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Redox phospholipidomics analysis reveals specific oxidized phospholipids and regions in the diabetic mouse kidney

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ABSTRACT

While it is generally accepted that oxidative stress impacts the diabetic kidney and contributes to pathogenesis, there is a substantial lack of knowledge about the molecular entity and anatomic location of a variety of reactive species. Here we provide a novel "oxidative stress map" of the diabetic kidney – the first of its kind, and identify specific, oxidized and other reactive lipids and their location. We used the db/db mouse model and Desorption Electrospray Ionization (DESI) mass spectrometry combined with heatmap image analysis. We analyzed a comprehensive array of phospholipid peroxide species in normal (db/m) and diabetic (db/db) kidneys using DESI imaging. Oxilipidomics heatmaps of the kidneys were generated focusing on phospholipids and their potential peroxidized products. We identified those lipids that undergo peroxidation in diabetic nephropathy. Several phospholipid peroxides and their spatial distribution were identified that were specific to the diabetic kidney, with significant enrichment in oxygenated phosphatidylethanolamines (PE) and lysophosphatidylethanolamine. Beyond qualitative and semi-quantitative information about the targets, the approach also reveals the anatomic location and the extent of lipid peroxide signal propagation across the kidney. Our approach provides novel, in-depth information of the location and molecular entity of reactive lipids in an organ with a very heterogeneous landscape. Many of these reactive lipids have been previously linked to programmed cell death mechanisms. Thus, the findings may be relevant to understand what impact phospholipid peroxidation has on cell and mitochondria membrane integrity and redox lipid signaling in diabetic nephropathy.

1. Introduction

Oxidative stress has long been recognized as one of the key players in the pathogenesis of diabetic kidney disease [1–4]. Much of the prior focus was placed on the superoxide radical anion, as the primary form of reactive oxygen species (ROS) produced mostly by mitochondria [5,6] or NADPH oxidases [7]. Indeed, overproduction of mitochondrial superoxide as a major form of oxidative stress and a primary event in diabetic/chronic kidney disease has been postulated [6,8–11]. Very often, the umbrella term "ROS" is also used to define several reactive species together with different kinetics and compartmentalization properties [12]. Charged free radicals (with few exceptions transported through ion channels) [13] do not cross membranes and cannot simply spread from one place to another in a cell [14,15]. Furthermore, the kidney is an organ with a rather heterogeneous cell landscape, where some cells may generate more free radicals or may be more sensitive to oxidative attack. Because of these limitations, a gap exists in the field to better understand how oxidative stress exactly impacts the diabetic kidney.

Lipid/phospholipid (hydro)peroxides are reactive species with longer half-life and many lipid peroxidation end products are also membrane diffusible. Lipid peroxidation and its basic tenets in biology are well established [16,17]. It is also well known that conditions like diabetes and kidney disease are associated with an increase in lipid peroxidation and that uncontrolled lipid peroxidation causes pathology [18,19]. Recent data from us and others [20–24] suggest that lipid peroxides, especially the electrophilic products (often termed as reactive lipids) are more than just byproducts or markers of disease, - they are mediators of multiple cellular processes and (patho)physiological conditions [20]. In contrast to superoxide, lipid peroxides have a unique biochemical attribute: they propagate, can get farther from their source and have preferential reaction affinity to target protein residues,

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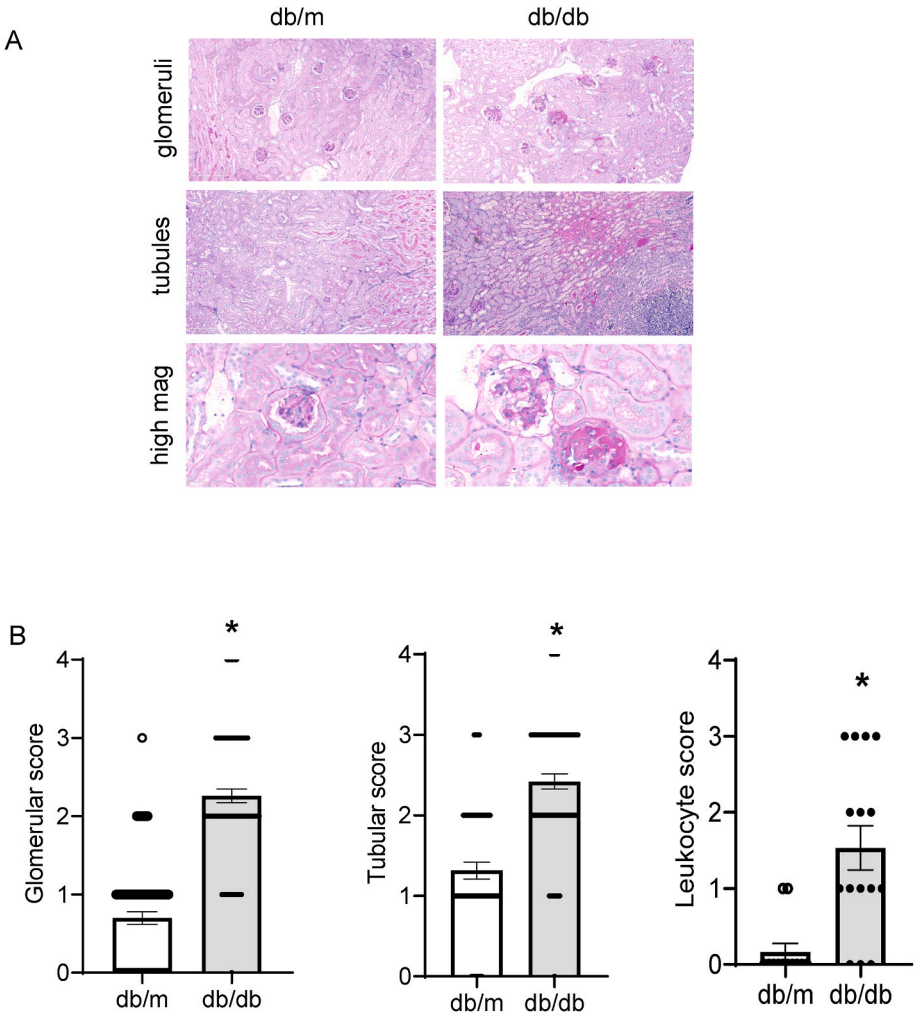

Fig. 1. Diabetic kidney disease in db/dbmice. (A) Representative microphotographs of PAS stained healthy (db/m) and diabetic (db/db) mouse kidneys at 24 weeks of age, showing characteristic glomerular scaring and tubular damage. 10×, high mag: 40×. (B) Glomerular and tubular scores as well as leukocyte infiltration scores were evaluated from the microphotographs. N = 3 mice/group, at least 30 viewing areas/slide ±SEM, $*P < 0.05$.

typically in the cysteine » histidine » lysine order [20]. Due to this distinct reactivity (electrophilic-nucleophilic), the pool of their potential molecular targets is specific. However, it is not well known what are the molecular entities and locations of oxidized lipids to begin with, that are increased in the kidney under diabetic conditions.

Here we provide a comprehensive oxilipidomics "map" of the diabetic kidney, focusing on lipid peroxide species and their distribution across regions of the kidney. We used Desorption Electrospray Ionization (DESI) mass spectrometry in negative ionization mode, combined with heatmap image analysis. DESI is a technique that provides chemical information obtained directly from a surface, such as an organ (kidney) slice. DESI provides qualitative information of the analyte, as well as information on the spatial distribution of molecules. Combined with an image heatmap analysis, it allows for answering the fundamental question in our case: where are the parent phospholipids and the oxidized derivatives in the kidney and which ones are changing in diabetes?

2. Materials and methods

Animals. 8 weeks old male control (db/m) and type 2 diabetic (db/db) mice were obtained from Jackson Laboratories (Bar Harbor, Me; strain:000642, BKS background). Mice were kept on chow (Purina 5001 rodent) and had access to drinking water ad libitum while housed at the Pennington Biomedical Comparative Biology facilities (12/12 h light dark cycle, air conditioning). Mice were kept until they reached 24 weeks of age by which time diabetic nephropathy (established but still not late stage) has developed. Blood glucose levels were monitored weekly using a small blood sample from tail and a One Touch Ultra Mini device. At 24 weeks, mice were euthanized by CO_2 asphyxiation method and kidney tissues were harvested. One kidney was flash frozen in liquid N_2, the other kidney was cut in halves, placed briefly in ice cold PBS for washing, then into buffered 10% formalin for fixation.

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals following protocol review and approval by the Institutional Animal Care and Use Committee at Pennington Biomedical.

Histology analysis. Paraffin embedded kidneys were cut into 5 μm sections. Sections were mounted on charged SuperFrost slides (Fisher Sci.), deparaffinized and stained with Periodic acid-Schiff (PAS) staining to evaluate general morphometrics, glomerular size, presence of sclerosis and proteinaceous casts and mesangial space expansion. At least 15 viewing areas per slide were evaluated on each section with a NanoZoomer Digital Pathology Virtual Slide Viewer and scored in a blinded fashion by trained laboratory members. Glomeruli were scored on a scale of 0–4, where 0 is a normal glomerulus, 1: <25% mesangial expansion and sclerosis, 2: 25–50%, 3: 50–75%, 4: >75% sclerosis. Tubular injury was scored as follows, based on the presence of tubular dilation, cast formation, brush border loss and loss of tubular epithelial structure: 0: <15%, 1: 15–30%, 2: 30–50%, 3: 50–75%, and 4: >75%.

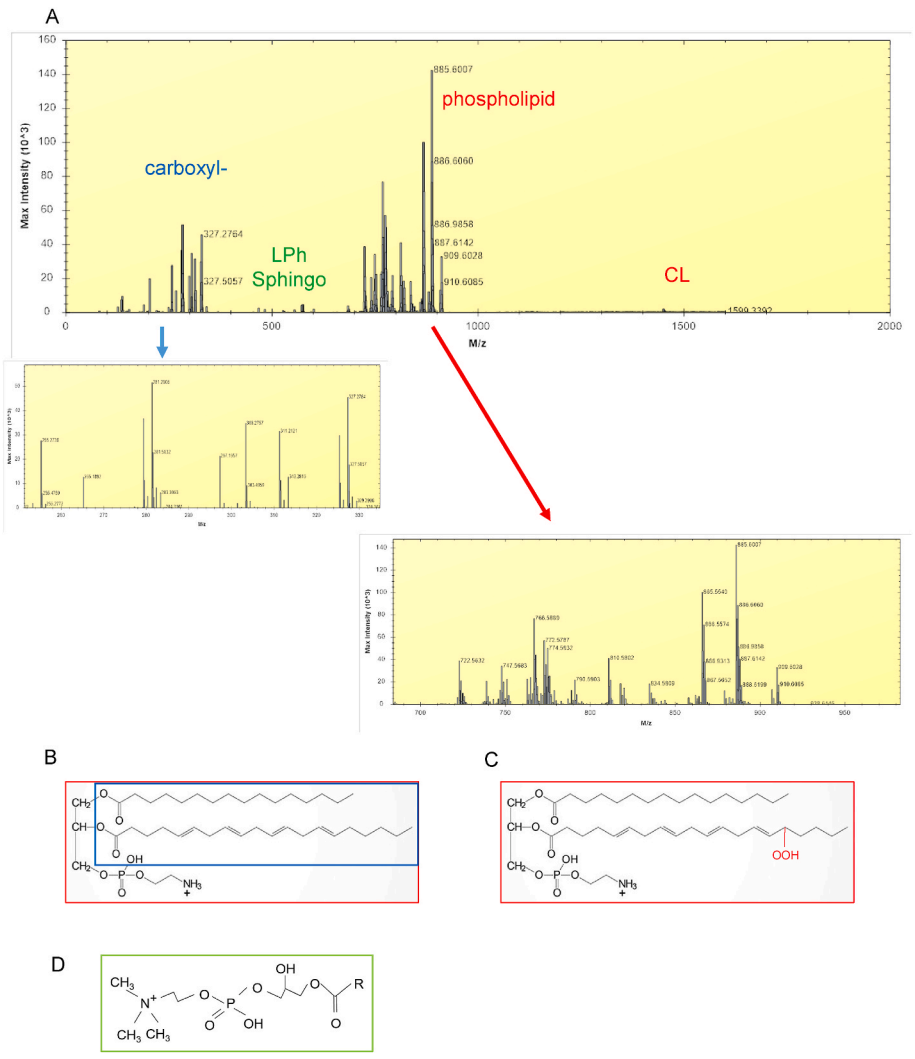

Fig. 2. DESI MS characterization of phospholipids and their oxidation products in kidneys, showing a complex array of targets (A). Each phospholipid species was identified based on their accurate mass. To confirm or exclude the possibility of various combinations of carbon chain lengths with identical molecular weight the detected carboxyl group accurate masses were considered (marked"carboxyl"onthespectrum), shown in detail in the inlet, blue arrow and Table1. (B) General structure of phospholipids, red and blue boxes indicate the full phospholipid molecule and the carboxyl chains, respectively. (C) Primary oxidation product of a general phospholipid molecule. (D) General structure of a lysophospholipid species. LPh: lysophospholipids, Sphingo:sphingolipids,CL:cardiolipin. (For interpretation of the reference to colour in this figure legend, the reader is referred to the Web version of this article.)

Leukocyte infiltration was scored in a similar fashion by evaluating the percentage of the viewing area (at 10×) having immune cell infiltration present.

Desorption Electrospray Ionization mass spectrometry (DESI). DESI mass spectrometry was performed using a DESI XS equipment (Waters Corp., Milford, MA) on a quadrupole time-of-flight mass spectrometer (Xevo G2-XS, Waters Corp). Briefly, kidneys were cut frozen at 30 μm thickness and kidney slices were stored in −80 C until analysis. The DESI equipment uses a jet of solvent focused at the surface of the sample, causing localized microextraction of molecules. The solvent then was desorbed from the surface via droplet pick-up and deflected into a mass spectrometer for analysis of phospholipid species. Negative ion polarity mode was used for MS analysis; resolution of DESI is 50 μm.

Analysis of mass spectrometry and heatmap image generation. The first 1000 most intense peaks were analyzed in each sample using the High Definition Imaging software (HDI v1.5., Waters Inc) from start mass: 100 m/z, end mass: 1500 m/z, m/z window: 0.02 Da.

Peaks of each phospholipid compounds and theoretical fragments were identified based on accurate mass using a Lipid Mass Database. To find or exclude each potential analyte of the same mass, the corresponding carboxyl groups present in the sample were also identified. Heatmap images of each compound were built in the software visualization tool. Image smoothing was applied using linear interpolation. Heatmap intensities were measured and control vs. diabetic groups were compared in square root (sqrt) composition scale mode.

Statistics. Control and diabetic groups for each measurement, analysis or amount of oxidized lipid species were compared using unpaired Student's t-test. Data were expressed as mean ± SEM and differences were considered statistically significant at $P < 0.05$.

Table 1
List of detected carboxyl groups, their accurate mass in negative ion mode and identity (C carbon chain length: number of double bonds).

m/z {M-H]-	Identity based on accurate mass
255.273	**C16:0**
279.275	**C18:2**
281.29	**C18:1**
283.31	**C18:0**
303.275	**C20:4**
311.21	**C20:0**
327.276	**C22:6**
329.29	**C22:5**

3. Results

Diabetic kidney disease in 24 weeks old db/db mice. Consistent with previous data in literature [25–27], db/db mice at 24 weeks of age developed characteristic features of diabetic kidney disease. When compared to age-matched db/m control mice, db/db mice had significant glomerular injury and scarring, tubular dilation and immune cell infiltration (Fig. 1).

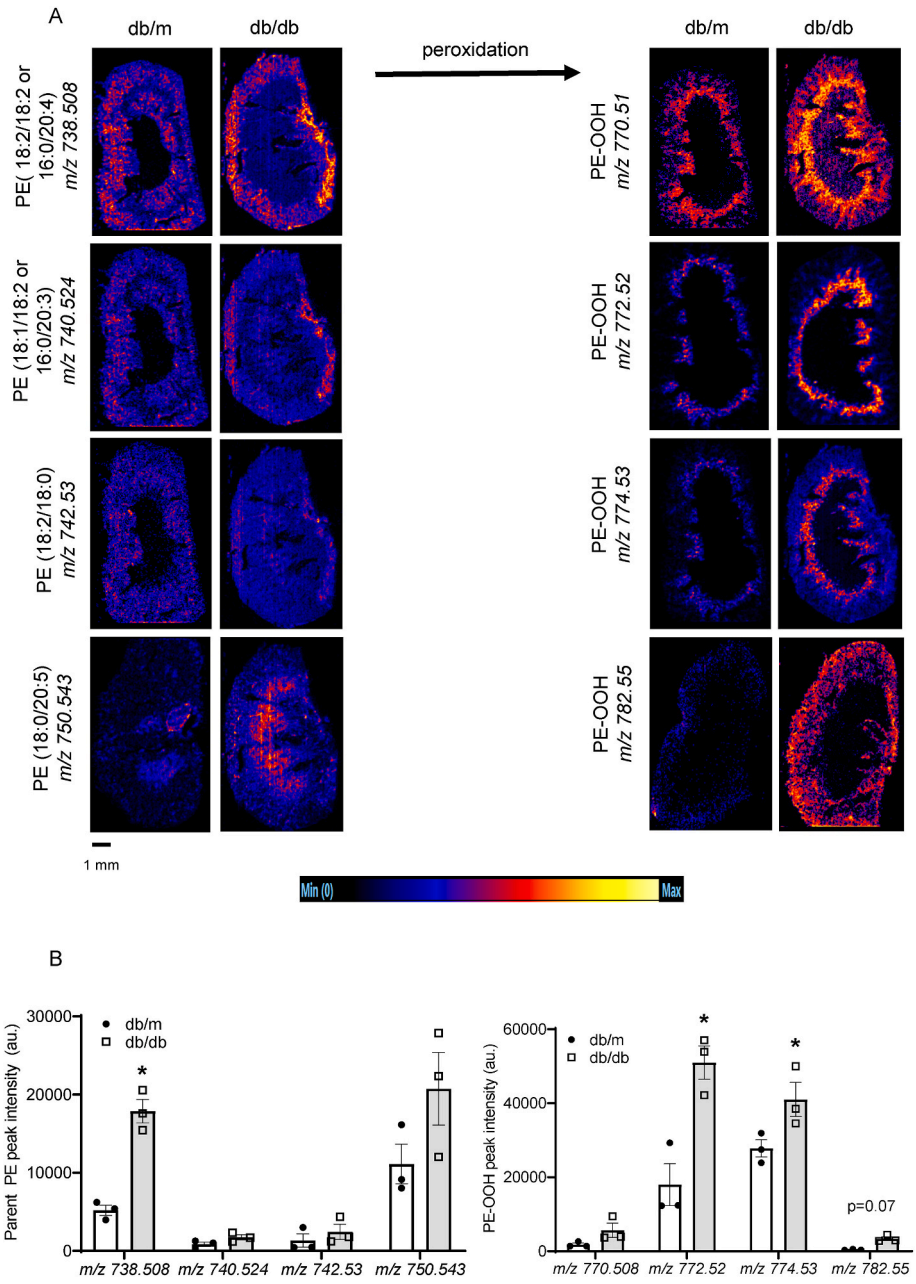

Fig. 3. Oxidized PE species and their parent PE lipids in diabetes. (A) Representative DESI-MS heatmap images of PE and corresponding peroxide species in healthy and diabetic kidneys. (B) Analysis of peak height intensities of each species. N = 3/group, bar graphs show peak intensity average (a.u.) of each oxidized species ±SEM, *P < 0.05.

Oxidized phospholipids unique to the diabetic kidney and their location. DESI/MS analysis revealed a wide array of parent phospholipid species in the kidney. A representative MS spectrum is shown in Fig. 2, with many of the detected phospholipids in the *m/z* 700–800 range. To assign acyl chain combinations for individual phospholipid species, the presence or absence of various carboxylated ions with *m/z* 255–300 were also verified or potential confounding carboxylated daughter ions excluded (Table 1). Expected primary peroxidized species (-OOH) were also identified and calculated from the *m/z* values of the corresponding parent phospholipids. From the first 1000 most intense peaks, phospholipids that underwent substantial oxidative modification were identified and mapped to anatomic locations in the diabetic kidney. Other, unique reactive phospholipid molecules (which were not peroxidized species) were also identified as detailed below.

Oxidized phosphatidylethanolamines (PE). Interestingly, PE species were the most impacted by oxidative modifications in the diabetic kidney. Fig. 3 shows the summary of our findings including the heatmap of each species at *m/z* 738.508, 740.524, 742.53, and 750.543 and their corresponding oxidized products at *m/z* 770.51, 772.52, 774.53, and 782.55. The majority of oxidized PE species were located in the inner cortex and outer medullar region, as judged from the heatmap images.

Oxidized phosphatidylserines (PS). Only two PS species of significance were detected in the diabetic kidneys (*m/z* 772.563 and 790.56) with their corresponding oxidized products (*m/z* 754.563 and 822.56). One of them showed a very marked medullar localization (Fig. 4).

Oxidized phosphatidylcholines (PC) We have found only one PC species (*m/z* 866.581) with a notable difference in the peroxidized PC levels (*m/z* 898.58) in diabetes (Fig. 5).

Other reactive lipids identified by DESI. Our analysis consistently found other phospholipid species elevated in the db/db kidneys at around the

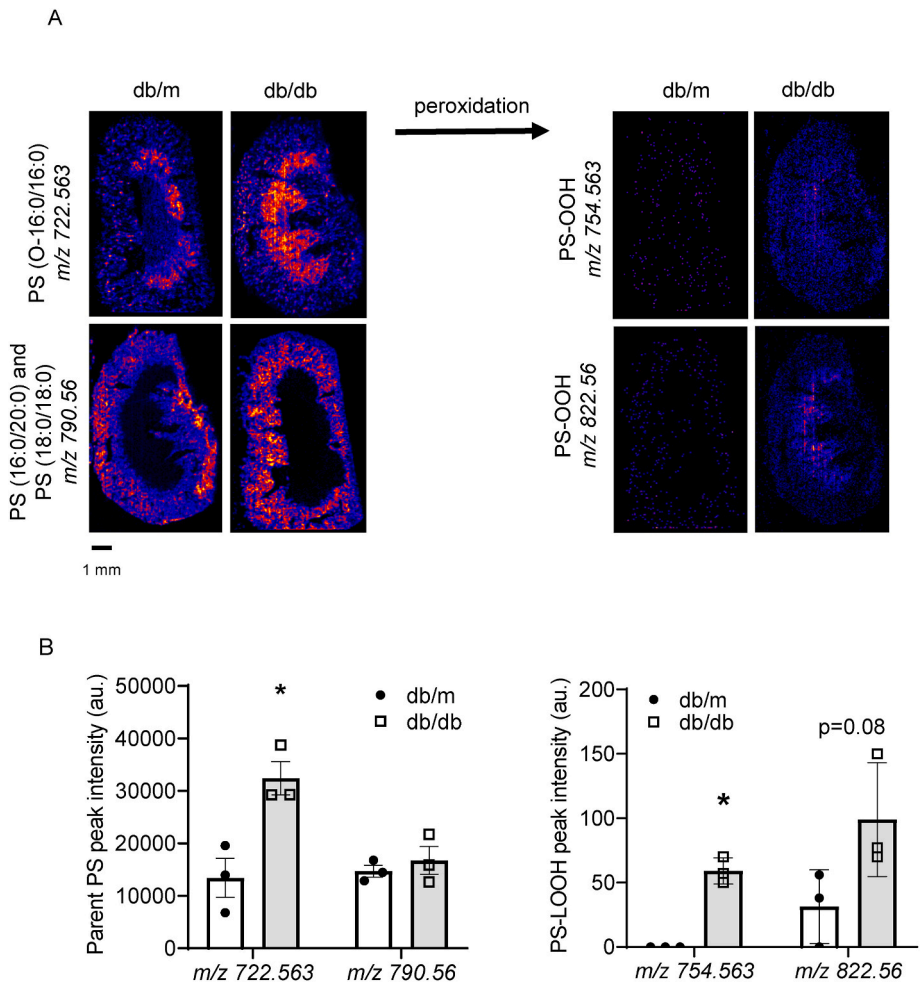

Fig. 4. Oxidized PS species and their parent PS lipids in diabetes. (A) Representative DESI-MS heatmap images of PS and corresponding peroxide species in healthy and diabetic kidneys. (B) Analysis of peak height intensities of each species. N = 3/group, bar graphs show peak intensity average (a.u.) of each oxidized species ±SEM, *P < 0.05.

m/z 450–575 range as well. These were identified as lysophosphatidylethanolamine (LPE, *m/z* 480.3) in the cortex and *N*-(tetradecanoyl)-deoxysphing-4-enine-1-sulfonate, which is a sphingolipid at *m/z* 572.3 in the outer medulla (Fig. 6). These are non-peroxidized reactive lipids, with known roles in regulation of cell function as it is further described in the Discussion section. It is also noteworthy, that we found some of the parent phospholipid species that were significantly or markedly increased in diabetic kidneys, but without any detectable oxidation products, such as: PE (18:0/20:4) at *m/z 766.54* with overwhelmingly present throughout the diabetic kidney, PC (18:0/18:0)/PC (16:0/20:0) at *m/z 788.54* in the cortex, and PE (20:4/22:6)/PS (18:4/20:4) at *m/z 810.581* in the outer medulla (Fig. 7), indicating a general change in phospholipid profile of db/db kidneys. To have a more complete view of various phospholipids in the kidney, we have also listed several other species in Table 2 that were detected but were not significantly different.

4. Discussion

While approaches like DESI-MS has been used previously to analyze normal and diabetic kidneys, for example alterations in lipid composition of PTCs or changes in glomerular and tubular lipid profiles [28,29], here we used the method to understand in deeper detail what oxidative stress means in a diabetic kidney and provide a novel "oxidative stress map" of diabetic kidneys – the first of its kind. We focused largely on phospholipid peroxidation for the reasons described above in the Introduction: the ability of lipid peroxides to propagate and travel larger distances in a complex organ and hit targets farther from their source of origin, thus being a form of a target-specific and more "chronic" oxidative stress in a chronic pathology like diabetes. Identifying these phospholipid peroxides will enrich knowledge in what phospholipids are most vulnerable and which regions of the kidney are impacted the most.

Our approach generated interesting and important findings beyond what is already known about oxidative stress in diabetic kidney disease:

1. Of the 1000 most intense peaks analyzed, oxygenated Pes, LPE and a sphingolipid were identified as either only present in the diabetic kidney or their levels significantly increased.
2. Some phospholipid species were not prone to oxidation or oxidized derivatives were not detected in diabetes.
3. Several oxidized species were localized at specific regions of the kidney.

Our analysis revealed PEs as the subclass with the most substantial oxidative modifications. We identified four different PE species in the diabetic kidney that were significantly oxidized (Fig. 3). The location and spread of these oxidized species was mostly in the inner cortex and outer medulla regions. As such, the presence of many of the oxidized PE species also correlated with areas of tubular immune-cell infiltration. We propose that these findings are significant for the following reasons. PE species – together with CL – make up about half of the phospholipids in the highly folded inner mitochondrial membrane in mammalian cells

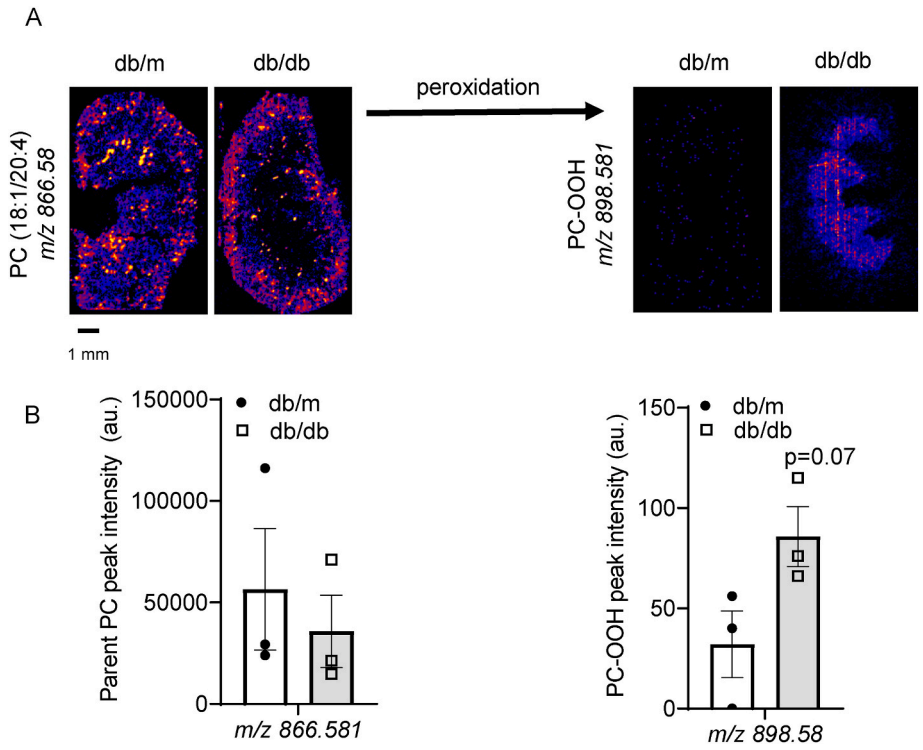

Fig. 5. Oxidized PC species and their parent PC lipids in diabetes. (A) Representative DESI-MS heatmap images of PC and corresponding peroxide species in healthy and diabetic kidneys. (B) Analysis of peak height intensities of each species. N = 3/group, bar graphs show peak intensity average (a.u.) of each oxidized species ± SEM, *P < 0.05.

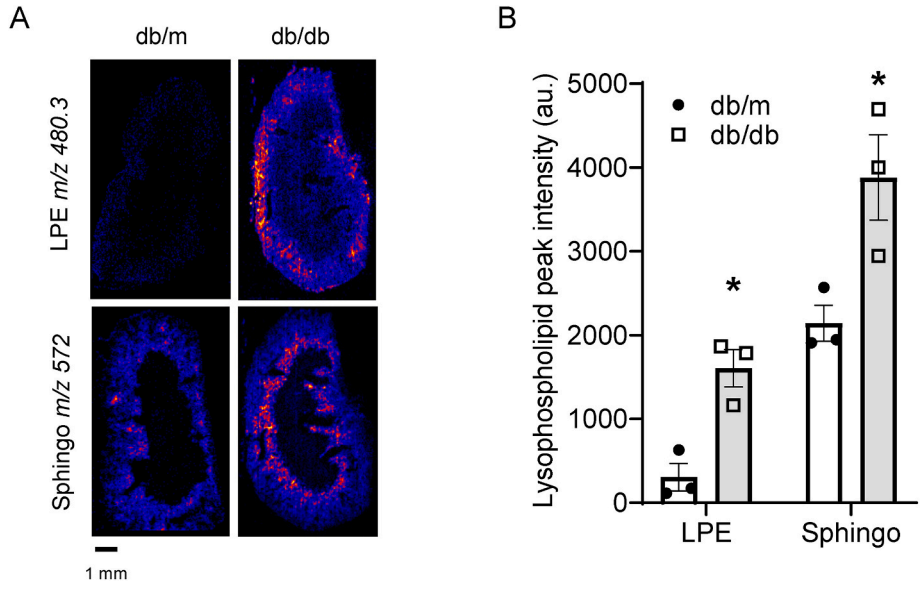

Fig. 6. Lysophospholipids and sphingolipids. Representative DESI-MS heatmap images of healthy and diabetic kidneys with lysophosphatidylethanolamine (LPE) and *N*-(tetradecanoyl)-deoxysphing-4-enine-1-sulfonate (Sphingo) location and levels. N = 3/group, bar graphs show peak intensity average (a.u.) of each oxidized species ±SEM, *P < 0.05, db/dbvs db/m group.

and they regulate the curvature of those membranes [30]. Their concentration is particularly high at the contact sites between inner and outer membranes [31–33]. Thus, oxidation of such PE species likely disrupts membrane curvature and fluidity in the mitochondria significantly, contributing to dysfunction of the organelle. The inner cortex-outer medullar region of the kidney is a PTC-rich region and these cells largely rely on mitochondria for their high energy need [34,35]. Any oxidative damage to mitochondrial membranes in these cells therefore can contribute to a decline in mitochondrial function in DN. In addition, it has recently been shown that 15-lipoxygenase can peroxidize PE species and these peroxidized PE serve as biomarkers of ferroptosis [36], a form of programmed cell death also relevant to kidney pathology [37]. Lastly, PE also has an important function in conjugating to LC3 protein, to ensure proper autophagosome formation and autophagic process [38]. Thus, oxidation of PE species in diabetes may affect autophagy as well. We also found an about 2-fold, significant increase in

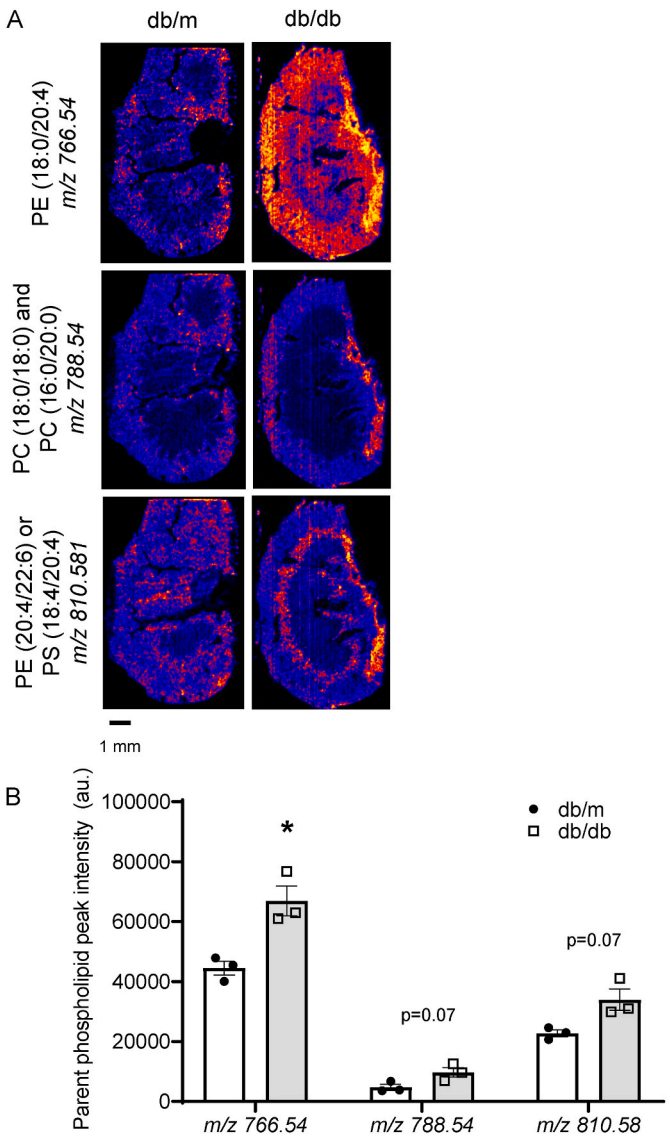

Fig. 7. Representative DESI-MS images of phospholipid species with significant difference in healthy vs diabetic kidneys but without significant oxidation products (not prone to oxidation). N = 3/group, bar graphs show peak intensity average (a.u.) of each oxidized species ±SEM, *P < 0.05, db/dbvs db/m group.

a parent PE species (PE (18:0/20:4) in diabetes. An explanation for such increase could be that as PE are important in membrane fluidity and curvature, increasing the levels may be an adaptation to the disease to protect remaining membrane intactness or a remodeling process in making new membranes. On the other hand, changes in PC/PE ratio can have an impact on cellular processes associated with health and disease. An increase in PE levels would decrease PC/PE molar ratio, and was shown to effect cell leakage, lipoprotein synthesis and VLDL secretion, impact mitochondrial respiration, oxidative capacity or contribute to mitochondrial fragmentation, and cause ER stress (reviewed in Ref. [39]). Many of these processes are known to have been linked to the development and pathogenesis of DKD [6,10,40–42].

Of the detected PS species, one (PS(O-16:0/16:0) was found to have increased in the diabetic medulla, with significantly more oxidation product detected as well. With regard to PS species, it is important to note that kidney injury molecule −1 (Kim-1) was shown to be a PS receptor, expressed on epithelial cells after injury [43]. Apoptotic cells expose membrane PS (and PE) species on the outer part of the plasma membrane. Thus, a possible explanation for our finding is that in the diabetic kidney, PS species contribute to the remodeling of injured epithelia through binding to Kim-1 during injury.

Table 2
List of other phospholipids detected by DESI-MS in healthy and diabetic kidneys with no significant difference nor significant oxidation products.

m/z {M-H]-	Tentative identity based on accurate mass	peak height db/m (au.)	peak height db/db (au.)	*P* value
742.53	PE (18:2/18:0)	1326.7 ± 843.7	2454 ± 964.6	*0.43*
747.568	PG (16:0/18:1)	22360.33 ± 4044	28,641 ± 2749	*0.28*
750.543	PE (O-18:0/20:5)	11115.7 ± 2535	20744.3 ± 4645	*0.143*
772.578	PE (20:0/18:1)	34081.33 ± 2397.7	45,964 ± 5608	*0.16*
774.593	PE (18:0/20:0) and PE (16:0/22:0)	27756.67 ± 2318	41,012 ± 4629	*0.09*
790.56	PS (16:0/20:0) and PS (18:0/18:0)	14711.7 ± 1140.7	16,762 ± 2650	*0.53*
834.581	PC(18:0/22:5)	8713.33 ± 941	10544.3 ± 3828.5	*0.68*
841.58	PI (P-16:0/20:4)	433.67 ± 411	1098.33 ± 957.5	*0.573*
850.596	PI (20:0/16:0)	172.33 ± 60.3	548.33 ± 192.55	*0.182*
865.5	PI (18:0/18:0) and PI (16:0/20:0)	80,770 ± 40,982	52,639 ± 23,976	*0.593*
909.603	PI (18:0/22:6)	35428.33 ± 1753.9	18203.33 ± 9628.7	*0.213*

Interestingly, we did not find any significant change or oxidation products of PI species in diabetic kidneys. One possible explanation for this is that PI species make up only a small fraction of cellular phospholipids [44]. Thus, with our settings, oxidized PI species may have been under the detection limit. Another important thing to consider is that while PI do not make up a large amount of phospholipids, they control several important aspects of cell life and death [44]. These include regulation of ion channels, pumps and transporters or exocytosis. It is then possible, that they are either not prone to oxidation or are protected by some mechanism in diabetes – at least in the db/db model and at time point in the disease we used herein - so that renal cells in the diabetic kidney can still maintain basic functions.

Furthermore, we also found two unique reactive lipids that were specific to the diabetic kidney: LPE and a sphingolipid species *N*-(tetradecanoyl)-deoxysphing-4-enine-1-sulfonate. Lysophospholipids (such as LPE) are a unique subclass of phospholipids in a sense that they are membrane-derived bioactive lipid mediators [45]. Lysophospholipids are generated from membrane phospholipids and sphingolipids by phospholipase-facilitated hydrolyzation. They elicit cellular responses including promoting cell survival, apoptosis, cell shape and motility, adhesion, migration, cytoskeletal arrangements or for example Ca^{2+} signaling [46], via G-protein coupled receptor-mediated pathways. Consequently, they can influence biological processes such as inflammation, healing, angiogenesis, immune processes, among many [47,48]. Many of these reactive lipids react with redox sensitive cysteine residues of small G-proteins like RhoA or Rac, thereby regulating cell function [49]. The increased levels of LPE in db/db kidneys –especially in the outer cortical region - suggest that under diabetic conditions, lysophospholipids may play a significant role in dysregulation of processes that rely on cytoskeletal dynamics, for example, podocyte foot process, cell motility or cell cycle. Indeed, lysophospholipids have also been suggested to have translational value to predict fast decliners with diabetic kidney disease [50]. Similar increases in LPE levels were found in an in vivo acute kidney injury model caused by ferroptosis where Gpx4 was deleted [37]. Pathological role for sphingolipids in diabetic kidney diseases has also been suggested before [51,52], thus it is not surprising that we found a specific species in diabetic kidneys of the db/db model. Similar to oxidized PE, the area where sphingolipids were identified

largely correlated with the area of immune cell infiltration areas.

In the current study we have not analyzed cardiolipin (CL) species or CL oxidation. It is important to recognize that CL plays a crucial role maintaining the stability of mitochondrial membrane and protecting several aspects of mitochondria health. Oxidation of CL is a well-established phenomenon with regards to mitochondrial dysfunction, including alterations related to diabetic kidney disease or acute kidney injury [53,54]. Peroxidation of CL has been established as essential for the release of pro-apoptotic factors from mitochondria into the cytosol [55]. Our limitation was simply technical: with the DESI-MS we used we did not observe high abundance of lipids over 1000 *m/z*. Thus, CLs (*m/z* = 1449.981, 1473.981, 1475.981) were either not detected in a reproducible manner with our current set up or were under the detection limit. Further studies should aim at identifying OOH-derivatives of CL species and the impact of such oxidation processes in the diabetic kidney.

A limitation of the DESI –based approach is that, while very informative about the presence of lip oxidative stress in various regions of the diabetic kidney, does not provide enough resolution to go to the single cell level to identify specific cell types. It would be also difficult to distinguish for example on a heatmap with the highest intensity in the cortical region, whether the cells are mostly of tubular or glomerular origin. Histology images from the same section however could be used to first identify glomeruli, and then superimposing this image with the heatmap of DESI could further enhance and refine analysis. Regardless, we suggest that our results will open new avenues to further research in a cell-specific manner to understand which cell types of the diverse kidney cell landscape are major sites or targets of oxidative stress. For example, the immuno-spin trapping approach (developed by Mason et al. [56,57] and successfully applied in vivo by our laboratory [58] and others [59–61]) can be used to capture and identify protein targets in diabetic renal cells where electrophile lipid peroxides react with cysteine residues of functionally relevant proteins [21]. Here, a specific spin trap, *5,5-dimethyl-1-pyrroline-N-oxide* (DMPO) is used to label and identify those residues that were modified by oxidative attack. DMPO is injected into mice. In vivo, it traps protein-centered radicals (oxidation happened on protein residues as a result of redox signaling). DMPO is specific to these redox reactions and would not label other residues. Kidney cells from regions identified on the DESI heatmap then can be separated through sieving or other established methods and the DMPO-labeled protein adducts analyzed by MS/MS. To identify a group of plausible targets with functional significance, we propose two approaches: cross-referencing targets detected in the MS/MS analysis with a) literature data of proteins with functional importance to T2DM, or b) transcriptomic analysis of genes corresponding to functionally relevant proteins in T2DM models. Such approach can enhance our understanding of redox regulation in renal cells. On one hand it can reveal specific, redox sensitive, functionally important and thus potentially druggable targets, on the other hand it can identify protective cell signaling pathways modulated by reactive lipids.

5. Conclusions

To summarize, here we provide a new, comprehensive mass spectrometry imaging analysis of oxilipidomics in the diabetic kidney. Our approach is a first step to go beyond the umbrella term of “ROS” and provide a more specific understanding of the identity and distribution of oxidized species, specifically lipid peroxides, as well as the regions that are more prone to oxidative stress and consequently, to cell or mitochondria phospholipid membrane injury in the kidney during diabetes.

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Data sharing statement

All data are included in the manuscript.

Declaration of competing interest

A. McCrimmon, S. Corbin and K. Stadler have no financial conflict of interest to disclose. B. Shresta, G. Roman and S. Dhungana were all employed at Waters Corporation when the manuscript was written.

Data availability

Data will be made available on request.

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