The Alterations of Erythrocyte Phospholipids in Type 2 Diabetes Observed after Oral High-Fat Meal Loading: The FTIR Spectroscopic and Mass Spectrometric Studies

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Summary  Little is known about the postprandial remodelling of erythrocytes phospholipids (PLs) in type 2 diabetes (T2DM). Therefore, this study aims to compare the alterations of erythrocyte PLs in T2DM to those of healthy subjects after ingestion of a high-fat meal. Eleven T2DM and ten healthy subjects underwent a high-fat meal loading. Erythrocytes were isolated from blood obtained after fasting and 4 h after the meal. Fourier Transform Infrared (FTIR) spectroscopy was initially used to screen erythrocyte PLs by monitoring C-H stretching vibrations. Phosphatidylcholine (PC) molecular species were further investigated by Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry (LC-ESI-MS). For the control group, FTIR revealed postprandial changes in C-H stretching vibrations, particularly of the olefinic band. These findings were supported by LC-ESI-MS data, showing marked changes in PC molecular species, especially of the PC34:1 (where 34 and 1 mean the summed number of carbons and double bonds, respectively). However, similar changes of those were not apparent in the T2DM group. Our results reveal marked postprandial alterations of erythrocyte PC species in healthy subjects whereas only mild alterations are observed in T2DM. The discrepant effects of high-fat meal loading suggest abnormal PC remodelling in the diabetic erythrocyte that may affect its membrane fluidity and integrity.

Key Words: erythrocyte phospholipids, high-fat meal, type 2 diabetes, FTIR spectroscopy, mass spectrometry

Introduction

Type 2 diabetes mellitus (T2DM) has emerged as a significant threat to human health in the 21st century. The number of adults with diabetes is dramatically increasing;
the global prevalence of T2DM was estimated to be 4.0% in 1995 and is projected to rise to 5.4% by 2025 [1, 2]. Among diabetic patients, macro- and microvascular complications are the most common complications that cause morbidity and mortality [3]. One of the potential factors contributing to these complications is abnormalities in the physical and biological properties of the blood cells [4]. Previous studies have shown that some phospholipids (PLs) are altered in erythrocytes of diabetic patients. These alterations were reported to be associated with rheological parameters that result in erythrocyte aggregation (EA) [5, 6], which is an independent risk factor for developing vascular complications in diabetic patients [7].

In general, PLs, which are the major lipid component of the biological membrane [8], are composed of many hundreds of molecular species whose physical and biological properties contribute to the overall properties of the membrane [9]. In the mature erythrocyte, membranes PLs are continuously renewed during circulation through the blood. The erythrocyte is unable to carry out de novo PLs biosynthesis; it regulates PL composition by passive exchange of intact PLs with plasma lipoproteins [10]. An alternative pathway of PL renewal involves acylation of fatty acids with either endogenous or exogenous lysophospholipids (LPC) followed by incorporation into the PL fraction of the erythrocyte [11]. Moreover, recent evidence obtained from healthy human subjects indicates that after a meal, fatty acids are rapidly incorporated into erythrocyte PLs fractions with preferential incorporation of meal linoleate. This mechanism may be important for the maintenance of membrane fluidity and integrity [12].

However, little is known about the remodelling of PLs in erythrocytes of T2DM during the postprandial period. We hypothesised that erythrocyte PL remodelling in diabetics is impaired during the postprandial period. Thus, the aim of the study was to compare the erythrocyte PLs in T2DM to those of healthy subjects after ingestion of a high-fat meal. In the present study, Fourier Transform Infrared spectroscopy (FTIR) was used to rapidly screen any changes in C-H stretching vibrations that exclusively arise from PLs in the erythrocyte [13]. Phosphatidylcholine (PC) is the predominant molecular species in erythrocyte PLs and is primarily involved in the PL remodelling process [14]. Hence, we employed a lipidomic approach using Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry (LC-ESI-MS) [15] to investigate alterations of PC content in erythrocytes obtained before and after ingestion of a high-fat meal. The results from this study should provide a better understanding of the PL remodelling process during the postprandial period in erythrocytes of diabetics.

Materials and Methods

Chemicals and solvents

1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC 14:1/14:1) (where 14 and 1 mean the summed number of carbons and double bonds, respectively) purchased from Avanti Polar Lipids (Alabaster, AL). With the exception of ammonia (25%), which was analytical grade, all solvents (e.g., hexane, isopropanol, formic acid, and others) were HPLC grade. All solvents were purchased from Merck (Darmstadt, Germany).

Subjects

Eleven subjects with T2DM were recruited from Theptarin hospital, Bangkok, Thailand. The inclusion criteria were as follows: 21–60 years of age, male or female, and diagnosed with T2DM. Patients with an acute infection, hepatic/renal disease, or were using insulin or lipid-lowering drugs were excluded. Ten age- and sex-matched control subjects with normal blood glucose and lipid levels were randomly selected from among the researchers’ laboratory staff. The informed consent forms and study protocol were in accordance with the Declaration of Helsinki and were reviewed and approved by the ethical committee on research involving human subjects from Theptarin Hospital, Bangkok, Thailand.

Study protocol

Subjects fasted overnight for 12 h prior to the designated study day. During the day of the study, no drugs or medications were taken until the experiment was completed. After submitting fasting blood samples, the subjects were given a single standard high-fat meal in the form of a milkshake and buttered bread. The meal contained 40 g fat/m^2 body surface area and had an energy distribution of 72% fat, 24% carbohydrate, and 4% protein. The polyunsaturated fatty acid (PUFA)/saturated fatty acid (SFA) ratio and monounsaturated fatty acid (MUFA)/SFA ratio were 0.08 and 0.46, respectively. The meal was ingested within 20 min and water was allowed ad libitum. Blood samples were collected again 4 h after ingestion of the meal. A 4 h time point was selected based on in vitro and in vivo studies in erythrocytes that showed rapid exchange or incorporation of meal-derived fatty acid into lipid fractions of erythrocytes [12, 16] and because postprandial triacylglycerol is at its peak (i.e., 4–5 h after a lipid-enriched meal administration) [17, 18].

Plasma biochemical parameters

Serum glucose and lipids were measured by standard enzymatic techniques. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation [19]. Hemoglobin A1C (HbA1C) was analysed by an immunoturbidity assay. Serum insulin was determined by

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using the immunochemiluminometric assay. Insulin resistance was estimated by the homeostasis model assessment insulin resistance (HOMA-IR) index derived from fasting plasma insulin and glucose concentrations (insulin (μU/ml) × glucose (mg/dl)/405) [20].

Isolation of erythrocytes

Venous blood samples were taken from subjects and placed into a tube pre-treated with EDTA. Plasma was immediately separated by centrifugation at 3,000 rpm for 10 min at 4°C. After removing plasma, the erythrocytes were washed with an ice-cold isotonic solution containing 0.15 M NaCl. The buffy coat was removed by aspiration after each wash. The packed erythrocyte sample was divided into small aliquots of 0.5 ml and kept under nitrogen at −80°C.

FTIR spectroscopy

A Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany), equipped with a high throughput extension (HTS-XT) accessory, was used. Erythrocytes were prepared according to the methods described by Petibois et al. with slight modification [21]. Twenty microlitres of washed, packed erythrocytes were diluted with 180 μl of water and mixed for 60 s. Duplicated dry films were made for each sample by applying 5 μl of the hemolysate to 96 wells in a silicon microplate. The hemolysate films were left to dry at 22°C after each wash. The packed erythrocyte sample was divided into small aliquots of 0.5 ml and kept under nitrogen at −80°C.

| Peak No. | Frequency (cm⁻¹) | Major assignments |
|----------|------------------|------------------|
| 1        | 3020–3000        | Olefinic = CH stretching: unsaturated lipids, phospholipids |
| 2        | 2990–2945        | CH: asymmetric stretching: phospholipids, cholesterol esters, fatty acids |
| 3        | 2945–2905        | CH: asymmetric stretching: phospholipids, long chain fatty acids |
| 4        | 2885–2860        | CH: symmetric stretching: phospholipids, fatty acids |
| 5        | 2860–2840        | CH: symmetric stretching: phospholipids, long chain fatty acids |

The profiling of PC molecular species by LC-ESI-MS

An aliquot of packed erythrocytes was prepared for lipid extraction according to the method of Blight and Dyer [23] with slight modification. PC 14:1/14:1 was added to the samples as an internal standard. After extraction, the organic phase was collected and dried by evaporation under a nitrogen stream. The lipid extracts were stored dry at −20°C until analysis. An HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) was used. PC was separated on the diol column Nucleosil 100-OH (Macherey-Nagel, Düren, Germany; 250 mm × 3.0 mm id. × 5-μm particle size). A linear solvent gradient was patterned after the method used by Wang [24] with slight modification. Hexane/isopropanol (4:1, v/v) was used as mobile phase A, and mobile phase B consisted of 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 and increasing to 60% over 22 min then maintained for another 2 min. After that, mobile phase B was increased to 80% over 11 min and maintained at that level for an additional 28 min. Finally, solvent B was decreased to 30% in the space of 2 min and the column was re-equilibrated for approximately 5 min prior to the next injection. The flow rate was 0.45 ml/min and the column temperature was 35°C. The injection volume was 5 μl.

The HPLC system was directly coupled to an electrospray ion-trap mass spectrometer or ESI-IT MS (Esquire HCT,
The ESI capillary potential was set at 4.5 kV. The dry nitrogen gas-flow rate was 8.0 L/min at 300°C. The MS data were collected in the negative ion mode. Mass range scan was set from 500–1,000 m/z at a rate of five spectra for each time point. All PC molecular species were well-detected as the formate adduct, \([M + 45]^−\), as previously described [25].

After a correction for overlap of the \(^{13}\)C isotope peak, each postprandial PC molecular species was calculated and expressed as a percent distribution relative to its total concentration in the fasting state. The data acquisition and analysis were expedited with DataAnalysis™ software version 3.2 (Bruker Daltonics GmbH, Bremen, Germany).

Statistical analysis

Values were expressed as the mean ± SEM. Normality was tested by using the Kolmogorov-smirnov test. Paired and two-sample \(t\) tests were used to compare parameters in the fasting state to the 4 h postprandial time point within groups and between the control and T2DM groups, respectively. A value of \(p<0.05\) was considered statistically significant. The statistical analyses were performed using SPSS software version 11.5 (SPSS Inc, Chicago, IL).

Results

Baseline characteristics and 4 h biochemical parameters of subjects

No differences in baseline characteristics were observed in the T2DM group compared to the control group (Table 2). Plasma biochemical parameters in the fasting state and 4 h after the meal are summarised in Table 3. As expected, fasting glucose, HbA1C, and HOMA-IR in T2DM subjects were significantly higher than those in control subjects; however, no differences in lipid parameters were observed between the two groups. Four hours after the meal, both groups showed a significant increase in triacylglycerol concentration compared to that in the fasting state. Postprandial glucose and insulin levels were also significantly greater among diabetic subjects than control subjects. Moreover, the change in insulin concentration relative to the fasting state was significantly higher in the diabetic group compared to that in the control.

Changes in the C-H stretching region of erythrocytes after the meal as measured by FTIR spectroscopy

The average second derivative spectra of erythrocytes

| Table 2. Baseline characteristics of subjects |
|-----------------------------------------------|
| Parameters                              | Control \((n = 10)\) | Type 2 diabetes \((n = 11)\) |
| Age (years)                             | 46.0 ± 1.7         | 48.4 ± 2.2         |
| Sex (M/F)                               | 4/6                | 4/7                |
| Duration of diabetes (years)            | —                  | 6.5 ± 1.0          |
| Weight (kg)                             | 59.3 ± 2.1         | 68.9 ± 5.0         |
| Body surface area (m²)                  | 1.61 ± 0.03        | 1.74 ± 0.07        |
| BMI (kg/m²)                             | 23.64 ± 0.72       | 25.89 ± 1.56       |
| Waist/Hip Ratio                         | 0.83 ± 0.02        | 0.86 ± 0.02        |

Values were expressed as the mean ± SEM or number of subjects.

| Table 3. Plasma biochemical parameters in fasting state and 4 h after the meal |
|-----------------------------------------------|
| 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* | 130.1 ± 9.1** | 132.6 ± 10.2** |
| Insulin (μU/ml)                | 5.20 ± 0.80 | 9.40 ± 1.80* | 7.55 ± 1.52      | 18.45 ± 3.59*** |
| Total cholesterol (mg/dl)      | 203.5 ± 13.6 | 204.1 ± 14.7 | 188.7 ± 7.0      | 198.6 ± 8.4*    |
| Triacylglycerol (mg/dl)        | 99.6 ± 11.9 | 239.2 ± 32.6* | 110.2 ± 14.8     | 270.1 ± 37.5*   |
| HDL cholesterol (mg/dl)        | 51.7 ± 3.6   | 51.7 ± 3.5   | 46.0 ± 2.8       | 45.8 ± 2.4      |
| LDL cholesterol (mg/dl)        | 131.8 ± 12.6 | 120.7 ± 7.2  | 7.3 ± 0.4**      | 2.37 ± 0.46**   |
| HbA1C (%)                      | 5.7 ± 0.2    | 7.3 ± 0.4**  |            |
| HOMA-IR                        | 1.17 ± 0.20  | 2.37 ± 0.46**|

Values were expressed as the mean ± SEM, 4 PP = 4 h postprandial period, * \(p<0.05\) fasting state vs 4 PP within group, ** \(p<0.05\) vs control group in the same period.
used to identify the C-H stretching region in control and diabetic groups are shown in Fig. 1A and 1B, respectively. The C-H stretching region arising from PLs in erythrocytes was originated mainly from olefinic $\nu = (\text{CH})$ (3020–3000 cm$^{-1}$), $\nu_{as}$ (CH$_3$) (2990–2945 cm$^{-1}$), $\nu_{as}$ (CH$_2$) (2945–2905 cm$^{-1}$), $\nu_{s}$ (CH$_3$) (2885–2860 cm$^{-1}$), and $\nu_{s}$ (CH$_2$) (2860–2840 cm$^{-1}$). The integrated areas of observed bands in the control and T2DM groups obtained from the fasting state and 4 h after the meal are shown in Table 4. After the experimental period, all C-H stretching bands in the control group seemed to increase whereas all were found to have decreased or remained unchanged in the T2DM group when compared to the fasting state. In addition, the percent change in olefinic $\nu = (\text{CH})$, $\nu_{as}$ (CH$_3$), $\nu_{as}$ (CH$_2$) and $\nu_{s}$ (CH$_2$) bands in the diabetic group were found to be significantly lower than those in the control group. Specifically, a marked difference was observed in the percent change in the integrated area of olefinic $\nu = (\text{CH})$ relative to the fasting state compared to what was seen in the control group ($-2.4 \pm 5.3$ vs. $74.3 \pm 9.0$, $p<0.001$, respectively).

Changes in PC molecular species of erythrocytes after the meal as measured by LC-ESI-MS

The typical profile of PC molecular species in erythrocytes obtained from LC-ESI-MS is shown in Fig. 2 Changes observed in the control and T2DM groups compared to the fasting state are presented in Fig. 3A and 3B, respectively. The predominant PC molecular species of erythrocytes from
both groups were PC 34:2 and PC 34:1, which accounted for approximately 28% and 24% of the total PC molecular species, respectively. In the T2DM group, four PC molecular species were found to significantly change after the high-fat meal. However, the control group showed marked changes of eight major PC molecular species, particularly PC34:1 (16:0/18:1 as major species). As shown in Fig. 4, the total PC molecular species with SFA and MUFA after the meal period in the control group significantly increased when compared to the fasting state, in which there were no significant differences in the T2DM group.

Table 4. Integrated areas and the percent changes in the C-H stretching region from erythrocytes obtained before and after the meal in the control and type 2 diabetic group

| Functional groups | Control (n = 10) | Type 2 diabetes (n = 11) |
|-------------------|----------------|------------------------|
|                   | Fasting        | 4 PP                   | ΔChange (%) | Fasting        | 4 PP                   | ΔChange (%) |
| 1. Olefinic – CH stretching, ν(CH) | −0.034 ± 0.002 | −0.058 ± 0.002* | 74.3 ± 9.0 | −0.025 ± 0.001** | −0.023 ± 0.001** | −2.4 ± 5.3** |
| 2. CH3 asym. stretching, νas(CH3) | −1.019 ± 0.006 | −1.053 ± 0.006* | 3.3 ± 0.9 | −1.032 ± 0.010 | −1.066 ± 0.006** | −2.4 ± 1.5** |
| 3. CH3 sym. stretching, νs(CH3) | −0.720 ± 0.007 | −0.732 ± 0.005 | 1.8 ± 1.2 | −0.738 ± 0.012 | −0.795 ± 0.006** | −4.1 ± 1.9** |
| 4. CH2 sym. stretching, νs(CH2) | −0.480 ± 0.002 | −0.482 ± 0.003 | 0.6 ± 1.3 | −0.492 ± 0.004** | −0.483 ± 0.003 | −1.7 ± 1.3 |
| 5. CH2 asym. stretching, νas(CH2) | −0.118 ± 0.003 | −0.116 ± 0.002 | −0.9 ± 3.0 | −0.128 ± 0.005 | −0.113 ± 0.002* | −9.8 ± 3.8** |

Values are expressed as the mean ± SEM, 4 PP = four hours postprandial period, Δ Change (%) was the percent change in integrated area relative to fasting state, * p<0.05 fasting vs 4 PP within group, ** p<0.05 control group vs type 2 diabetics in the same period.

Fig. 2. LC-ESI-MS of phosphatidylcholine (PC) molecular species from erythrocytes; (A) Base peak chromatogram (B) Negative-ion mass spectrum of PC molecular species. The LC-ESI-MS conditions are described in Materials and Methods. All PC molecular species were detected as [M + 45]− and the internal standard was PC 14:1/14:1, m/z 718.5.
Discussion

After the meal period, both groups showed a significant increase in triacylglycerol concentration. This finding was consistent with the other studies that have showed that a meal intake containing 40–50 g of fat results in significant lipemia in healthy adults [26, 27]. Moreover, the plasma insulin concentration at 4 h after the meal was in agreement with the same studies. A previous study demonstrated that plasma insulin of T2DM was still high 3–4 h after lipid-enriched meal administration [28].

The preliminary results from FTIR spectroscopy showed that all integrated areas of C-H stretching bands in the diabetic group seemed to decrease or maintain the status quo when compared to those in the fasting state. This finding contrasts with observations of the control group, which exhibited apparent changes in those bands, especially a significant increase in the olefinic band ($\nu$ (CH)). The olefinic band is an indicator of the relative concentration of double bond-containing unsaturated lipids in the cell [29]. The findings suggested that unsaturated lipids in the PLs of a diabetic’s erythrocytes remain unchanged after a high-fat meal. Since the changes in the olefinic band are closely related to the modifications occurring with erythrocyte PLs that contain unsaturated acyl chains [30], we hypothesised that there may be mild changes in PLs with unsaturated fatty acids, especially in the PC content of erythrocytes in diabetic patients. To support this hypothesis, we further investigated the changes in PC molecular species in erythrocytes obtained from both the fasting and postprandial states compared to control subjects by the LC-ESI-MS.

Data from LC-ESI-MS demonstrated that, 4 h after intake of the meal, there were apparent changes in PC molecular species in the control group, but not in T2DM; in particular, a significant increase in PC 34:1 (16:0/18:1 as major species) was observed. During the postprandial period, the rapid exchange of intact PLs or incorporation of meal-derived fatty acids into erythrocyte PL fractions has been described for healthy subjects [12]. The predominant fatty acids of the high-fat meal were palmitic acid (16:0) and oleic acid (18:1), and the predominant PC molecular species was PC34:1 (data not shown). Thus, the results of this study suggest that the increase in PC species may be from the exchange of intact PC molecules from plasma lipoproteins [10] and/or ATP-dependent acylation of ingested fatty acids with either endogenous erythrocytes or plasma lysophosphatidylcholine (LPC) and further incorporated into the PC fraction of the erythrocyte. On the other hand, our findings revealed that there were only mild changes in the PC molecular species of T2DM, indicating abnormalities in the PC remodelling process of diabetic erythrocytes during the postprandial period. The abnormal behaviour may be a consequence of impaired activities of enzymes participating in the exchange or renewal processes. The principal enzymes involved in such processes are phosphatidylcholine-transfer proteins [31, 32], acyl-CoA synthetases, and lysophosphatidylcholine acyl-CoA transferases (LAT). The latter two enzymes have a major role in acylation of ingested fatty acids to LPC. In T2DM humans, there is no evidence that these enzymes are defective. Nevertheless, there is evidence in streptozotocin-induced diabetic rats of alterations in membrane fatty acid turnover by such enzymes as LAT and phospholipase A:
In addition, these abnormalities can be restored to control levels by ATP and insulin treatment [34]. However, such experiments were done using a type 1 diabetes model, which was defined as “insulin deficiency”. Further evidence has been presented regarding a prolonged incubation of high-insulin concentration, which suppressed the activities of the phospholipid transfer protein and cholesteryl ester transfer protein in HepG2 cells [35, 36]. Enzymatic deficiencies and high insulin levels may play a vital role in the impairment of lipid transfer protein activities. Therefore, it can be speculated that high postprandial insulin concentrations in T2DM impairs or suppresses the principal enzyme activities of PL remodelling in the erythrocyte. In addition, other possible mechanisms may involve glycation of amino-phospholipids in the erythrocyte membrane, which would cause changes in the structure and stability of the membrane proteins [37]. Glycation of amino-phospholipids, particularly phosphatidylethanolamine (PE), was reported to be higher in diabetic erythrocytes [38, 39]. In the present study, some PE and PS molecular species in the diabetic erythrocytes obtained during the fasting state were significantly lower than that of normal subjects (data not shown). A reduction of such species may be transformed to the glycated species through the Maillard reaction. This affects erythrocyte membrane properties, resulting in the alteration of membrane-bound enzymes. Thus, further research is needed to clarify the relationship between high postprandial insulin levels and glycated aminophospholipids on the altered activities of enzymes involved in PLs remodelling in the T2DM model.

In conclusion, the results of the present study indicate that there are abnormalities in postprandial remodelling of erythrocyte PLs in T2DM. Abnormal acylation of unsaturated fatty acids with LPC and/or passive exchange of intact PC in the postprandial period directly altered the erythrocyte PLs composition. This phenomenon can contribute to the impairment of erythrocyte fluidity and deformability, resulting in the elevated blood viscosity [40, 41]. This eventually caused the vascular changes in diabetics [42]. Therefore, the abnormalities in postprandial remodelling of erythrocyte PLs in diabetics could be a potential risk factor for the development of microvascular complications in diabetic patients.

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Abbreviations

FTIR, Fourier Transform Infrared spectroscopy; LC-ESI-MS, Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry; PLs, Phospholipids, T2DM, Type 2 diabetes.
Author Disclosures

Sukrit Sirikwanpong, Winai Dahlan, Sathaporn Ngamukote, Siriporn Sangsuthum, Sirichai Adisakwattana, Vanida Nopponpunth, and Thep Himathongkam declare that there are no conflicts of interest.

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