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Lipidomic Profiling on Oxidized Phospholipids in Type 2 Diabetes Mellitus Model Zebrafish

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Type 2 diabetes mellitus is a serious metabolic disorder in the world. Oxidative stress, as a key role on the pathogenesis of diabetes, also results in the oxidation of phospholipids. However, studies on phospholipid oxidation in diabetes, especially directly focusing on oxidized and degraded phospholipid species, are quite limited. In this study, phospholipid profiles of diabetic zebrafish plasma were characterized by LC-HRMS and MS/MS, and the total amounts of each lipid class were compared. Furthermore, the key molecular species as biomarkers in distinguishing control and diabetic samples were investigated by orthogonal partial least squares discriminant analysis. Among the identified 114 phospholipid species in total, there were 11 hydroperoxides, 7 aldehydes, and 19 lysophospholipids found significantly elevated along with the increasing blood glucose, which were known as oxidation or degradation products. Furthermore, lysophosphatidylcholine 20:5 and lysophosphatidylcholine 22:6 were assessed as potential biomarkers in diabetic zebrafish. The current work would not only help to gain further insights into diabetes, but also contribute to find new clinical parameters for the screening of the promising antioxidant agents for its therapies.

Keywords Lipid oxidation, oxidized phospholipids, oxidative stress biomarkers, type 2 diabetes mellitus, zebrafish, LC-MS

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Introduction

Diabetes mellitus, as a prevalent metabolic disorder, is characterized by hyperglycemia due to the inability to secrete insulin or use it effectively. Among all of the diabetes cases, more than 90% are type 2 diabetes mellitus.¹ It is known that oxidative stress plays a key role in the pathogenesis of diabetes. Hyperglycemia stimulates several biochemical pathways that produce reactive oxygen species (ROS), such as oxidative phosphorylation, glucose autooxidation, advanced glycation end products (AGE) formation, and glucosamine pathway.² Oxidative stress, in turn, leads to promote insulin disturbance that contribute to diabetes development. For example, it is known that ROS can damage mitochondria and significantly attenuate insulin secretion.³ Moreover, oxidative stress alters Ca²⁺ homeostasis to cause an increase in Ca²⁺ influx, and triggers

the activation of phospholipase to produce arachidonic acid. Furthermore, the prostaglandins derived from arachidonic acid are considered to be predominantly proinflammatory, which is the risky factor of oxidative stress.^{4,5}

Since the oxidative stress in diabetes disrupts the balance between free radicals and the antioxidant defense system of cells, the relationship between lipid oxidation and diabetic complication draws researchers' attention. Studies have been reported that diabetic complication is associated with an elevation of the lipid peroxidation products, such as comparative studies on thiobarbituric acid reactive substances (TBARS), malondialdehydes (MDA), and isoprostanes (IsoPs) in diabetic individuals.^{6,7} Those compounds serve as products of the lipid peroxidation process. A better way to investigate lipid peroxidation would be to directly analyze the original product, lipid hydroperoxides (L-OOH), rather than its secondary products. However, investigations directly targeted on the oxidized lipid molecules are quite limited.

Thanks to the development of mass spectrometry (MS) technologies, especially coupling it to high-performance liquid chromatography (LC-MS) and tandem MS (MS/MS) in the recent years, researchers can successfully determine various

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analytes in different samples.^{8–11} For the oxidized phospholipids, there have been reports on the determination of different kinds of oxidized phospholipids in various biological samples, including the hydroperoxides of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL), as well as PC aldehydes (PC-CHO).^{12–18} Nevertheless, with regard to lipidomics in diabetes, although there have been studies on lipidomics *via* LC-MS,¹⁹ there are seldom reports concerning on the changes of these oxidized phospholipids. In our laboratory, previous studies on various lipid hydroperoxides (*e.g.* PC-OOH, TG-OOH, CE-OOH, CL-OOH) in different biological samples (*e.g.* blood, cell, lipoprotein) have been carried out. The investigated hydroperoxides were known as biomarkers for the oxidation degree of the sample matrix, and their LC-MS data could be the basis of further studies.^{13,14,20,21} Therefore, whether oxidized lipids could be considered as biomarkers for type 2 diabetes mellitus is of our great interest. The finding of reliable biomarkers will not only help to clinical diagnosis, but also promote elucidating the mechanism of type 2 diabetes mellitus, and in addition contribute to screening the potential agents for diabetic therapies. There is one report concerning on the positive association between serum PC-OOH levels and blood glucose control in type 2 diabetes patients by Nagashima *et al.*,²² yet there are more phospholipid oxidation products to be clarified.

An appropriate animal model is important to in-depth studies on diabetes and anti-diabetic drug development. Most of the available models are rodent-based, which is labor-intensive, and, because of ethical issues, only small groups of animals can be used. Due to the high similarity to mammals in organ physiology and metabolism along with the less labor intensity, zebrafish (*Danio rerio*) has been considered to be favorable animal to explore diabetes and its related diseases, being a favorable alternative to rodent.^{23,24} Recently Zang *et al.* developed a novel zebrafish model for type 2 diabetes mellitus, which was considered to be a promising platform for mechanic and therapeutic studies of diet-induced glucose intolerance and insulin resistance.²⁵ In the present work, the omics analysis for phospholipids and oxidized phospholipids was performed to investigate the characteristics in the plasma from diabetic zebrafish. With the help of HPLC coupled to high-resolution MS (HRMS) combined with multivariate statistical analysis, the levels of specific lipid molecules were semi-quantitatively compared, and the potential biomarkers for diabetic diagnosis were found.

Experimental

Chemicals

LC-MS grade chloroform, isopropanol, methanol, acetonitrile and water were purchased from Wako Pure Chemical (Osaka, Japan). Ammonium acetate and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were of analytical grade and purchased from Kanto Chemical Industry (Tokyo, Japan) unless specified.

Plasma sample of the type 2 diabetic zebrafish

All animal procedures were approved by the Ethics Committee of Mie University, and performed according to the Japanese animal welfare regulation “Act on Welfare and Management of Animals” (Ministry of Environment of Japan) and complied with international guidelines.

The type 2 diabetes mellitus model zebrafish were prepared as reported previously.²⁵ Briefly, male healthy zebrafish of *AB* and *Tg(-1.0ins:EGFP)^{sc1}* strain (the Zebrafish International Resource Center, Eugene, OR) were maintained in Graduate School of Regional Innovation Studies, Mie University according to established protocols²⁶ before modeling. The zebrafish were divided into a control group (Con) and an overfed group, both of which were fed with the commercial fish food Otohime B2 (Marubeni Nisshin Feed, Tokyo, Japan). The zebrafish in the overfed group were conducted using an automated feeding system (Marukan, Osaka, Japan) for modeling.²⁵

The fasting blood glucose level varied among the overfed zebrafish due to individual differences. Based on their fasting blood glucose, the overfed zebrafish were grouped into a medium blood glucose group (MBG, with fasting blood glucose less than 60 mg/dL) and a high blood glucose group (HBG, with fasting blood glucose more than 60 mg/dL), as previously reported.²⁵ In order to compare the influence of the overfed period, two groups were investigated: 1 month for short-term, and 7 months for long-term. For the 1-month experiment, $n = 10$ (Con), 7 (MBG), and 11 (HBG), respectively, and for the 7-month experiment, $n = 10$ for each group. The detailed blood glucose data are shown in Fig. S1 (Supporting Information).

After being fed or overfed for 1 month and 7 months, respectively, all of the zebrafish were fasted overnight prior to anesthetization by 2-phenoxyethanol (500 ppm). Then blood collection was performed by a heparinized needle, and the blood glucose level was determined by a handheld glucometer (Glutest Neo Super; Sanwa Kagaku Kenkyusha, Nagoya, Japan).²⁷ Due to the blood volume limitation, for plasma lipid profiling analysis, blood from the same group was combined and pooled in micro centrifuge tubes. The pooled blood samples were centrifuged of 680g, 4°C for 10 min, and the plasma were obtained (approximately 60 μ L). All of the plasma samples were stored in -80°C until use.

Total lipids extraction

The extraction procedure was according to Bligh and Dyer.²⁸ In Brief, 20 μ L of zebrafish plasma sample was extracted with 160 μ L of cold chloroform/methanol 1:1 (v/v, with 0.002% BHT) twice, followed by the combination of extract and dryness under vacuum. Then, the dried lipids were dissolved in 150 μ L of methanol, centrifuged at 2500g 4°C for 15 min to remove any insoluble material, and thereafter stored at -80°C until analysis. All of the sample pretreatment was completed within 1 h to avoid lipid degradation and auto-oxidation. All of the lipid extracts were prepared in triplicate.

LC-MS conditions

Lipid extracts were separated on an Atlantic T3 C18 column (2.1 \times 150 mm, 3 μ m, Waters, Milford, MA) connected to a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan). A flow rate of 200 μ L/min was used for the analysis, and the column and sample tray were held at 40 and 4°C, respectively. MS analysis was carried out using an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc., San Jose, CA). The MS capillary voltage was set at 3.0 kV, the sheath gas (nitrogen) flow was set to 50 units, and the auxiliary gas (nitrogen) was set to 5 units. The MS¹ data was obtained in the Fourier transform mode with resolving power of 60000 and 2 Hz scan speed, while the tandem MS data was acquired using the collision-induced dissociation in ion-trap mode and data-dependent acquisition, which included scans on the most intense ions in MS² (collision energy of 35 V) and MS³ (collision energy of 45 V), respectively. These conditions were held constant for

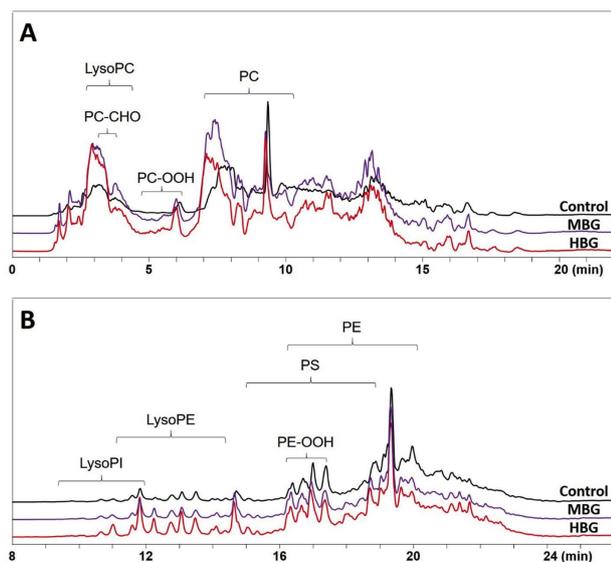


Fig. 1 TIC of zebrafish plasma phospholipid profiles in ESI-positive (A) and negative (B) modes. Control, MBG, and HBG samples are drawn in different colors.

both positive- and negative-ionization mode acquisitions.

In the positive mode, LC gradient elution was performed with a mobile phase of (A) 5 mM aqueous ammonium acetate, (B) isopropanol, and (C) methanol. The following gradient system was utilized: 0 – 1 min 4% A, 6% B, and 90% C; 1 – 10 min 2% A, 83% B, 15% C, and this ratio was kept to 19 min; 19 – 20 min returned to the initial ratio for re-equilibration. The MS¹ scan range was *m/z* 150 – 1100. In the negative mode, the LC mobile phase was changed to (A) methanol/acetonitrile/water 1:1:3 (v/v/v, with 5 mM ammonium acetate), (B) isopropanol, and (C) methanol/acetonitrile 1:1 (v/v, with 5 mM ammonium acetate). The gradient program was as follows: 0 – 2 min 90% A, 0% B, and 10% C; 2 – 22 min 3% A, 82% B, 15% C; this ratio was kept to 27 min; 27.01 – 30 min returned to the initial ratio for re-equilibration. The MS¹ scan range was *m/z* 250 – 1000.

Data analysis

The extracted ion chromatograms (EICs) were drawn within the mass tolerance of 5.0 ppm by Xcalibur 2.2 (Thermo-Fisher Scientific Inc.). The LC-MS identification of lipid molecules was executed with the help of LipidBlast²⁹ as well as an in-house library.^{18,30} The alignment, peak extraction, and EIC peak area integration from the raw data were utilized by the MS-label free differential analysis software package SIEVE 2.0 (Thermo-Fisher Scientific Inc.), of which the main parameters were set as follows: frames, 10 K; *m/z* window, 0.01 Da; retention time window, 1.00 min; intensity threshold, 10000. The exported EIC peak area data were treated as the intensities of analytes and used for further statistical analysis. The hierarchical cluster analysis (HCA) was performed using JMP 10.0 (SAS Institute Inc., Cary, NC), in which the Euclidean distance was used as a dissimilarity, and Ward's minimum variance was used as a linkage rule.³¹ Also, the orthogonal projection to latent structures discriminant analysis (OPLS-DA) was processed using R software and the muma package,³² in which the S-plot was generated after Pareto scaling and mean-centering.³³

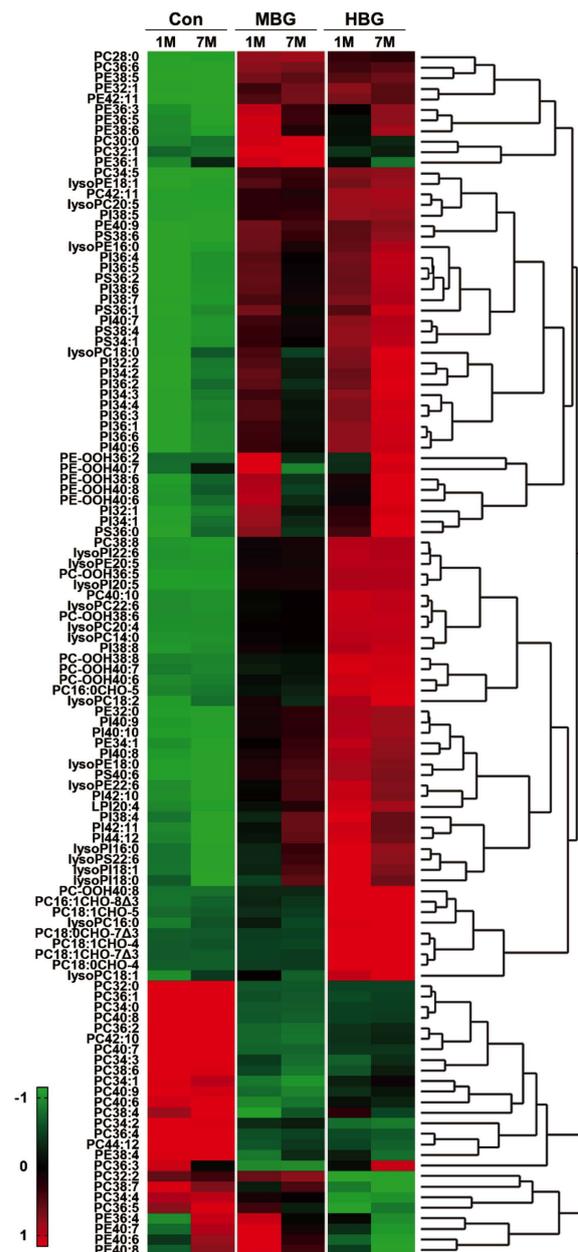


Fig. 2 Heat map with cluster analysis of the phospholipid profiles among the control, MBG, and HBG zebrafish. The colors from green to red indicate the means of relative contents. 1M and 7M represent the 1-month and 7-month overfeeding, respectively.

Results and Discussion

Phospholipid profiles of zebrafish plasma

The representative LC-MS total ion chromatogram (TIC) of the zebrafish plasma lipid under positive and negative modes are shown in Figs. 1A and 1B, respectively. A total of 114 phospholipid species, including the choline type of 29 PC, 6 PC-OOH, 7 PC-CHO, 8 lysoPC, the ethanolamine type of 15 PE, 5 PE-OOH, 5 lysoPE, the inositol type of 25 PI, 6 lysoPI, and the serine type of 7 PS and 1 lysoPS were detected and identified based on their retention behavior on a reversed-phase column as well as the protonated/deprotonated ion peaks on HRMS. The LC-MS data are shown in Table S1 (Supporting Information).

In order to search significant variables in large dynamic range

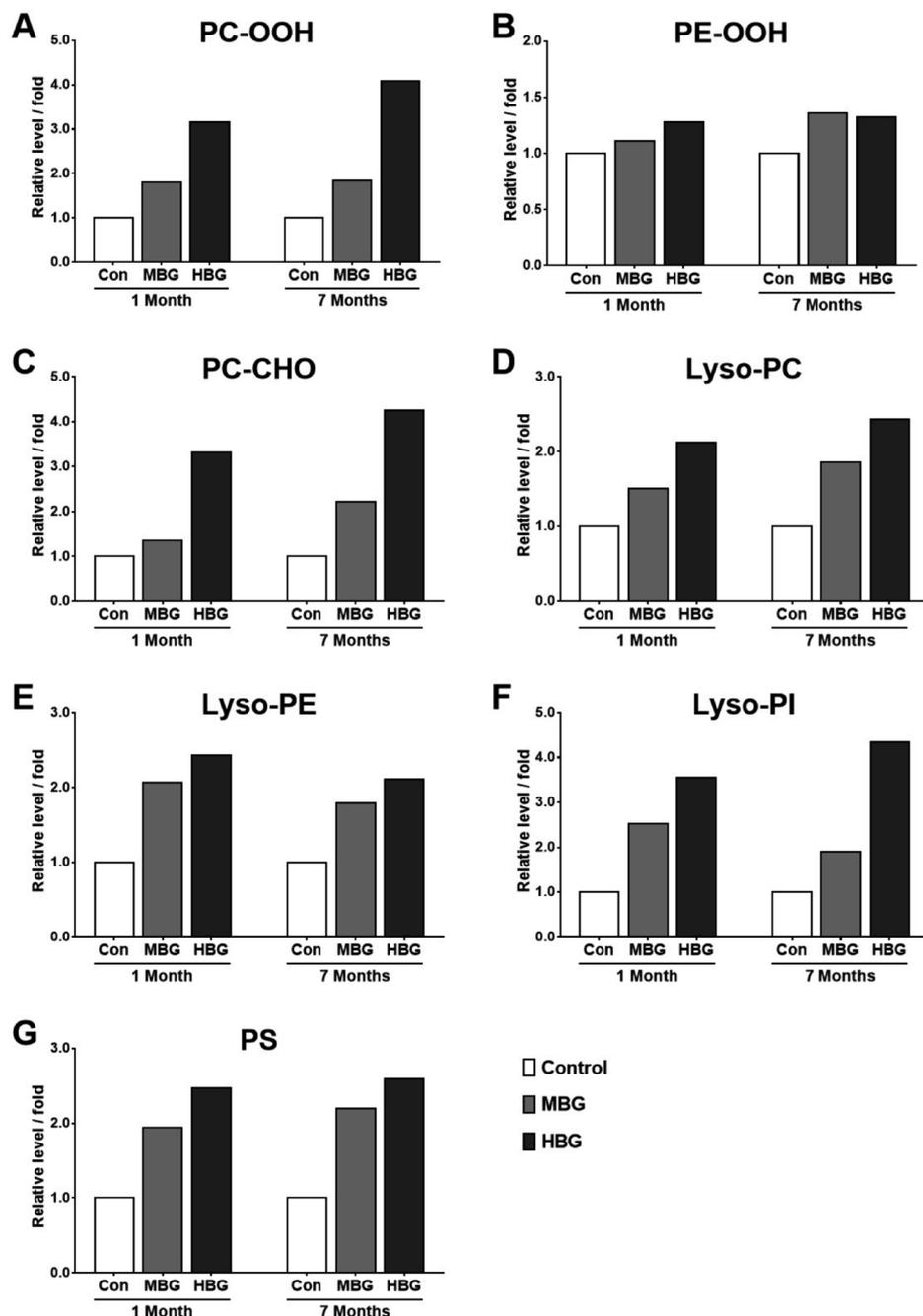


Fig. 3 Comparison of each class of phospholipid oxidation and degradation products based on their intensities among Con, MBG, and HBG zebrafish, including PC-OOH (A), PE-OOH (B), PC-CHO (C), lysoPC (D), lysoPE (E), lysoPI (F), and PS (G).

and to avoid noisy data efficiently, we performed normalization and centralization of the EIC peak area of all detected phospholipids using the R software,³⁴ followed by generating a heat map by JMP. Specific patterns of correlation between groups and phospholipid variables are shown in Fig. 2. The HCA dendrogram expressed different distribution patterns of each phospholipid species, and the red squares indicated relative higher contents in each row. The variables of higher abundance in overfed groups (MBG and HBG of both 1-month and 7-month) mainly consisted of phospholipid hydroperoxides, aldehydes, and lysophospholipids, which suggested that their elevated levels might be related to increased blood glucose levels. Some reports have suggested that lipid species containing

less carbon atoms and less double bonds are associated with an increased risk of diabetes or other metabolic syndromes,^{35,36} but in our results there seemed to be no significant differences in the specific pattern of the acyl compositions between the different test groups.

Variation of phospholipid oxidation and degradation products between control and diabetic zebrafish

Since the variables that became elevated in diabetic zebrafish mainly comprised of phospholipid oxidation and degradation products, based on the heat map (Fig. 2) the sum of the abundance for each related phospholipid class was calculated, and the relative intensities of the three groups of 1-month and of

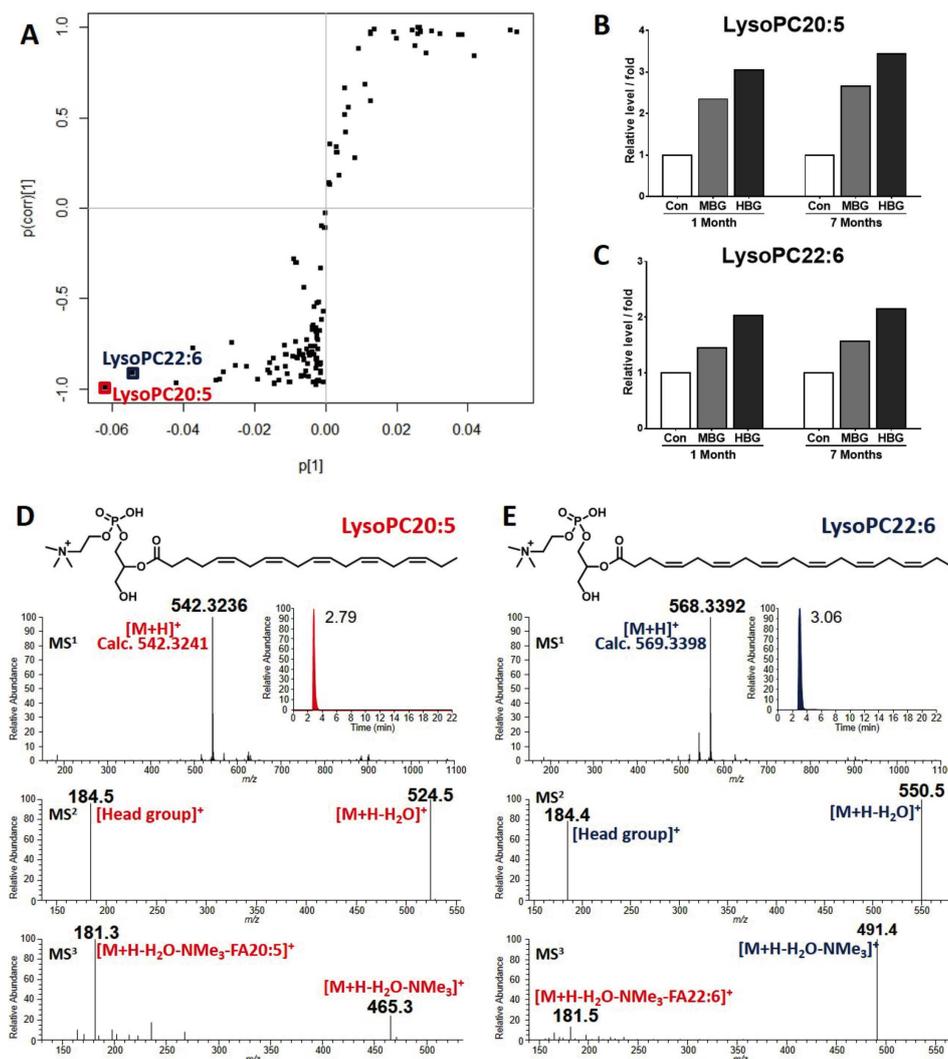


Fig. 4 OPLS-DA reveals lysoPC 20:5 and lysoPC 22:6 as potential biomarkers. These two variables are marked in S-plot (A); their levels among Con, MBG, and HBG zebrafish are compared (B and C), and the MS fragment signals are interpreted (D and E), respectively.

7-month were subsequently compared. As one of the major products of lipid peroxidation, PC-OOH was evaluated in HBG (increased to 3.16-fold after 1-month overfeeding, and increased to 4.09-fold after 7-month overfeeding, compared with control group, respectively) (Fig. 3A). It is reported that lipid hydroperoxides act as toxic mediators, and relate to various pathologic effects, *e.g.* PC-OOH accumulation in the plasma is involved in diabetes as well as hyperlipidemia, Alzheimer's disease, and other metabolic diseases.³⁷ Our result concerning of PC-OOH was in favor of Nagashima *et al.* who demonstrated that PC-OOH in serum was increased in type 2 diabetic patients.²² While for the other detected hydroperoxide class, PE-OOH, the variation between groups seemed to be similar (for 1-month: 1.11-fold in MBG, 1.28-fold in HBG; and for 7-month: 1.36-fold in MBG, 1.33-fold in HBG) (Fig. 3B). The levels of phospholipid oxidation and degradation products presented no obvious difference between the short-term (1 month) and long-term (7 months) groups of overfeeding, which fit the data of the fasting blood glucose levels. It seems that phospholipid oxidation might be related to the development of hyperlipidemia and diabetes.

As the end product of the PC oxidation reaction, PC-CHO

also increased significantly in this experiment. After 1 month of overfeeding, the sum of these aldehydes increased to 1.35 and 3.31-fold in MBG and HBG, respectively. Also, for 7 months of overfeeding, the values became higher (2.21 and 4.25-fold, respectively) (Fig. 3C). There are quite a few reports on the PC-CHO variation in hyperglycemia up to now, although it is considered to be an oxidation product of PC. Continuous research has been conducted by Itabe *et al.* since 20 years ago, who focused on the analysis of PC-CHO and other PC oxidation products in oxidized lipoprotein, and demonstrated them to be chemical markers of oxidative stress.³⁸⁻⁴⁰ In addition, Nakanishi *et al.* found that all of the PC-CHO species together with other ox-PC were in higher amounts in the ischemic myocardium than in the non-ischemic myocardium, indicating the correlation between these oxidized phospholipids and oxidative stress-related diseases.⁴¹ A recent study by Khoury *et al.* successfully identified a diversity of phospholipid aldehydes. The achieved results enriched the oxidized phospholipids information for databases, and the authors proposed it to be the strategy of analyzing these new biomarkers.¹⁸ In our study, PC-CHO showed a consistent tendency with PC-OOH, supporting the oxidation and degradation pathway in type 2 diabetes mellitus.

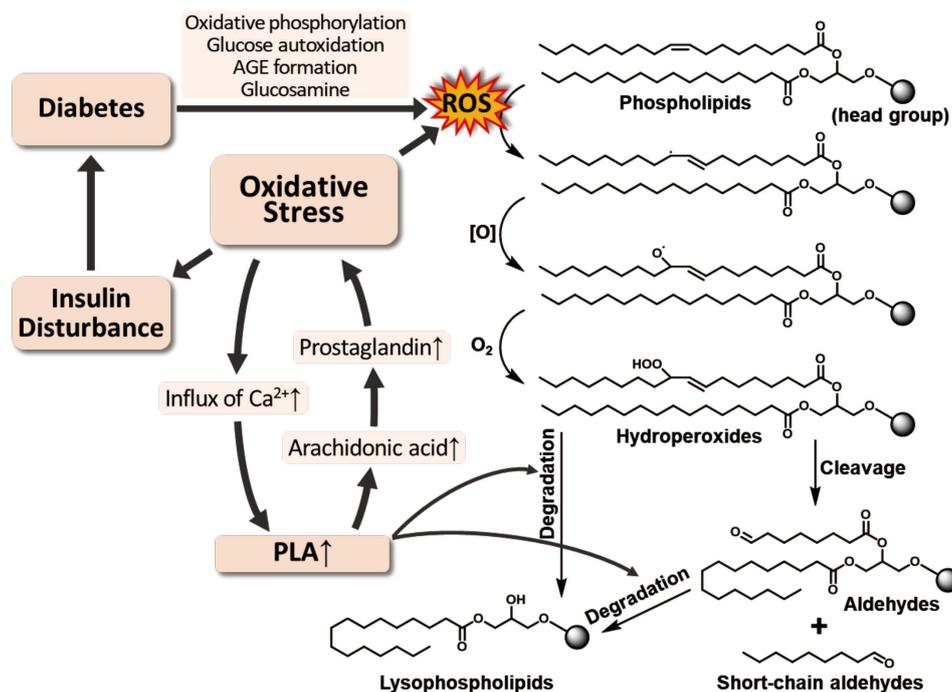


Fig. 5 Putative process of phospholipid peroxidation and degradation related to diabetes and oxidative stress.

Lysophospholipid also showed a comparable increase at the same time. For the total lysoPC, 1 month of overfeeding resulted in 1.51-fold in MBG and 2.12-fold in HBG, while 7 months of overfeeding resulted in 1.86 and 2.44-fold, respectively (Fig. 3D). A similar pattern was observed for lysoPE and lysoPI (Figs. 3E and 3F). Our results are in accordance with that of Ha *et al.*, who disclosed that the 6 lysoPC and 2 lysoPE species significantly increased in male subjects with newly diagnosed type 2 diabetes mellitus.⁴² Besides, the present result of PS level elevation in MBG and HBG (Fig. 3G) agreed with Lappas *et al.*, who reported that PS served as one of the significant risk factors for the development of type 2 diabetes mellitus in women with a previous history of gestational diabetes.⁴³ Within these series of lipid molecules, the most representative species as potential diabetic biomarkers is to be discovered.

LysoPC20:5 and lysoPC22:6 as potential diabetic biomarkers

To reveal potential key phospholipid species differences between the normal group (Con) and the diabetic group (MBG + HBG), OPLS-DA was performed based on the EIC peak area from phospholipid profile after Pareto scaling and mean-centering, and the S-plot was generated (Fig. 4A). The X-axis represented the contribution to the group differences of a particular variable, while the Y-axis indicated the confidence of the variable's contribution. The top-two important variables were marked in the S-plot, which were identified as lysoPC 20:5 (Fig. 4D) and lysoPC 22:6 (Fig. 4E) by MS/MS data. Taking lysoPC 20:5 as an example, HR-MS¹ gave the singly charged $[M+H]^+$ signal of the $C_{28}H_{49}O_7NP^+$ ion with m/z 542.3236 (calculated: 542.3241, $\Delta ppm = -0.92$). The MS² gave two characteristic peaks at m/z 524.5 and m/z 184.5, which were assigned as $[M+H-H_2O]^+$ and the PC head group, respectively. Also, the MS³ of the precursor ion m/z 524.5 gave a continuous fragmentation of trimethylamine cleavage (m/z 465.3) and acyl cleavage (m/z 181.3). These diagnostic ions contributed to a

certain identification.

The relative content variations among the three groups were observed (Figs. 4B and 4C). LysoPC 20:5 increased to 2.35-fold for MBG and 3.05-fold for HBG after 1 month of overfeeding, and 2.66-fold/3.45-fold after 7 months. While the significant increase of lysoPC 22:6 expressed 1 month of 1.45-fold/2.03-fold and 7 months of 1.57-fold/2.15-fold, respectively. The two lysoPC species showed no significant difference between 1 and 7 months of overfeeding, which was in accordance with other phospholipid oxidation and degradation products as well as fasting blood glucose levels. There have been several studies that found lysoPC 18:2 as a target in human diabetic diagnosis,⁴⁴⁻⁴⁶ while Wang *et al.* demonstrated lysoPC 16:0 and lysoPC 18:0 as potential biomarkers for distinguishing diabetic patients.⁴⁷ The present result was consistent with those investigations in terms of the phospholipid class, but differed in molecular species. This may be due to the diversity of the fatty acid composition in different animals (or in human). Fang *et al.* investigated atherogenic zebrafish larvae under high-cholesterol diet feeding, to characterize 18:0, 18:1, 20:5, and 22:6 as significantly elevated lysoPC species.¹⁷ Despite the varieties of animal overfeeding, the current work suggested that lysoPC acted as a meaningful chemical indicator toward oxidative stress-related metabolic diseases. The subsequent research design could include the oxidized phospholipid variation among different experimental objects (including diabetic patients), and the exploration/validation of oxidized phospholipid profile variation/reverse by a certain diabetic treatment.

To the best of our knowledge, there has been very limited investigation of lipidomic analysis in type 2 diabetes mellitus model zebrafish. In terms of lipidomic studies on diabetic samples, such factors as the polar head group, the linkage and unsaturation degree of the acyl chains, are accepted as important roles in diabetes, obesity, and other cardiovascular diseases, since they influence the biophysical properties of the cellular membrane, including the membrane fluidity.³⁵ On the other side,

oxidative stress is known to take a crucial part in diabetes, which is not only involved in these pathological processes above, but also resulted in the oxidation of phospholipid that participates in wide-ranging physiological processes. As the initiation of the chain reaction, ROS attacks the unsaturated fatty acyl in the phospholipid, and produces a variety of oxidized structures, such as phospholipid peroxides and their cleavage products aldehydes. The oxidized phospholipids are degraded to lyso-PL under enzyme catalysis by phospholipase.^{48,49} Thus, the putative process of phospholipid peroxidation and degradation, which are related to oxidative stress in diabetic zebrafish, was uncovered (Fig. 5). Along with the fact that oxidized phospholipids are increasingly recognized as markers of oxidative stress and of various diseases associated with inflammation, the current work suggested that oxidized phospholipids and the related products accumulated because of ROS attack, which fit the theory that a high degree of oxidative stress happens in the pathogenesis of diabetes.

Conclusion

In summary, the lipidomic analysis of phospholipids variations in type 2 diabetes mellitus model zebrafish plasma was systematically studied in the present work. The oxidative stress-related oxidized phospholipids (PC-OOH, PI-OOH, PC-CHO) and phospholipid degradative products (lysoPC, lysoPE, lysoPI) exhibited significant elevation, which could be correlated to rising blood glucose levels. Among them, lysoPC 20:5 and lysoPC 22:6 were found to be two potential biomarkers in diabetic zebrafish. Herein, these findings would help to gain further insights into type 2 diabetes mellitus. Furthermore, using these potential targets as clinical parameters might contribute to the screening of promising antioxidant agents for diabetic therapies.

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Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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