

centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was collected and evaporated, and the dried sample was used for further extraction by the Bligh and Dyer (BD) method (Bligh and Dyer, 1959). Briefly, to 1 ml of the original volume of supernatant, 0.08 ml of water and 0.3 ml of chloroform/methanol (1:2) were added and mixed. Next, 0.1 ml of chloroform was added and mixed, followed by the adding and mixing of 0.1 ml of water. The suspension was then centrifuged at 3000 rpm for 5 min at 4 °C. The organic phase was collected and evaporated, and then reconstituted in appropriate volume of methanol.

Extraction from urine was also performed by the BD method. Briefly, 0.3 ml of chloroform/methanol (1:2) was added to 0.08 ml of urine, and the extraction was performed as above.

These extracted samples were then used for MALDI-QIT-TOF-MS or liquid chromatography-linear ion trap quadrupole-Orbitrap-MS (LC-LTQ-Orbitrap-MS).

Unilateral urethral obstruction

For allowing urine to stagnate, 28-week-old Balb/c mice received unilateral urethral obstruction (UUO), as previously described (Li et al., 2010). Briefly, the left mid-ureter was obstructed by two-point ligations with silk sutures. The mice were sacrificed 2 weeks later.

MALDI-QIT-TOF-MS

All analyses were performed in positive ion mode and in mid-mass range by using a MALDI-QIT-TOF-type mass spectrometer (AXIMA-QIT;

Shimadzu Corporation and Kratos Analytical, Manchester, UK), equipped with a 337 nm nitrogen laser, as previously described (Hayasaka et al., 2008; Sugiura and Setou, 2009). An external calibration method was performed with ions from angiotensin II and its decomposition products covering from m/z 680 to 900.

Data acquisition and imaging

The data acquisition and processing were controlled by Launchpad software (Kratos Analytical). All the spectra were recorded with the standard instrument settings for optimum transmission. Each raster scan was performed in square regions of 5000 μm to 8000 μm with a measurement pitch of 100 μm automatically. Laser irradiation consisted of 10 shots in each spot, and the power setting was 50 to 60 as appropriate. The raw data were converted by using free software (Axima2Analyze; Novartis, Basel, Switzerland), to apply the BioMap (Novartis). Mass signals between m/z 600 and m/z 1000 were analyzed with an interval of 0.1 and a tolerance of 0.05, resulting in 4001 images for each experiment. The ion image highlighted the signal intensity obtained from a specific molecule.

Tandem MS analysis

The instrument settings were changed to correspond to the ions from the molecules of interest. The power and collision-induced dissociation (CID) settings were adjusted from 50 to 60 and from 200 to 400, respectively.

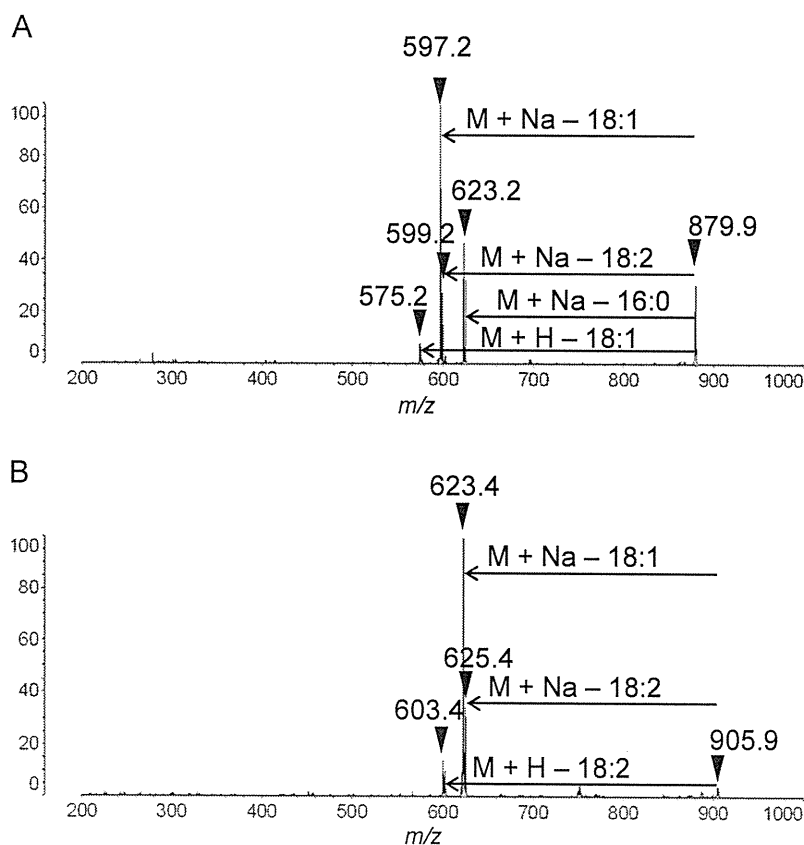


Fig. 2. Two triacylglycerols are present in the renal hilum. The fragmentation patterns of the molecules m/z 879.9 (A) and m/z 905.9 (B) in tandem MS analysis were involved with the loss of fatty acids and thereby identified as the triacylglycerols [TAG 16:0/18:2/18:1 + Na] + (A) and [TAG 18:1/18:2/18:1 + Na] + (B), respectively.

MS analysis of extracted lipids from kidney homogenates or urine

Approximately, 5 to 10 μ l of lipid extracts were dried on ITO slides and coated by spraying the matrix solution. For inferring the adduct ion, 10 mM (final concentration) sodium or potassium acetate was added to extracted samples since excessive sodium or potassium ions have been shown to modify the signal intensity of polar lipids (Sugiura and Setou, 2009). The procedure was performed, as described above.

LC-LTQ-Orbitrap-MS

LC-LTQ-Orbitrap-MS analysis using an Agilent 1200 system was performed to determine the primary structures of the molecules. All procedures were performed in Kazusa DNA Research Institute, Kisarazu, using a detailed protocol, described previously (Iijima et al., 2008). Briefly, the extracted sample reconstituted in methanol was applied to a TSK-GEL Octyl-80Ts (TOSOH Corporation, Tokyo, Japan). Water (solvent A) and acetonitrile (solvent B) were used as the mobile phase with 0.1% v/v formic acid added to both solvents. The gradient program was as follows: 80% B (0 min), 90% B (20 min), 95% B (40 min), 97% B (60 min), 98% B (80 min), 99% B (100 min), 99% B (110 min), 80% B (110.1 min), and 80% B (120 min). The flow rate was set to 0.015 ml/min, and the column oven temperature at 40 °C; 20 μ l of each sample was injected.

Data were analyzed with a molecular formula calculation by using the following databases: KEGG (<http://www.genome.jp/kegg/>), KNApSAcK (<http://kanaya.naist.jp/KNApSAcK/>), Flavonoid Viewer (<http://www.metabolome.jp/software/FlavonoidViewer/>), and LIPID MAPS (<http://www.lipidmaps.org/>). Molecular species were confirmed by tandem MS of selected peaks and by collating each peak's accurate mass measurement with those from the databases.

Results

Molecular distribution of lipids in the HIGA kidney differs from that in the control kidney

Eight mass peaks— m/z 792.6, m/z 814.6, m/z 830.6, m/z 840.6, m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6—were strongly represented in the HIGA kidney (Figs. 1N–U), but not in the control (Figs. 1A–H). However, 2 significant mass peaks, m/z 844.6 and m/z 868.6, in the peripheral (cortex) area (Figs. 1I, J, V, W) and 2 significant mass peaks, m/z 879.9 and m/z 905.9, in the hilum area (Figs. 1K, L, X, Y) were observed in both the HIGA and control kidneys.

Two triacylglycerols identified in the hilum

Tandem MS analysis revealed that the fragmentation patterns of the molecules m/z 879.9 and m/z 905.9 were involved in the loss of fatty acids and were identified as the triacylglycerols (TAGs) [TAG (16:0/18:2/18:1) + Na]⁺ and [TAG (18:1/18:2/18:1) + Na]⁺, respectively, by collating them with existing TAGs in the database (Fig. 2).

Several lipids in the cortex are phosphatidylcholines

LC-LTQ-Orbitrap-MS analysis showed that m/z 806.57 and m/z 830.57 were commonly seen in lipids extracted from Balb/c and HIGA kidneys (Figs. S1A, B). Tandem MS identified these peaks to correspond to [PC(16:0/22:6) + H]⁺ and [PC(18:2/22:6) + H]⁺, respectively (Figs. S1C, D). In addition, m/z 844.6 and m/z 868.6 were estimated to be carrying a potassium ion as the adduct ion (Fig. 3), and thus, were considered to correspond to [PC(16:0/22:6) + K]⁺ and [PC(18:2/22:6) + K]⁺, respectively.

HIGA-specific lipids in the tubular lesions are O-phosphatidylcholines that are possibly related to phosphatidylcholines in the cortex area

LC-LTQ-Orbitrap-MS also revealed that m/z 792.59 and m/z 818.61 were seen specifically in HIGA (Fig. S1B), and tandem MS analysis determined that they corresponded to [PC(O-16:0/22:6) + H]⁺ and [PC(O-18:1/22:6) + H]⁺, respectively (Figs. S1E, F). In addition, m/z 814.6 and m/z 840.6 were estimated to be carrying a sodium ion as an adduct ion, while m/z 830.6 was estimated to be carrying a potassium ion (Fig. 3). Accordingly, m/z 792.6, m/z 814.6, m/z 830.6, and m/z 840.6 were considered to correspond to [PC(O-16:0/22:6) + H]⁺, [PC(O-16:0/22:6) + Na]⁺, [PC(O-16:0/22:6) + K]⁺, and [PC(O-18:1/22:6) + Na]⁺, respectively.

LC-LTQ-Orbitrap-MS failed to identify the corresponding molecules of m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 because the mass peaks compatible to them were absent; however, the adduct ion of these molecules were speculated to be a sodium ion (Fig. 3). Moreover, the fragmentation patterns analyzed by on-section tandem MS analysis revealed that these molecules possessed a regularity of arrangement and were part of orderly structures (Fig. 4).

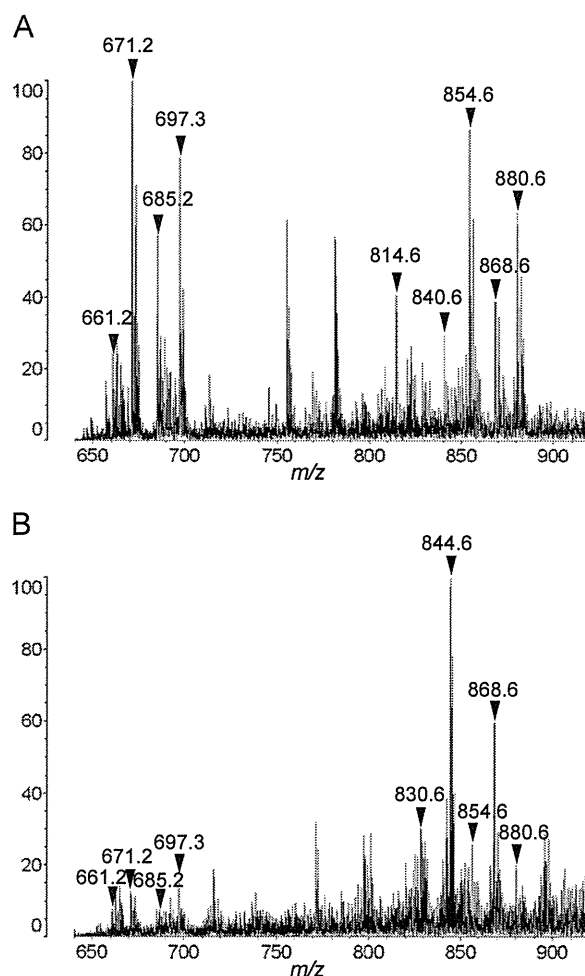


Fig. 3. Addition of sodium or potassium ions in the lipid extracts implied the identity of the adduct ions. The mass peak signals m/z 814.6, m/z 840.6, m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 are intensified when sodium ions are added in the lipid extracts of HIGA kidneys (A); m/z 830.6, m/z 844.6, and m/z 868.6 are intensified when potassium ions are added (B); but m/z 792.6 was not intensified in either condition. Thus, these results imply that the adduct ion of m/z 814.6, m/z 840.6, m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 might be sodium, and the adduct ion of m/z 830.6, m/z 844.6, and m/z 868.6 might be potassium.

Urinary stagnation causes HIGA-specific distribution of lipids in the tubular area

For understanding the source from where HIGA-specific molecules were derived, it was important to investigate whether these lipids were excreted in urine. Mass peaks of lipids extracted from urine were investigated using MALDI-QIT-TOF-MS and compared with those from the kidney. The results showed that significant mass peaks in the urine matched the HIGA-specific mass peaks in the kidney (Fig. 5). Thus, we verified that the HIGA-specific molecules were lipids that were normally excreted in urine. We also found that the artificial urinary stagnation due to UUO in Balb/c mice could reproduce the HIGA-specific distribution of lipids in the tubular area (Fig. 6) and that these molecules were, therefore, derived from the urine.

Discussion

We revealed the molecular distribution of lipids in the kidneys, hypothesized on the underlying mechanisms causing the HIGA-specificity, and determined the identity of several molecules by using IMS in combination with LC-LTQ-Orbitrap-MS (Table 1).

We noticed a similarity among PC(16:0/22:6), PC(18:2/22:6), PC(O-16:0/22:6), and PC(O-18:1/22:6) (Fig. 7). All these molecules have 22:6 lipid side chains. Molecules that were common in both the HIGA and normal kidneys were regular PCs and were distributed in the cortex area; while HIGA-specific molecules were O-PCs and distributed in the tubular areas. In addition, the only difference between PC(16:0/22:6) and PC(O-16:0/22:6) was an oxygen atom in the 16:0 side chains. Similarly, the only differences between PC(18:2/22:6) and PC(O-18:1/22:6) were an

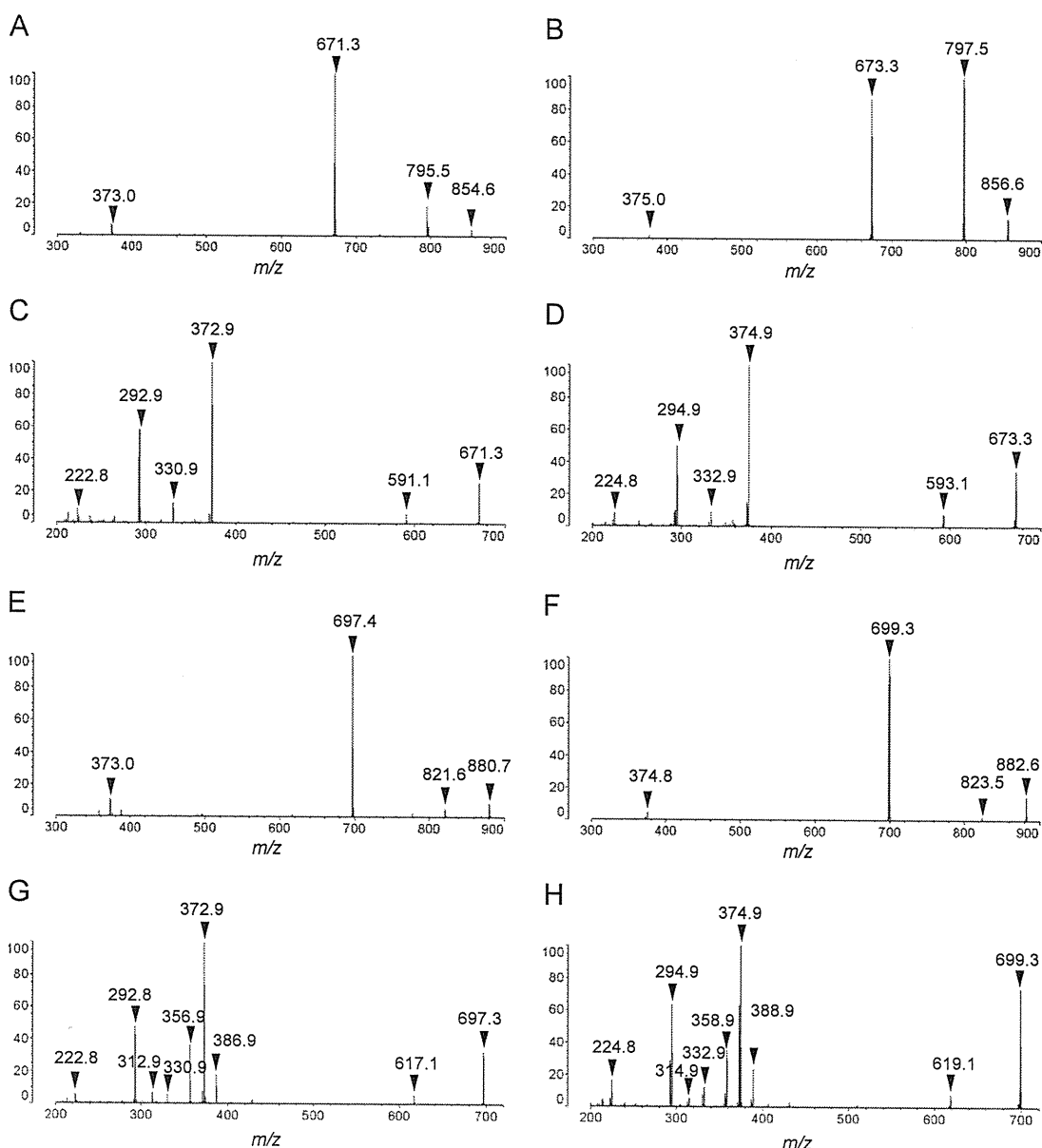


Fig. 4. Tandem MS analysis of HIGA kidney-specific mass peaks m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 shows their molecular relationship. The fragmentation patterns of the molecules of m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 by MS-MS analysis are involved with the loss of m/z 59 Da and m/z 183 Da (A,B,E,F). Subsequent MS3 analysis failed to identify these molecules (C,D,G,H).

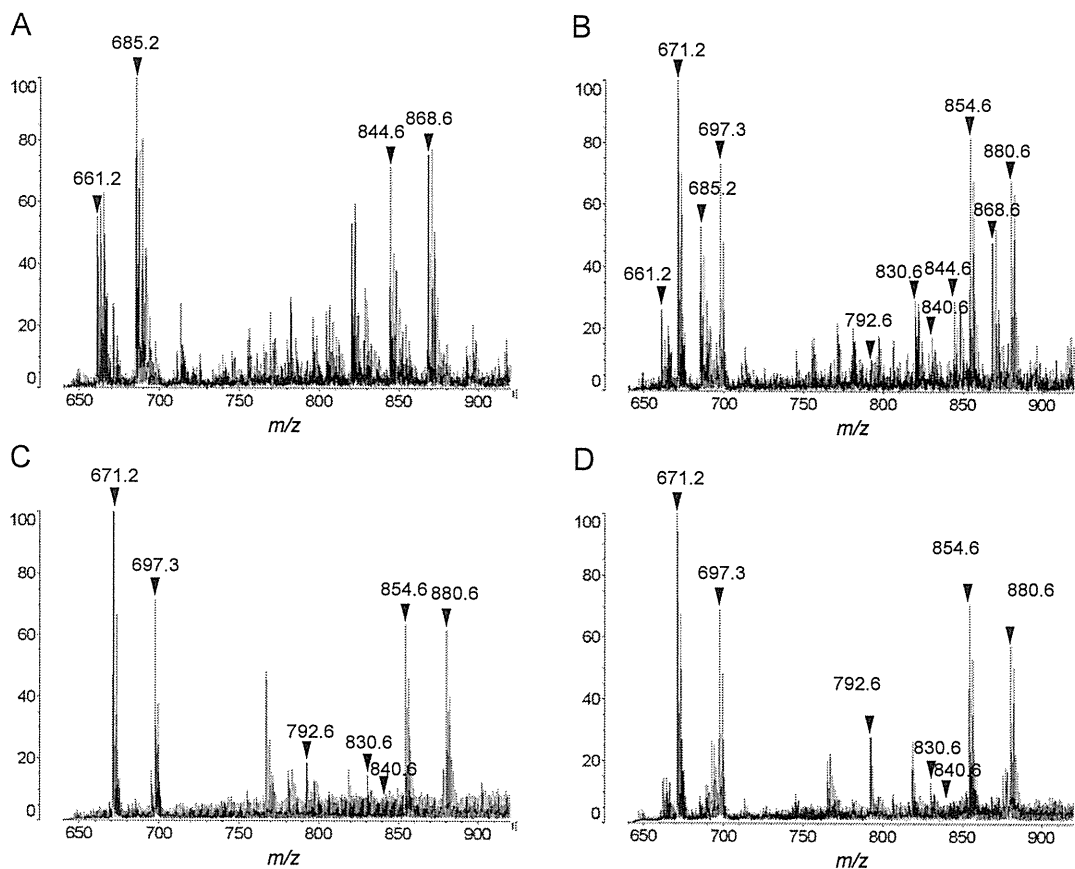


Fig. 5. HIGA-specific lipids are derived from urine. MALDI-QIT-TOF-MS-analyzed mass peaks of lipids extracted from control kidney (A), HIGA kidney (B), control urine (C), and HIGA urine (D) are shown. HIGA-specific mass peaks are seen in both the HIGA and control urine, while the mass peaks that are common in both kidneys are absent in urine.

oxygen atom and a saturation of the side chain. These findings suggest that these molecules may have a novel, unidentified renal function.

Although, we were unable to determine the chemical structures of 4 specific molecules corresponding to m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6, tandem MS still provided some interesting findings. First, the fragments of m/z 854.6 and m/z 856.6 always had a difference of 2 Da, which was also true for m/z 880.6 and m/z 882.6. Second, m/z 854.6 and

m/z 880.6, as well as m/z 856 and m/z 882 generated several common fragments. Third, PC(O-16:0/22:6) and PC(O-18:1/22:6) had a difference of 26 Da, while m/z 854.6 (or m/z 856.6) and m/z 880.6 (or m/z 882.6) also had one. Collectively, the results suggested that these lipids have common structural skeletons. In addition, m/z 854.6, m/z 856.6, m/z 880.6, and m/z 882.6 were speculated to be related to PC(O-16:0/22:6) and PC(O-18:1/22:6).

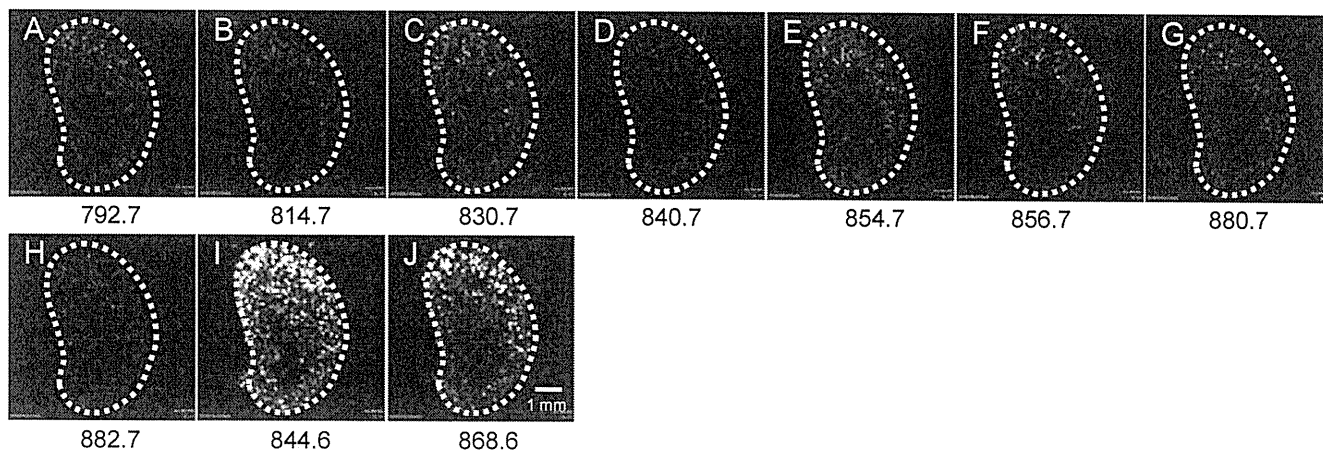


Fig. 6. Artificial urinary stagnation causes HIGA-specific distribution of lipids in tubular area. HIGA-specific mass peaks were detected in the kidneys of a unilateral urethral obstruction model (UUO) in which urine was artificially stagnated (A–H). Images of common mass peaks, m/z 844.6 and m/z 868.6, are also shown for comparison (I, J). Scale bar indicates a length of 1 mm.

Table 1
Distribution of identified lipid molecules in the kidney.

m/z	Distribution	Specificity	Molecules	Urine
844.6	Cortex	Common	[PC(16:0/22:6) + K] ⁺	
868.6	Cortex	Common	[PC(18:2/22:6) + K] ⁺	
879.7	Hilum	Common	[TAG(16:0/18:2/18:1) + Na] ⁺	
905.9	Hilum	Common	[TAG(18:1/18:2/18:1) + Na] ⁺	
792.6	Medulla	HIGA-specific	[PC(O-16:0/22:6) + H] ⁺	Yes
814.6	Medulla	HIGA-specific	[PC(O-16:0/22:6) + Na] ⁺	Yes
830.6	Medulla	HIGA-specific	[PC(O-16:0/22:6) + K] ⁺	Yes
840.6	Medulla	HIGA-specific	[PC(O-18:1/22:6) + Na] ⁺	Yes
854.6	Medulla	HIGA-specific	Not determined	Yes
856.6	Medulla	HIGA-specific	Not determined	Yes
880.6	Medulla	HIGA-specific	Not determined	Yes
882.6	Medulla	HIGA-specific	Not determined	Yes

The lesions in the tubular areas of HIGA mouse have been recognized but not well investigated. One of the reasons for this lack of investigation is that the lesions have not been considered a primary defect in IgA nephropathy in human diseases (Coppo et al., 2010). Another reason is absence of a technique that could identify the molecules. In this study, we detected molecular differences between HIGA and Balb/c mice. These findings could be associated with the histological differences that we have already indicated.

We also revealed that HIGA-specific lipids distributed in the tubular area are derived from urine and succeeded in reproducing the same findings by artificial urinary stagnation using the UUO model; this suggests that urinary stagnation causes HIGA-specific distribution. Urinary stagnation seems not to be the primary cause of IgA because it seems unrelated to glomerular lesions. This finding suggests that urinary stagnation and deposition of these molecules in the tubular area could affect the pathogenesis of the nephropathy and could be a clue for understanding other pathologic mechanisms such as acute renal failure.

Although we now have a better understanding of the mechanisms underlying the HIGA-specific molecular distribution of lipids in the kidney, the following issues need to be investigated: determination of the unidentified molecules, understanding the excretion system of lipids in urine, and discovery of the molecular differences in glomerular lesions of IgA nephropathy. Moreover, PC(O-16:0/22:6) and PC(O-18:1/22:6) are also known as analogues of platelet-activating factor (PAF) and a plasmalogen, respectively, and it will be worth investigating whether these molecules are related to the pathogenesis in IgA nephropathy (Coppo et al., 2010; Robinson et al., 1988).

Conclusion

In conclusion, our data are the first report that sheds light on the molecular distribution of lipids in the HIGA mouse kidney. In addition, IMS might provide the dynamic physiological function of kidneys in addition to the distributional information so that it might be applied to understanding the pathophysiology of various diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexmp.2011.07.002.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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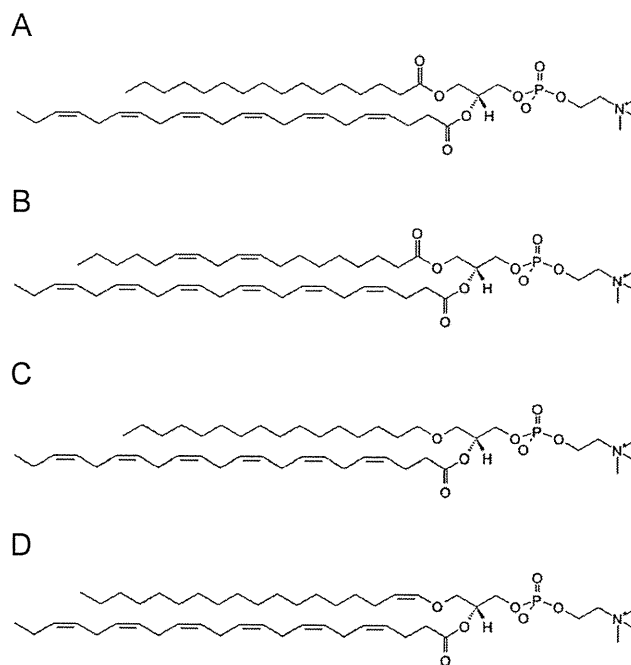


Fig. 7. Phosphatidylcholines that are seen in the control are structurally similar to HIGA-specific O-phosphatidylcholines. Determined or speculated primary structures of selected molecules are shown. Two common phosphatidylcholines that were seen in the cortex area, PC(16:0/22:6) (A) and PC(18:2/22:6) (B), are structurally similar to the HIGA-specific O-phosphatidylcholines, PC(O-16:0/22:6) (C) and PC(O-18:1/22:6), that are seen in the tubular areas and urine (D). The position of the double bonds in the side chains 18:2 and 18:1 were not determined, and therefore the most possible structures, PC(18:2/22:6) and PC(O-18:1/22:6), are shown.

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