion of triacylglycerol-phospholipid complexes in man: effect on fatty acid pattern of plasma and on erythrocyte membrane lipid composition. Doctoral dissertation, Department of surgery, Faculty of medicine, Universite Libre De Bruxelles, 1989.

Dallongeville, J. and Fruchart, J. C. Postprandial dyslipidemia: a risk factor for coronary heart disease. Ann. Nutr. Metab. 42 (1998): 1-11.

Dang, A. Q. ; Faas, F. H. and Carter, W. J. Effects of streptozotocin-induced diabetes on phosphoglyceride metabolism of the rat liver. Lipids 19 (1984): 738-748 (Abstract).

Davis, B. ; Koster, G. ; Douet, L. J., et al. Electrospray ionization mass spectrometry identifies substrates and products of lipoprotein-associated phospholipase A2 in oxidized human low density lipoprotein. J. Biol. Chem. 283 (2008): 6428-6437.

Davis, T. M. E. ; Pramukkul, P. ; Suputtamongkola, Y. ; Chaowagul, W. and Levyd, J. Glucose tolerance in rural diabetic Thais, first-degree relatives and non-diabetic controls. Diabetes Res. Clin. Pract. 27 (1995): 171-180.

Decsi, T. ; Minda, H. ; Hermann, R., et al. Polyunsaturated fatty acids in plasma and erythrocyte membrane lipids of diabetic children. Prostaglandins Leukot. Essent. Fatty Acids 67 (2002): 203-210.

Dullaart, R. P. and van Tol, A. Twenty four hour insulin infusion impairs the ability of plasma from healthy subjects and Type 2 diabetic patients to promote cellular cholesterol efflux. Atherosclerosis 157 (2001): 49-56.

Eckel, R. H. ; Grundy, S. M. and Zimmet, P. Z. The metabolic syndrome. Lancet 365 (2005): 1415-1428.

Ellis, D. I. and Goodacre, R. Metabolic fingerprinting in disease diagnosis: biomedical applications of infrared and Raman spectroscopy. Analyst 131 (2006): 875-885.

Enriquez, Y. R. ; Giri, M. ; Rottiers, R. and Christophe, A. Fatty acid composition of erythrocyte phospholipids is related to insulin levels, secretion and resistance in obese type 2 diabetics on Metformin. Clin. Chim. Acta 346 (2004): 145-152.

Eriksson, J. W. ; Buren, J. ; Svensson, M. ; Olivecrona, T. and Olivecrona, G. Postprandial regulation of blood lipids and adipose tissue lipoprotein lipase in type 2 diabetes patients and healthy control subjects. Atherosclerosis 166 (2003): 359-367.

Esposito, K. ; Ciotola, M. ; Sasso, F. C., et al. Effect of a single high-fat meal on endothelial function in patients with the metabolic syndrome: role of tumor necrosis factor-alpha. Nutr. Metab. Cardiovasc. Dis.17 (2007): 274-279.

Esposito, K. ; Nappo, F. ; Giugliano, F., et al. Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. Am. J. Clin. Nutr. 78 (2003): 1135-1140.

Exton, J. H. Phosphatidylcholine breakdown and signal transduction. Biochim. Biophys. Acta 1212 (1994): 26-42.

Ferreira, A. ; Peter, A. A. ; Mendez, A. J., et al. Postprandial Hypertriglyceridemia Increases Circulating Levels of Endothelial Cell Microparticles. Circulation 110 (2004): 3599-3603.

Folsom, A. R. ; Ma, J. ; McGovern, P. G. and Eckfeldt, H. Relation between plasma phospholipid saturated fatty acids and hyperinsulinemia. Metabolism 45 (1996): 223-228.

Friedewald, W. T. ; Levy, R. T. and Fredrickson, D. S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem. 18 (1972): 499-502.

Gaenzer, H. ; Sturm, W. ; Neumayr, G., et al. Pronounced postprandial lipemia impairs endothelium-dependent dilation of the brachial artery in men. Cardiovasc. Res. 52 (2001): 509-516.

Galgani, J. ; Aguirre, C. and Díaz, E. Acute effect of meal glycemic index and glycemic load on blood glucose and insulin responses in humans. Nutr. J. 5 (2006): 5-22.

Gao, F. ; Tian, X. ; Wen, D., et al. Analysis of phospholipid species in rat peritoneal surface layer by liquid chromatography/electrospray ionization ion-trap mass spectrometry. Biochim. Biophys. Acta 1761 (2006): 667-676.

Genge, B. R. ; Wu, L. N. Y. and Wuthier, R. E. Separation and quantification of chicken and bovine growth plate cartilage matrix vesicle lipids by high-performance liquid chromatography using evaporative light scattering detection. Anal. Biochem. 322 (2003): 104-115.

Giannattasio, C. ; Zoppo, A. ; Gentile, G., et al. Acute Effect of High-Fat Meal on Endothelial Function in Moderately Dyslipidemic Subjects. Arterioscler. Thromb. Vasc. Biol. 25 (2005): 406-410.

Ginsberg, B. H. ; Jabour, J. and Spector, A. A. Effect of alterations in membrane lipid unsaturation on the properties of the insulin receptor of Ehrlich ascites cells. Biochim. Biophys. Acta 690 (1982): 157-164.

Ginsberg, H. N. and Illingworth, D. R. Postprandial dyslipidemia: an atherogenic disorder common in patients with diabetes mellitus. Am. J. Cardiol. 88 (2001): 9H-15H.

Glomset, J. A. The plasma lecithins:cholesterol acyltransferase reaction. J. Lipid Res. 9 (1968): 155-167.

Greenfield, J. R. ; Samaras, K. ; Chisholm, D. J. and Campbell, L. V. Effect of postprandial insulinemia and insulin resistance on measurement of arterial stiffness (augmentation index). Int. J. Cardiol. 114 (2007): 50-56.

Gregoriou, V. G. ; Jayaraman, V. ; Hu, X. and Spiro, T. G. FT-IR difference spectroscopy of hemoglobins A and Kempsey: evidence that a key quaternary interaction induces protonation of Asp beta 99. Biochemistry 34 (1995): 6876-6882.

Griglio, S. ; Sultan, F. and Lagrange, D. Role of hepatic lipase in the catabolism of chylomicron remnants in the rat (Abstract). Diabetes Metab. 18 (1992): 150-155.

Groot, P. H. ; Stiphout, W. A. V. ; Krauss, K. H., et al. Postprandial lipoprotein metabolism in normolipidemic men with and without coronary artery disease. Arterioscler .Thromb. 11 (1991): 653-662.

Guerci, B. ; Paul, J. L. ; Hadjadj, S., et al. Analysis of the postprandial lipid metabolism: use of a 3-point test. Diabetes Metab. 27 (2001): 449-457.

Gurr, M.I. ; Harwood, J.L. and Frayn, K.N.Lipid biochemistry, 5th ed. UK: Blackwell Publishing, 2002.

Gylling, H. and Miettinen, T. A. Cholesterol absorption, synthesis, and LDL metabolism in NIDDM. Diabetes Care 20 (1997): 90-95.

Haffner, S. M. ; Lehto, S. ; Ronnemaa, T. ; Pyorala, K. and Laakso, M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. N. Engl. J. Med. 339 (1998): 229-234.

Haffner, S. M. ; Miettinen, H. ; Gaskill, S. P. and Stern, M. P. Decreased insulin secretion and increased insulin resistance are independently related to the 7-year risk of NIDDM in Mexican-Americans. Diabetes 44 (1995): 1386-1391.

Han, X. ; Abendschein, D. R. ; Kelley, J. G. and Gross, R. W. Diabetes-induced changes in specific lipid molecular species in rat myocardium. Biochem. J. 352 (2000): 79-89.

Han, X. and Gross, R. W. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. Proc. Natl. Acad. Sci. U.S.A. 91 (1994): 10635-10639.

Harris, J. W. and Kellermeyer, R. W. The red cell. Production, metabolism, destruction: normal and abnormal. Cambridge Massachusetts: Harward University Press, 1972.

Heeley, E. L. ; Hohlfeld, J. M. ; Krug, N. and Postle, A. D. Phospholipid molecular species of bronchoalveolar lavage fluid after local allergen challenge in asthma. Am. J. Physiol. Lung Cell Mol. Physiol. 278 (2000): 305-311.

Hettihewa, L. M. ; Dharmasiri, L. P. ; Ariyaratne, C. D., et al. Significant correlation between BMI/BW with insulin resistance by McAuley, HOMA and QUICKI indices after 3 months of pioglitazone in diabetic population. Int. J. Diab. Dev. Ctries. 27 (2007): 87-92.

Hodson, L. ; Mcquaid, S. E. ; Karpe, F. ; Frayn, K. N. and Fielding, B. A. Differences in partitioning of meal fatty acids into blood lipid fractions: a comparison of linoleate, oleate and palmitate. Am. J. Physiol. Endocrinol. Metab. 296 (2009): E64-E71.

Howard, B. Insulin Resistance and Lipid Metabolism. Am. J. Cardiol. 84 (1999): 28-32.

Hsuan, J. and Cockcroft, S. The PITP family of phosphatidylinositol transfer proteins. Genome Biol. 2 (2001): 3011.1–3011.8.

Hyson, D. ; Rutledge, J. C. and Berglund, L. Postprandial lipemia and cardiovascular disease. Curr. Atheroscler. Rep. 5 (2003): 437-444.

Iovine, C. ; Vaccaro, O. ; Gentile, A., et al. Post-prandial triglyceride profile in a population-based sample of Type 2 diabetic patients. Diabetologia 47 (2004): 19-22.

Isomaa, B. ; Almgren, P. ; Tuomi, T., et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care 24 (2001): 683-689.

Ivanova, P. T. ; Milne, S. B. ; Forrester, J. S. and Brown, H. A. LIPID arrays: new tools in the understanding of membrane dynamics and lipid signaling. Mol. Interv. 4 (2004): 86-96.

Jand, K. and Yu, S. Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. Acta Biochim. Biophys. Sin. 39 (2007): 549-559.

Karamanos, B. G. ; Thanopoulou, A. C. and Roussi-Penesi, D. P. Maximal post-prandial triglyceride increase reflects post-prandial hypertriglyceridaemia and is associated with the insulin resistance syndrome. Diabet. Med. 18 (2001): 32-39.

Kaser, S. ; Foger, B. ; Ebenbichler, C. F., et al. Influence of leptin and insulin on lipid transfer proteins in human hepatoma cell line, HepG2. Int. J. Obes. 25 (2001): 1633-1639.

Kim, H. Y. ; Wang, T. C. and Ma, Y. C. Liquid chromatography/mass spectrometry of phospholipids using electrospray ionization. Anal. Chem. 66 (1994): 3977-3982.

King, H. ; Aubert, R. E. and Herman, W. H. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 21 (1998): 1414-1431.

King, M.W. Phospholipid synthesis [Online]. 2009. Available from http:// themedicalbiochemistrypage.org/lipid-synthesis.html#phospholipids [2009, October, 23]

Koivusalo, M. ; Haimi, P. ; Heikinheimo, L. ; Kostiainen, R. and Somerharju, P. Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. J. Lipid Res. 42 (2001): 663-672.

Kolosova, M. V. ; Novitskii, V. V. ; Stepovaya, E. A. and Kravets, E. B. Protein Spectrum and State of Lipid Bilayer in Erythrocyte Membranes in Children with Insulin-Dependent Diabetes Mellitus: Polyacrylamide Gel Electrophoresis and Fluorescence Analysis Data. Bull. Exp. Biol. Med. 3 (2000): 261-263.

Kondepati, V. R. ; Heise, H. M. ; Oszinda, T., et al. Detection of structural disorders in colorectal cancer DNA with Fourier-transform infrared spectroscopy. Vib. Spectrosc. 46 (2008): 150-157.

Kovar, J. ; Fejfarova, V. ; Pelikanova, T. and Poledne, R. Hyperglycemia downregulates total lipoprotein lipase activity in humans. Physiol. Res. 53 (2004): 61-68.

Krachler, B. ; Norberg, M. ; Eriksson, J. W., et al. Fatty acid profile of the erythrocyte membrane preceding development of Type 2 diabetes mellitus. Nutr. Metab. Cardiovasc. Dis. 18 (2008): 503-510.

Kuroda, K. and Ohnishi, S. Phosphatidylcholine transfer activity in human erythrocyte hemolysate. J. Biochem. 94 (1983): 1809-1813.

Labrouche, S. ; Freyburger, G. ; Gin, H. ; Boisseau, M. R. and Cassagne, C. Changes in phospholipid composition of blood cell membranes (erythrocyte, platelet, and polymorphonuclear) in different types of diabetes- clinical and biological correlations. Metabolism 45 (1996): 57-71.

Land, W. E. Metabolism of glycerolipids II. The enzymatic acylation of lysolecithin. J. Biol. Chem. 235 (1960): 2233-2237.

Larsen, A. ; Uran, S. ; Jacobsen, P. B. and Skotland, T. Collision-induced dissociation of glycero phospholipids using electrospray ion-trap mass spectrometry. Rapid Commun. Mass Spectrom. 15 (2001): 2393-2398.

Le Petit-Thevenin, J. ; Nobili, O. and Boyer, J. Decreased acylation of phosphatidylcholine in diabetic rat erythrocytes. Diabetes 37 (1988): 142-146 (Abstract).

Leahy, J. L. Pathogenesis of Type 2 Diabetes Mellitus. Arch. Med. Res. 36 (2005): 197-209.

Leidl, K. ; Liebisch, G. ; Richter, D. and Schmitz, G. Mass spectrometric analysis of lipid species of human circulating blood cells. Biochim. Biophys. Acta 1781 (2008): 655-664.

Lemieux, I. ; Couillard, C. ; Pascot, A., et al. The small, dense LDL phenotype as a correlate of postprandial lipemia in men. Atherosclerosis 153 (2000): 423-432.

Levy, Y. ; Leibowitz, R. ; Aviram, M. ; Brook, J. G. and Cogan, U. Reduction of plasma cholesterol by lovastatin normalizes erythrocyte membrane fluidity in patients with severe hypercholesterolaemia. Br. J. Clin. Pharmacol. 34 (1992): 427-430.

Lillioja, S. ; Mott, D. M. ; Spraul, M., et al. Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. N. Eng. J. Med. 329 (1993): 1988-1992.

Lindman, A. S. ; Müller, H. ; Seljeflot, I., et al. Effects of dietary fat quantity and composition on fasting and postprandial levels of coagulation factor VII and serum choline-containing phospholipids. Br. J. Nutr. 90 (2003): 329-336.

Liu, K. ; Tsang, K. ; Li, C. ; Shaw, R. and Mantsch, H. Infrared spectroscopic identification of beta-thalassemia. Clin. Chem. 49 (2003): 1125-1132.

Liu, K. Z. ; Bose, R. and Mantsch, H. H. Infrared spectroscopic study of diabetic platelets Vib. Spectrosc. 28 (2002): 131-136.

Liu, K. Z. ; Shi, M. H. and Mantsch, H. H. Molecular and chemical characterization of blood cells by infrared spectroscopy: a new optical tool in hematology. Blood Cells Mol. Dis. 35 (2005): 404-412.

Lowe, G. D. O. ; Lowe, J. M. ; Drummond, M. M. and et al. Blood viscosity in young male diabetics with and without retinopathy. Diabetologia 18 (1980): 359-363.

Malmstrom, R. ; Packard, C. J. ; Caslake, M., et al. Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. Diabetologia 40 (1997): 454-462.

Mamo, J. C. ; Szeto, L. and Steiner, G. Glycation of very low density lipoprotein from rat plasma impairs its catabolism (Abstract). Diabetologia 33 (1990): 339-345.

Marcus, A. J. and Hajjar, D. P. Vascular transcellular signaling. J. Lipid Res. 34 (1993): 2017-2031.

Marques, F. ; Crespo, M. E. ; Silva, Z. I. and Bicho, M. Insulin and high glucose modulation of phosphatase and reductase enzymes in the human erythrocytes: a comparative analysis in normal and diabetic states. Diabetes Res. Clin. Pract. 47 (2000): 191-198.

Matthews, D. R. ; Hosker, J. P. ; Rudenski, A. S., et al. Homeostasis model assessment: insulin resistance and B-cell function from fasting plasma glucose and insulin concentration in man. Diabetologia 28 (1985): 412-419.

Mawatari, S. ; Saito, K. ; Murakami, K. and Fujino, T. Absence of correlation between glycated hemoglobin and lipid composition of erythrocyte membrane in type 2 diabetic patients. Metabolism 53 (2004): 123-127.

Mero, N. ; Syvanne, M. and Taskinen, M. R. Postprandial lipid metabolism in diabetes. Atherosclerosis 141 (1998): S53-S55.

Miller, J. A. ; Gravallese, E. and Bunn, H. F. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. J. Clin. Invest. 65 (1980): 896-901.

Milne, S. ; Ivanova, P. ; Forrester, J. and Brown, H. A. Lipidomics: An analysis of cellular lipids by ESI-MS. Methods 39 (2006): 92-103.

Mohanlal, N. and Holman, R. R. A Standardized Triglyceride and Carbohydrate Challenge The oral triglyceride tolerance test Diabetes Care 27 (2004): 89-94.

Moore, D. ; Sills, R. ; Patel, N. and Mendelsohn, R. Conformational order of phospholipids incorporated into human erythrocytes: an FTIR spectroscopy study. Biochemistry 35 (1996): 229-235.

Mouritsen, O. G. Life — As a Matter of Fat, The Emerging Science of Lipidomics: Springer Berlin Heidelberg, 2005.

Muderhwa, J. M. and Brockman, H. L. Lateral lipid distribution is a major regulator of lipase activity. Implications for lipid-mediated signal transduction. J. Biol. Chem. 267 (1992): 24184-24192.

Myher, J. J. ; Kuksis, A. and Pind, S. Molecular species of glycerophospholipids and sphingomyelins of human erythrocytes: improved method of analysis. Lipids 24 (1989): 396-407.

Nappo, F. ; Esposito, K. ; Cioffi, M., et al. Postprandial endothelial activation in healthy subjects and in type 2 diabetic patients: role of fat and carbohydrate meals. J. Am. Coll. Cardiol. 39 (2002): 1145-1150.

Nigro, J. ; Osman, N. ; Dart, A. M. and Little, P. J. Insulin Resistance and Atherosclerosis. Endocrine Reviews 27 (2006): 242-259.

Nikkila, E. A. ; Huttunen, J. K. and Ehnholm, C. Postheparin plasma lipoprotein lipase and hepatic lipase in diabetes mellitus. Relationship to plasma triglyceride metabolism. Diabetes 26 (1977): 11-21 (Abstract).

Nikolic, M. ; Stanic, D. ; Antonijevic, N. and Niketic, V. Cholesterol bound to hemoglobin in normal human erythrocytes: a new form of cholesterol in circulation? Clin. Biochem. 37 (2004): 22-26.

Nikolic, M. ; Stanic, D. ; Baricevic, I., et al. Efflux of cholesterol and phospholipids derived from the haemoglobin-lipid adduct in human red blood cells into plasma. Clin. Biochem. 40 (2007): 305-309.

Noto, D. ; Rizzo, M. ; Barbagallo, C. M., et al. Low-density lipoproteins generated during an oral fat load in mild hypertriglyceridemic and healthy subjects are smaller, denser, and have an increased low-density lipoprotein receptor binding affinity. Metab. Clin. Exp. 55 (2006): 1308- 1331.

Oliveira, M. M. and Vaughan, M. Incorporation of fatty acids into phospholipids of erythrocyte membrane. J. Lipid Res. 5 (1964): 156-162.

Petibois, C. and Deleris, G. Evidence that erythrocytes are highly susceptible to exercise oxidative stress: FT-IR spectrometric studies at the molecular level. Cell Biol. Int. 29 (2005): 709-716.

Petibois, C. and Deleris, G. Analysis and monitoring of oxidative stress in exercise and training by FTIR spectrometry. Int. J. Sports Physiol. Perform. 3 (2008): 119-130.

Petropoulos, I. K. ; Margetis, P. I. ; Antonelou, M. H., et al. Structural alterations of the erythrocyte membrane proteins in diabetic retinopathy. Graefes Arch. Clin. Exp. Ophthalmol. 245 (2007): 1179-1188.

Popp-Snijders, C. ; Schouten, J. A. ; van Blitterswijk, W. J. and van der Veen, E. A. Changes in membrane lipid composition of human erythrocytes after dietary supplementation of (n-3) polyunsaturated fatty acids. Maintenance of membrane fluidity. Biochim. Biophys. Acta 854 (1986): 31-37 (Abstract).

Postle, A. D. ; Wilton, D. C. ; Hunt, A. N. and Attard, G. S. Probing phospholipid dynamics by electrospray ionisation mass spectrometry. Prog. Lipid Res. 46 (2007): 200-224.

Pradhan, A. D. ; Manson, J. E. ; Rifai, N. ; Buring, J. E. and Ridker, P. M. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 286 (2001): 327-334.

Pruneta-Deloche, V. ; Ponsin, G. ; Groisne, L., et al. Postprandial increase of plasma apoAV concentrations in Type 2 diabetic patients. Atherosclerosis 181 (2005): 403-405.

Pruneta-Deloche, V. ; Sassolas, A. ; Dallinga-Thie, G. M., et al. Alteration in lipoprotein lipase activity bound to triglyceride-rich lipoproteins in the postprandial state in type 2 diabetes. J. Lipid. Res. 45 (2004): 859-865.

Rader, D. J. Effect of Insulin Resistance, Dyslipidemia, and Intra-abdominal Adiposity on the Development of Cardiovascular Disease and Diabetes Mellitus. Am. J. Med. 120 (2007): S12-S18.

Rashid, S. ; Uffelman, K. D. and Lewis, G. F. The mechanism of HDL lowering in hypertriglyceridemic, insulin-resistant states. J. Diabetes Complicat. 16 (2002): 24-28.

Rashid, S. ; Watanabe, T. ; Sakaue, T. and Lewis, G. F. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. Clin. Biochem. 36 (2003): 421-429.

Rasmussen, O. ; Gregersen, S. and Hermansen, K. Influence of the amount of starch on the glycaemic index to rice in non-insulin-dependent diabetic subjects. Br. J. Nutr. 67 (1992): 371-377.

Ravand, i. A. and Kuksis, A. Phospholipids of plasma liproproteins, red blood cells and atheroma. Encyclopedia of Analytical Chemistry (2006): 1-39.

Rekila, H. O. ; Ramstedt, B. ; Leppimaki, P. and Slotte, J. P. Cholesterol interactions with phospholipids in membranes. Prog. Lipid Res. 41 (2002): 66-97.

Renooij, W. and Van Golde, L. M. The exchange of phospholipids between rat erythrocytes and plasma, and the translocation of phosphatidylcholine across the red cell membrane, are temperature dependent processes. FEBS Lett. 71 (1976): 321-324.

Roderick, S. L. ; Chan, W. W. ; Agate, D. S., et al. Structure of human phosphatidylcholine transfer protein in complex with its ligand. Nat. Struct. Biol. 9 (2002): 507-511.

Rodriguez, Y. and Christophe, A. B. Long-chain omega6 polyunsaturated fatty acids in erythrocyte phospholipids are associated with insulin resistance in non-obese type 2 diabetics. Clin. Chim. Acta 354 (2005): 195-199.

Rondeau, P. ; Armenta, S. ; Caillens, H. ; Chesne, S. and Bourdon, E. Assessment of temperature effects on beta-aggregation of native and glycated albumin by FTIR spectroscopy and PAGE: relations between structural changes and antioxidant properties. Arch. Biochem. Biophys. 460 (2007): 141-150.

Ruge, T. ; Svensson, A. ; Eriksson, J. W. ; Olivecrona, T. and Olivecrona, G. Food deprivation increases post-heparin lipoprotein lipase activity in humans. Eur. J. Clin. Invest. 31 (2001): 1040-1047.

Saheki, S. ; Hitsumoto, Y. ; Takeuchi, N., et al. Lipase activities in post-heparin plasma and tissues, and susceptibilities of lipoproteins in experimental diabetic rats. J. Atheroscler. Thromb. 2 (1996): 87-95 (Abstract).

Sanders, T. A. Dietary fat and postprandial lipids. Curr. Atheroscler. Rep. 5 (2003): 445-451.

Satoh, M. ; Imaizumi, K. ; Bessbo, T. and et al. Increased erythrocyte aggregation in diabetes mellitus and its relationship to glycosylated haemoglobin and retinopathy. Diabetologia 27 (1984): 517-521.

Saxenaa, R. ; Madhub, S. V. ; Shuklaa, R. ; Prabhua, K. M. and Gambhira, J. K. Postprandial hypertriglyceridemia and oxidative stress in patients of type 2 diabetes mellitus with macrovascular complications. Clin. Chim. Acta 359 (2005): 101-108.

Serhan, C. N. Mediator lipidomics. Prostaglandins Other Lipid Mediat. 77 (2005): 4-14.

Sethi, S. ; Gibney, M. J. and Williams, C. M. Postprandial lipoprotein metabolism. Nutr. Res. Rev. 6 (1993): 161-183.

Severcan, F. ; Gorgulu, G. ; Gorgulu, S. T. and Guray, T. Rapid monitoring of diabetes-induced lipid peroxidation by Fourier transform infrared spectroscopy: evidence from rat liver microsomal membranes. Anal. Biochem. 339 (2005): 36-40.

Shohet, S. B. Release of Phospholipid Fatty Acid from Human Erythrocytes. J. Clin. Invest. 49 (1970): 1668-1678.

Shohet, S. B. The apparent transfer of fatty acid from phosphatidylcholine to phosphatidylethanolamine in human erythrocytes. J. Lipid Res. 12 (1971): 139-142.

Shohet, S. B. ; Nathan, D. G. and Karnovsky, M. L. Stages in the incorporation of fatty acids into red blood cells. J. Clin. Invest. 47 (1968): 1096-1108.

Sills, R. H. ; Moore, D. J. and Mendelsohn, R. Erythrocyte peroxidation: quantitation by Fourier transform infrared spectroscopy. Anal. Biochem. 218 (1994): 118-123.

Sittiwicheanwong, R. In vitro and in vivo studies on low-density liprotein subpopulation and their distributions influenced by other lipoproteins. Doctoral dissertation, Graduate studies, Mahidol University, 2006.

Siuzdak, G. Mass spectrometry for biotechnology. London: Academic Press, 1995.

Skelton, M. S. ; Kremer, D. E. ; Smith, E. W. and Gladden, L. B. Lactate influx into red blood cells from trained and untrained human subjects. Med. Sci. Sports Exerc. 30 (1998): 536-542.

Soupene, E. ; Fyrst, H. and Kuypers, F. A. Mammalian acyl-CoA:lysophosphatidylcholine acyltransferase enzymes. Proc. Natl. Acad. Sci. USA 105 (2008): 88-93.

Spooner, P. M. ; Chernick, S. S. ; Garrison, M. M. and Scow, R. O. Development of lipoprotein lipase activity and accumulation of triacylglycerol in differentiating 3T3-L1 adipocytes. Effects of prostaglandin F2alpha, 1-methyl-3-isobutylxanthine, prolactin, and insulin. J. Biol. Chem. 254 (1979): 1305-1311.

Stampfer, M. J. ; Krauss, R. M. ; Ma, J. and et al. A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction. JAMA 276 (1996): 882-888.

Stewart, M. W. ; Laker, M. F. ; Dyer, R. G., et al. Lipoprotein compositional abnormalities and insulin resistance in type II diabetic patients with mild hyperlipidemia. Arterioscler. Thromb. 13 (1993): 1046-1052.

Stipanuk, M. Biochemical and physiological aspects of human nutrition: W.B. Sanders Company, 2000.

Straczkowski, M. ; Kowalska, I. ; Nikolajuk, A., et al. Relationship Between Insulin Sensitivity and Sphingomyelin Signaling Pathway in Human Skeletal Muscle. Diabetes 53 (2004): 1215-1221.

Stubbs, C. D. and Smith, A. D. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim. Biophys. Acta 779 (1984): 89-137.

Summers, L. K. ; Fielding, B. A. ; Ilic, V. ; Quinlan, P. T. and Frayn, K. N. The effect of triacylglycerol-fatty acid positional distribution on postprandial metabolism in subcutaneous adipose tissue. Br. J. Nutr. 79 (1998): 141-147.

Summers, S. A. ; Garza, L. A. ; Zhou, H. and Birnbaum, M. J. Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. Mol. Cell Biol. 18 (1998): 5457-5464.

Syvanne, M. ; Vuorinen-Markkola, H. ; Hilden, H. and Taskinen, M. R. Gemfibrozil reduces postprandial lipemia in non-insulin-dependent diabetes mellitus (Abstract). Arterioscler. Thromb. 13 (1993): 286-295.

Szostek, R. ; Kucuk, O. ; Lis, L. J., et al. Effect of inserted oxysterols on phospholipid packing in normal and sickle red blood cell membranes. Biochem. Biophys. Res. Commun. 180 (1991): 730-734.

Tan, K. C. B. ; Cooper, M. B. ; Ling, K. L. E., et al. Fasting and postprandial determinants for the occurrence of small dense LDL species in non-insulin-dependent diabetic patients with and without hypertriglyceridaemia: the involvement of insulin, insulin precursor species and insulin resistance. Atherosclerosis 13 (1995): 273-287.

Tanford, C. The hydrophobic effects. New York: Wiley, 1973.

Taskinen, M. Diabetic dyslipidemia: from basic research to clinical practice. Diabetologia 46 (2003): 733-49.

Teno, S. ; Uto, Y. ; Nagashima, H. and et al. Association of postprandial hypertriglyceridemia and carotid intima-media thickness in patients with type 2 diabetes. Diabetes Care 23 (2000): 1401-1406.

Teruel, T. ; Hernandez, R. and Lorenzo, M. Ceramide Mediates Insulin Resistance by Tumor Necrosis Factor in Brown Adipocytes by Maintaining Akt in an Inactive Dephosphorylated State Diabetes 50 (2001): 2563-2571.

Tomasevic, N. ; Nicolic, M. ; Klappe, K. ; Hoekstra, D. and Niketic, V. Insulin-induced lipid binding to hemoglobin. J.Sereb.Chem.Soc. 68 (2003): 25-33.

Toyran, N. ; Lasch, P. ; Naumann, D. ; Turan, B. and Severcan, F. Early alterations in myocardia and vessels of the diabetic rat heart: an FTIR microspectroscopic study. Biochem. J. 397 (2006): 427-436.

Tushuizen, M. E. ; Diamant, M. and Heine, R. J. Postprandial dysmetabolism and cardiovascular disease in type 2 diabetes. Postgrad. Med. J. 81 (2005): 1-6.

Uran, S. ; Larsen, A. ; Jacobsen, P. B. and Skotland, T. Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry. J. Chromatogr. B Biomed. Sci. Appl. 758 (2001): 265-275.

Van Berkel, T. J. C. ; Kruijt, J. K. ; Scheek, L. M. and Groot, P. H. E. Effect of apolipoproteins E and C-III on the interaction of chylomicrons with parenchymal and non-parenchymal cells from rat liver. Biochem. J. 216 (1983): 71-80.

van den Berg, J. J. ; Op den Kamp, J. A. ; Lubin, B. H. and Kuypers, F. A. Conformational changes in oxidized phospholipids and their preferential hydrolysis by phospholipase A2. a monolayer study. Biochemistry 32 (1993): 4962-4967 (Abstract).

van Wijk, J. P. ; Halkes, C. J. M. ; Erkelens, D. W. and Cabezas, M. C. Fasting and Daylong Triglycerides in Obesity With and Without Type 2 Diabetes. Metabolism 52 (2003): 1043-1049.

Van Wijk, R. and Van Solinge, W. W. The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. Blood 106 (2005): 4034-4042.

Vu, T. T. Standardization of body surface area calculations. J. Onco. Pharm. Practice 8 (2002): 49-54.

Wakelam, M. J. ; Pettitt, T. R. and Postle, A. D. Lipidomic analysis of signaling pathways. Methods Enzymol. 432 (2007): 233-246.

Wang, C. ; Kong, H. ; Guan, Y., et al. Plasma Phospholipid Metabolic Profiling and Biomarkers of Type 2 Diabetes Mellitus Based on High-Performance Liquid Chromatography/ Electrospray Mass Spectrometry and Multivariate Statistical Analysis. Anal. Chem. 77 (2005): 4108-4116.

Wang, C. ; Xie, S. ; Yang, J. ; Yang, Q. and Xu, G. Structural identification of human blood phospholipids using liquid chromatography/quadrupole-linear ion trap mass spectrometry. Anal. Chim. Acta 525 (2004): 1-10.

Wang, W. Q. and Gustafson, E. J. One-dimensional thin-layer chromatographic separation of phospholipids and lysophospholipids from tissue lipid extracts. J. Chrom. 581 (1992): 139-142.

Wautier, J. L. ; Le Blanc, H. ; Wautier, M. P. and et al. Erythrocyte adhesion to cultured endothelium and glycaemic control in type I insulin-dependent diabetic patients. Diabetologia 29 (1986): 151-155.

Wells, J. M. and McLuckey, S. A. Collision-induced dissociation (CID) of peptides and proteins. Methods Enzymol. 402 (2005): 148-185.

Westphal, S. ; Taneva, E. ; Kästner, S., et al. Endothelial dysfunction induced by postprandial lipemia is neutralized by addition of proteins to the fatty meal. Atherosclerosis 185 (2006): 313-319.

Weyer, C. ; Hanson, R. L. ; Tataranni, P. A. ; Bogardus, C. and Pratley, R. E. A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia. Diabetes 49 (2000): 2094-2101.

Wolf, C. and Quinn, P. J. Lipidomics: practical aspects and applications. Prog. Lipid Res. 47 (2008): 15-36.

Wolff, S. P. and Dean, R. T. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem. J. 245 (1987): 243-250.

Yamashita, A. ; Sugiura, T. and Waku, K. Acyltransferases and transacylases involved in fatty acid remodeling of phospholipids and metabolism of bioactive lipids in mammalian cells. J. Biochem. 122 (1997): 1-16.

Yoshino, G. ; Hiranob, T. and Kazumi, T. Dyslipidemia in diabetes mellitus. Diabetes Res. Clin. Pract. 33 (1996): 1- 14.

Younsi, M. ; Quilliot, D. ; Al-Makdissy, N., et al. Erythrocyte membrane phospholipid composition is related to hyperinsulinemia in obese nondiabetic women: effects of weight loss. Metabolism 51 (2002): 1261-1268.

Zancan, P. and Sola-Penna, M. Regulation of human erythrocyte metabolism by insulin: cellular distribution of 6-phosphofructo-1-kinase and its implication for red blood cell functions. Mol. Genet. Metab. 86 (2005): 401-411.

Zeghari, N. ; Younsi, M. ; Meyer, L., et al. Adipocyte and erythrocyte plasma membrane phospholipid composition and hyperinsulinemia: a study in nondiabetic and diabetic obese women Int. J. Obes. Relat. Metab. Disord. 24 (2000): 1600-1607.

APPENDICES

APPENDIX A Ethic approval for the experiment

เทพธารินทร์ Theptarin Hospital
3850 ถนนพระราม 4 คลองเตย กรุงเทพฯ 10110
3850 Rama 4 Road, Klongtoey, Bangkok 10110
Tel +66 2 348 7000 Fax +66 2 249 8774
www.theptarin.com

Documentary Proof of Ethical Committee on Researches Involving Human Subjects.

Theptarin Hospital

No. 1/2007

Title of Project : The influence of postprandial hypertriacylglycerolemic state on modification of erythrocyte membrane phospholipids subspecies in type 2 diabetes with varied insulin resistance.

Protocol Number : -

Principle Investigator : Mr.Sukrit Sirikwangpong

Name of Department : Doctor of Philosophy Program in Biomedical Sciences
Graduate School, Chulalongkorn University

The Aforementioned Project and informed consent have been reviewed and approved by Ethical Committee on Researches Involving Human Subjects, based on the Declaration of Helsinki.

Signature of Chairman
Ethical Committee on Researches .....
Involving Human Subjects Prof.Rajata Rajatanavin, M.D.,F.A.C.E.

Date of Approval 05-07-02

Excellence
Teamwork
Hospitality
Integrity
Continuous Improvement
Social Responsibility

APPENDIX B Patient Information Sheet

คำแนะนำสำหรับผู้เข้าร่วมโครงการวิจัย

- **ก่อนเข้ารับการทดสอบ** (Oral fat load test)
    1. หลีกเลี่ยงการออกกำลังกายอย่างหนัก หรือ กิจกรรมที่ใช้พลังงานมาก
    2. งดการรับประทานยาลดไขมันล่วงหน้าก่อนทำการทดสอบอย่างน้อย 3 อาทิตย์
    3. รับประทานอาหารได้ตามปกติ รวมถึงยา antihyperglycemic drugs ที่ใช้อยู่ประจำ
    4. ก่อนเข้ารับการทดสอบ ผู้เข้าร่วมโครงการต้องอดอาหารเป็นเวลาอย่างน้อย 12 ชั่วโมง เพื่อเจาะเลือดตรวจวิเคราะห์ค่าต่างๆทางห้องปฏิบัติการ,ค่า HOMA รวมทั้งองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงเพื่อใช้เป็นค่าพื้นฐาน (Baseline blood parameters)
    5. ผู้เข้าร่วมโครงการจะได้รับการตรวจเลือดเพื่อประเมินสุขภาพเป็นพิเศษ (การทำงานของไต, ตับ, ระดับไขมัน และ Uric acid)
- **การทดสอบ** Oral fat load test
    1. ผู้เข้าร่วมโครงการจะได้รับอาหารที่ทางผู้วิจัยจัดเตรียมไว้ให้ ซึ่งอาหารที่ให้จะมีปริมาณไขมัน 40 กรัม ต่อ ตารางเมตรพื้นที่ผิวของร่างกาย
    2. ผู้เข้าร่วมโครงการจะต้องรับประทานอาหารที่เตรียมไว้ภายใน 20 นาทีและสามารถดื่มน้ำได้ในระหว่างทำการทดสอบ
- **หลังการทดสอบ** (Oral fat load test)
    1. ห้ามรับประทานอาหารหรือเครื่องดื่มที่นอกเหนือจากที่ผู้วิจัยจัดไว้ให้ภายในระยะเวลาที่ทดสอบ **ยกเว้น** น้ำเปล่า
    2. ช่วงระยะเวลา 4 ชั่วโมงหลังจากได้รับอาหาร ผู้เข้าร่วมโครงการจะได้เข้าร่วมกิจกรรมที่ผู้วิจัยได้จัดเตรียมไว้
    3. เมื่อครบเวลาการทดสอบ 4 ชั่วโมง ผู้เข้าร่วมโครงการต้องเจาะเลือดเพื่อตรวจวิเคราะห์ค่าต่างๆทางห้องปฏิบัติการ, ค่า HOMA รวมทั้งองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงเพื่อใช้เป็นค่าหลังการทดสอบ
- **การเจาะเลือด**

ผู้เข้าร่วมโครงการต้องเจาะเลือดเพื่อใช้ในการวิจัยทั้งหมดจำนวน 4 หลอด คือ Clotted blood 4 ml จำนวน 1 หลอดและ EDTA blood 6 ml จำนวน 3 หลอด ทั้งก่อนและหลังการทดสอบ

เอกสารชี้แจงข้อมูล/คำแนะนำแก่ผู้เข้าร่วมโครงการ
(Patient Information Sheet)

ชื่อโครงการ

การศึกษาอิทธิพลของภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารต่อการเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกัน

ชื่อผู้วิจัย นายสุกฤต ศิริขวัญพงศ์ นิสิตปริญญาเอก หลักสูตรสหสาขาชีวเวชศาสตร์ บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

สถานที่วิจัย โรงพยาบาลเทพธารินทร์

ผู้สนับสนุนการวิจัย รศ.ดร.วินัย ดะห์ลัน คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
อ.ดร.สุวิมล ทรัพย์วโรบล คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ความเป็นมาของโครงการ

ปัจจุบันภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารนั้นมีความสำคัญต่อความผิดปกติของเซลล์ต่างๆมากมายโดยเฉพาะผู้ป่วยโรคเบาหวานซึ่งมีภาวะดื้อต่ออินซูลิน การเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงเป็นความผิดปกติอย่างหนึ่งซึ่งอาจทำให้เซลล์เม็ดเลือดแดงมีโครงสร้างผิดปกติไปอันนำไปสู่การสูญเสียหน้าที่ต่างๆของเซลล์ เนื่องจากเม็ดเลือดแดงเป็นเซลล์ที่เปลี่ยนแปลงได้ง่ายต่อสภาวะที่เปลี่ยนไปมากกว่าเซลล์ชนิดอื่น การวิจัยนี้จึงมุ่งเน้นไปที่การศึกษาอิทธิพลของภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารต่อการเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกัน

วัตถุประสงค์

เพื่อที่จะศึกษาการเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงที่ถูกเหนี่ยวนำโดยภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกัน

รายละเอียดที่จะปฏิบัติต่อผู้เข้าร่วมโครงการ

หากผู้เข้าร่วมโครงการมีคุณสมบัติเหมาะสมและยินยอมที่จะเข้าร่วมในโครงการวิจัยนี้ ผู้เข้าร่วมโครงการจะต้องอดอาหารเป็นเวลา 12 ชั่วโมงจากนั้นจะเจาะเลือดเพื่อใช้วิเคราะห์ค่าต่างๆทางห้องปฏิบัติการ, ค่า HOMA รวมทั้งองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงเพื่อใช้เป็นค่าพื้นฐาน จากนั้นผู้เข้าร่วมโครงการจะได้รับอาหารที่ทางผู้วิจัยจัดเตรียมไว้ให้ ซึ่งอาหารที่ให้จะมีปริมาณไขมัน 40 กรัม ต่อ ตารางเมตรพื้นที่ผิวของร่างกาย โดยที่พลังงานของอาหารจะขึ้นอยู่กับปริมาณของไขมันที่ได้รับ ผู้เข้าร่วมโครงการจะต้องรับประทานอาหารที่เตรียมไว้ภายใน 20 นาทีและสามารถดื่มน้ำได้ในระหว่างทำการทดลอง หลังจากนั้น 4 ชั่วโมง ผู้เข้าร่วมโครงการต้องเจาะเลือดเพื่อวิเคราะห์ค่าต่างๆทางห้องปฏิบัติการ, ค่า HOMA รวมทั้งองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงเพื่อใช้เป็นค่าหลังการทดสอบ จากนั้นผู้วิจัยจะนำข้อมูลทั้งหมดไปวิเคราะห์ต่อไป

ค่า Homeostasis model assessment (HOMA)

ค่า HOMA หรือ homeostasis model assessment เป็นค่าที่แสดงถึงภาวะดื้อต่ออินซูลิน ซึ่งสามารถประเมินได้จากการคำนวณ โดยใช้สูตรคำนวณดังนี้

HOMA-IR = = FIRI x FPG /22.5

โดยที่ FIRI คือ ค่า fasting immunoreactive insulin มีหน่วยเป็น uU/mL

FPG คือ ค่า fasting plasma glucose level มีหน่วยเป็น mmol/L

ประโยชน์และผลข้างเคียงที่จะเกิดแก่ผู้เข้าร่วมโครงการ

ประโยชน์ที่จะเกิดแก่ผู้เข้าร่วมโครงการ

ผู้เข้าร่วมโครงการจะได้รับการตรวจเลือดทางห้องปฏิบัติการ คือ ตรวจน้ำตาล, HbA1C, ระดับไขมันในเลือด ตลอดจน ระดับของภาวะดื้อต่ออินซูลิน และได้ทราบถึงการเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงทั้งก่อนและหลังทำการทดลอง ซึ่งการตรวจดังกล่าว ผู้เข้าร่วมโครงการสามารถนำผลที่ได้มาพบแพทย์เพื่อประเมินภาวะสุขภาพอันจะเป็นประโยชน์แก่ผู้เข้าร่วมโครงการอีกทางหนึ่ง

ข้อพิจารณาด้านจริยธรรม (ETHICAL CONSIDERATION)

ความเสี่ยงที่ได้รับจากการเจาะเลือด

ผู้เข้าร่วมโครงการมีโอกาสที่จะเกิดอาการเจ็บ เลือดออก ช้ำจากการเจาะเลือด อาการบวมบริเวณที่เจาะเลือดหรือหน้ามืดและโอกาสที่จะเกิดการติดเชื้อบริเวณที่เจาะเลือดพบได้น้อยมาก ทั้งนี้ไม่มีความแตกต่างจากการเจาะเลือดตรวจร่างกายตามปกติแต่อย่างใด

ผู้วิจัยได้เตรียมดำเนินการ ดังนี้ : อธิบายให้ผู้เข้าร่วมโครงการเข้าใจเพื่อคลายความวิตกกังวล

ความเสี่ยงหลังจากการได้รับอาหารที่ผู้วิจัยจัดเตรียมไว้ให้

ผู้เข้าร่วมโครงการมีโอกาสเกิดความไม่สบาย หรือ ท้องเสีย หลังจากที่รับประทานอาหารที่ผู้วิจัยจัดเตรียมไว้ให้แต่พบได้น้อยมากเนื่องจากสิ่งที่ให้ผู้เข้าร่วมโครงการรับประทานเป็นอาหารซึ่งจะมีสารอาหารเหมือนอาหารที่รับประทานกันปกติเพียงแต่จะมีสัดส่วนของไขมันค่อนข้างสูงกว่าอาหารปกติ จุดประสงค์เพื่อต้องการทำให้เกิดภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารเท่านั้น ซึ่งจะไม่มีอันตรายเนื่องจากภาวะนี้เกิดเป็นตามปกติหลังจากที่รับประทานอาหารเข้าไปใหม่ๆ

ผู้วิจัยได้เตรียมดำเนินการ ดังนี้ : อธิบายให้ผู้ถูกทดลองเข้าใจว่าสิ่งที่ให้ผู้เข้าร่วมโครงการรับประทานเป็นเอาหารซึ่งมีสารอาหารเหมือนอาหารที่รับประทานกันปกติเพียงแต่จะมีสัดส่วนของไขมันค่อนข้างสูงกว่าอาหารปกติเท่านั้น ซึ่งหากเกิดอาการไม่สบาย หรือ ท้องเสีย ก็สามารถหยุดการทดลองเพื่อให้คลายความวิตกกังวล

ความเสี่ยงที่ไม่ทราบแน่นอน

ผู้เข้าร่วมโครงการอาจเกิดอาการข้างเคียงอื่นๆนอกเหนือจากที่แสดงในเอกสารฉบับนี้ ซึ่งอาการข้างเคียงเหล่านี้เป็นอาการที่ไม่เคยพบมาก่อน ทั้งนี้ผู้วิจัยจะให้การดูแลผู้เข้าร่วมโครงการอย่างใกล้ชิดตลอดการดำเนินงาน

ผู้วิจัยได้เตรียมดำเนินการ ดังนี้ : อธิบายให้ผู้เข้าร่วมโครงการแจ้งผู้วิจัยให้ทราบทันทีเมื่อเกิดความผิดปกติใดๆขึ้น เพื่อผู้วิจัยจะได้ดำเนินการต่อไป

หลักเกณฑ์ในการยุติการวิจัย

ผู้เข้าร่วมโครงการอาจขอถอนตัวออกจากโครงการนี้เมื่อไรก็ได้ตามต้องการ

การเก็บข้อมูลเป็นความลับ

ข้อมูลในการวิจัยรวมถึงข้อมูลทางการแพทย์ของผู้เข้าร่วมโครงการที่อาจนำไปสู่การเปิดเผยตัวของท่าน จะได้รับการปกปิดและจะไม่เปิดเผยแก่สาธารณชน ในกรณีที่ผลการวิจัยได้รับการตีพิมพ์ ชื่อและที่อยู่ของท่านจะต้องได้รับการปกปิดอยู่เสมอ โดยจะใช้เฉพาะรหัสประจำโครงการวิจัยของท่าน ข้อมูลของผู้เข้าร่วมโครงการจะผ่านกระบวนการต่างๆ เช่น การเก็บข้อมูล การบันทึกข้อมูลในคอมพิวเตอร์ การตรวจสอบ การวิเคราะห์และการรายงานเพื่อวัตถุประสงค์ทางวิทยาศาสตร์ รวมทั้งการใช้ข้อมูลทางการแพทย์ในอนาคตหรือการวิจัยทางการแพทย์เท่านั้น

APPENDIX C Patient Informed consent

Participant No.

หนังสือยินยอมโดยได้รับการบอกกล่าว

ชื่อโครงการ **การศึกษาอิทธิพลของภาวะไตรเอซิลกลีเซอรอลในเลือดสูงหลังมื้ออาหารต่อการเปลี่ยนแปลงองค์ประกอบของฟอสโฟลิพิดบนเยื่อหุ้มเซลล์เม็ดเลือดแดงในผู้ป่วยเบาหวานชนิดที่ 2 ที่มีภาวะดื้อต่ออินซูลินต่างกัน**

ชื่อผู้วิจัย **นายสุกฤต ศิริขวัญพงศ์**

ชื่ออาสาสมัครผู้เข้าร่วมโครงการวิจัย .....

อายุ..... เลขที่เวชระเบียน.....

คำยินยอมของผู้เข้าร่วมโครงการวิจัย

ข้าพเจ้า นาย/นาง/นางสาว ..... ได้ทราบรายละเอียดของโครงการ การรักษาและทดลองตลอดจนประโยชน์และข้อเสี่ยงที่จะเกิดขึ้นต่อผู้เข้าร่วมโครงการวิจัยจากผู้ดำเนินงานวิจัยแล้วอย่างชัดเจน ไม่มีสิ่งใดปิดบังซ่อนเร้น และยินยอมให้ทำการรักษาและทดลองในโครงการที่มีชื่อข้างต้น และข้าพเจ้ารู้ว่าถ้ามีปัญหาหรือข้อสงสัยเกิดขึ้น ข้าพเจ้าสามารถถามผู้ดำเนินงานวิจัยได้ และข้าพเจ้าสามารถออกจากโครงการวิจัยนี้เมื่อใดก็ได้ โดยไม่มีผลกระทบต่อการรักษาที่ข้าพเจ้าพึงได้รับ

ลงชื่อ..... (ผู้เข้าร่วมโครงการวิจัย)

..... (พยาน)

..... (พยาน)

วันที่ .....

คำอธิบายของผู้ดำเนินงานวิจัย

ข้าพเจ้าได้อธิบายรายละเอียดของโครงการ ตลอดจนประโยชน์ของการรักษา และการทดลองรวมทั้งข้อเสี่ยงที่อาจจะเกิดขึ้นแก่ผู้เข้าร่วมโครงการวิจัยทราบแล้วอย่างชัดเจน โดยไม่มีสิ่งใดปิดบังซ่อนเร้น

ลงชื่อ.....(ผู้วิจัย)

วันที่ .....

BIOGRAPHY

Mr. Sukrit Sirikwanpong was born on September, 11st, 1980 in Bangkok, Thailand. He graduated with Bachelor degree of Sciences in Medical Technology with second class honours from Faculty of Allied Health Sciences, Chulalongkorn University in 2003. Then, he has been accepted as a qualified recipient for scholarship under The Commission on Higher Education Staff Development Project for studying in Ph.D Program in Biomedical Sciences (Interdisciplinary Program), Faculty of Graduate School, Chulalongkorn University.