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Mass Spectrometric Imaging Reveals Temporal and Spatial Dynamics of Bioactive Lipids in Arteries Undergoing Restenosis

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Abstract

Restenosis, or renarrowing of the arterial lumen, is a common recurrent disease following balloon angioplasty and stenting treatments for cardiovascular disease. A major technical barrier for deciphering restenotic mechanisms is the dynamic, spatial profiling of bioactive lipids in the arterial wall, especially in small animals. Here, applying matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI), we conducted the first lipidomic study of temporal-spatial profiling in a small animal model of angioplasty-induced restenosis. Cross sections were collected 3, 7, and 14 days after balloon angioplasty of rat carotid arteries. MALDI-MSI analyses showed that diacylglycerols (DAGs), signaling lipids associated with restenosis, and lysophosphatidylcholines (LysoPCs), whose function was uncharacterized in restenosis, dramatically increased at postangioplasty day 7 and day 14 in the neointimal layer of balloon-injured arteries compared to uninjured controls. In contrast, sphingomyelins (SMs) did not increase, but rather decreased at day 3, day 7, and day 14 in injured arteries versus the uninjured control arteries. These results revealed previously unexplored distinct temporal-spatial lipid

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ASSOCIATED CONTENT

Supporting Information

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Supporting figures and tables (PDF)

Notes

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MALDI MSI data is publicly available through METASPACE (<https://metaspace2020.eu/>). Project: Restenosis, Uploader: Jillian Johnson.

dynamics in the restenotic arterial wall. Additionally, we employed time-of-flight secondary ion mass spectrometry (TOF-SIMS) tandem MS imaging for both molecular identification and imaging at high spatial resolution. These imaging modalities provide powerful tools for unraveling novel mechanisms of restenosis involving lipids or small signaling molecules.

Graphical Abstract

Keywords

MALDI-MSI; TOF-SIMS; vascular disease; restenosis; neointima; lipids

INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide.¹ The most common treatment to resume circulation is balloon angioplasty, performed in over a million patients each year. Although acutely effective, this intervention can injure the artery leading to the development of neointimal lesion in the inner vessel wall that renarrows the lumen—a recurrent disease known as restenosis. Even with stent implantation, rates of restenosis remain at ~12–30%.^{2,3} It is therefore imperative to better understand mechanisms of restenosis for achieving more effective treatments.

The artery is composed of three distinct layers: adventitia, media, and the inner lining of endothelium, which would be removed due to angioplasty followed by the development of neointima lesion. Three major cell types, fibroblasts, smooth muscle cells (SMCs), and endothelial cells reside in these three layers, respectively. The networks formed by these cells and their signaling molecules are crucial to the pathogenesis and progression of restenosis. While a wealth of knowledge is available on many pathways involved in restenosis,^{4–6} spatiotemporal dynamics of signaling molecules and their networks remain poorly understood due to technological limitations. Particularly challenging is the spatial profiling of small molecules including bioactive lipids in the vessel wall of small animal models, the thickness of which is typically in the submillimeter range.

Bioactive lipids are important signaling molecules involved in a variety of biological processes.^{7,8} A number of studies have implicated abnormal synthesis and distribution of these lipids associated with a wide range of diseases, particularly in the cardiovascular system.^{9,10} However, the majority of these studies have relied on lipid detection in whole

tissue homogenates or body fluid. Critical information concerning lipid spatial dynamics is missing. Aside from lability, chemical diversity and complexity, and generally low abundance of bioactive lipids in the arterial wall, the small arteries in experimental animals (e.g., ~1 mm diameter in rats) impose an additional technical challenge. The emerging technology of mass spectrometric imaging (MSI) brings about a unique opportunity to overcome these long-standing hurdles in spatially profiling lipids in the arterial wall. MSI spatial profiling of lipids has been reported, mostly in lipid-rich organs such as brain, kidney, lung, and some types of tumors,^{11–17} and also in arteries of human patients, but not in arteries from small animals.

Here, we describe the first MSI-based in situ lipidomic imaging in a rat model mimicking angioplasty-elicited clinical restenosis. This model features well-characterized stages of disease progression and corresponding dynamic signaling events with a variety of bioactive lipids involved. Our data reveals distinct temporal-spatial profiles of different bioactive lipid species during the development of restenosis, including those not previously linked to restenosis.

EXPERIMENTAL SECTION

Chemicals and Reagents

Methanol and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA). Difco gelatin was purchased from Becton, Dickinson and Company (Sparks, MD). 2,5-Dihydroxybenzoic acid (DHB) was from Acros Organics (Morris Plains, NJ). Microscope glass slides were from VWR international, LLC (Radnor, PA). Reagents used for Verhoeff–Van Gieson staining were purchased from Sigma-Aldrich (St. Louis, MO). Distilled water was Milli-Q water from a Millipore filtration system (Bedford, MA). All reagents were used without additional purification.

Animal Experiments

Animal experiments were conducted following institutional guidelines (University of Wisconsin-Madison IACUC). Balloon angioplasty for carotid arteries was performed in male Sprague–Dawley rats (350–400 g) as previously described.¹⁸ Briefly, rats were anesthetized with isoflurane followed by a longitudinal incision in the neck to expose carotid arteries. A 2-F balloon catheter (Edwards Lifesciences, Irvine, CA) was inserted through an arteriotomy on the left external carotid artery to the common carotid artery. To produce arterial injury, the balloon was inflated at a pressure of 2 atm and withdrawn from proximal common carotid artery to the carotid bifurcation. This action was repeated three times. The external carotid artery was then permanently ligated, and blood flow was resumed. A total of 9 rats were used for these experiments. Three injured and three control arteries were collected for each condition: day 3, day 7, and day 14 post balloon angioplasty.

Tissue Collection and Sectioning

For each rat, the balloon angioplasty-treated left common carotid artery was collected while the contralateral common carotid artery without angioplasty was harvested as control. Collected carotid arteries were embedded in gelatin (100 mg/ mL in warm Milli-Q water)

and snap-frozen on dry ice immediately after dissection. The frozen tissues were stored at -80°C until cryosectioning into $12\text{ }\mu\text{m}$ thick slices on a cryostat (Thermo Scientific Microm HM 525, Waltham, MA). A total of 18 tissue slices (3 from each condition) were mounted onto two microscope glass slides ($75 \times 25 \times 1\text{ mm}$). The glass slides were then dried in a desiccator for 30 min at room temperature to avoid condensation of water vapor.

Matrix Application

A robotic sprayer system (TM sprayer, HTX Technologies, Carrboro, NC) was used to homogeneously apply matrix on two tissue slides: 24 passes of DHB matrix (40 mg/mL in 0.1% FA/methanol/water (50:50, v:v)) was applied with a flow rate of 0.05 mL/min, nozzle temperature of 80°C , and a moving velocity of 800 mm/min. The slides were dried in a desiccator at room temperature following matrix application and stored at -80°C until analysis.

MALDI MS Imaging

All MALDI-MSI experiments were performed on a MALDITQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a 60 Hz 337 nm N_2 laser. Full MS experiment was performed in positive-ion mode with m/z range of 450–1000, mass resolution of 30 000 (at m/z 400), using a laser energy of 20 μJ and 2 microscans/step. A spatial resolution of $75\text{ }\mu\text{m}$ was used for all acquisitions. The precursor ions of target lipids in the full MS scan were isolated and subsequently fragmented by higher-energy collisional dissociation (HCD) with the normalized collisional energy of 25. Instrumental methods were set up in Xcalibur software (Thermo Scientific, Bremen, Germany) and the imaging position file was defined with TunePlus software (Thermo Scientific, Bremen, Germany).

Histology

After MALDI-MSI acquisition, artery sections mounted onto glass slides were fixed in 4% paraformaldehyde solution at 4°C overnight. Slides were then air-dried in a chemical hood and rinsed in a series of ethanol solutions (100%, 95%, and 75%). Gelatin was removed by incubating the slides at 37°C for 45 min. Verhoeff–Van Gieson (VVG) staining was performed as we previously described.¹⁹ Briefly, processed sections were stained in a working solution containing alcoholic hematoxylin, ferric chloride, and Verhoeff's iodine solution for 15 min, followed by rinsing in running water for 5 min. Slides were differentiated in fresh ferric chloride solution before mounted with a coverslip. Images were acquired on a Nikon Eclipse TE2000-inverted microscope (Minato, Tokyo, Japan) at 4 $\times$ magnification.

Data Processing and Statistical Analysis

MS data was processed using Xcalibur (Thermo Scientific, Bremen, Germany) and ImageQuest (Thermo Scientific, Bremen, Germany). MS images were analyzed in MSiReader (North Carolina State University, Raleigh, NC) following exporting data into imzML in ImageQuest. Images were normalized to total ion current (TIC) and the mass tolerance window for image construction is 5 ppm. MS data as an imzML format was then

loaded into SCiLS software 2018 (Bruker Daltonics, Bremen, Germany) for statistical analysis. Using SCiLS, discriminative analysis was used to calculate Receiver Operator Characteristic (ROC) curves and Area Under the Curve (AUC) values. Each AUC value represents the ability of a single m/z value to act as binary classifier for the injured versus control arteries. Additionally, hypothesis testing using t tests was used to calculate the p -value for the differences in the mean spectra between the control and injured arteries. SCiLS software was also used for spatial analysis by co-registration of the VVG stained histology to the TIC of the artery, annotating regions of interest based on the histology, and then performing hypothesis testing on the 3 layers of the injured arteries: the adventitia, media, and neointima based on the histological staining overlay. METLIN metabolite database (Scripps Center for Metabolomics, La Jolla, CA) was used for accurate mass matching and lipid assignment with 5 ppm tolerance.

TOF-SIMS Tandem MS Imaging for MS/MS Identification of Lipids

The analyses were performed using a PHI nanoTOF II TOFSIMS Parallel Imaging MS/MS instrument (Physical Electronics, Minnesota, USA). A detailed description of this TOF-TOF instrument has been reported previously.^{20–22} In the present study, all the spectra and images were recorded with electrostatically bunched pulses of a 30 keV Bi_3^+ primary ion beam of which the DC current was ≈ 16 nA. The ion beam was operated in the high resolution-squared (HR^2) mode to achieve $1\ \mu\text{m}$ lateral resolution at high mass resolving power. The field-of-view of each analytical area was $500\ \mu\text{m} \times 500\ \mu\text{m}$ divided by 256×256 image pixels; to image the entire tissue cross section a stage raster motion was used with software stitching of 4×4 imaging tiles to view a total image area of $2.0\ \text{mm} \times 2.0\ \text{mm}$ encompassing the tissue section. TOF-SIMS data were acquired over a m/z range of 0–2000 in positive ion mode, and the ion fluence for each acquisition was at least an order of magnitude below the static limit of analysis. For the TOF-SIMS analysis, the m/z 0–2000 range was used because that was the duty cycle (approximately 121 μs) required for stable charge neutralization and which also encompasses the mass range of the targeted lipids. During the analyses, low energy electrons (15 eV) and low energy Ar^+ ions (10 eV) were applied for charge compensation. The lateral resolution (Δ) was measured using 80/20 bounds to be $\approx 1\ \mu\text{m}$; however, the pixel size ($500\ \mu\text{m}/256$ pixels) was $1.95\ \mu\text{m}$. Data acquisition was achieved using PHI SmartSoft-TOF software and data processing was performed using PHI TOF-DR (Physical Electronics, MN) software.

RESULTS AND DISCUSSION

MALDI imaging MS was applied to investigate the spatiotemporal changes of lipids during the progress of restenosis (Figure 1A). We used the balloon angioplasty technique to model restenosis. Typically, a balloon catheter is inserted into the rat carotid artery and then inflated and pulled. This action inflicts injury by overstretching the artery wall while damaging the endothelium. Disruption of this endothelial barrier between blood and the vessel wall exposes SMCs in the medial layer to various stimulants in circulation such as cytokines and growth factors that trigger a myriad of signaling events. Consequently, SMCs become migratory and proliferative forming a new layer of tissue called neointima, which occupies and narrows the lumen space (so-called restenosis). To illustrate the distinct layers

of the arterial wall structure and neointima formation, diagrams in Figure 1B show the different layers of the artery present in the 3 different injured conditions and in the uninjured condition. In contrast to the uninjured control and injured day 3 arteries, the injured artery at day 7 and day 14 postangioplasty has an additional thick neointima layer.

This widely used balloon angioplasty model recapitulates distinct stages of postangioplasty development of restenosis and associated dynamic signaling events. Samples including injured and uninjured (sham control) carotid arteries were collected at three postangioplasty time points that are well-defined in the literature.²³ The day 3 time point features acute activation of proliferation, as well as apoptosis of SMCs in the medial layer, with neointima not yet formed. The day 7 time point represents a highly active signaling stage marked by diminished cell death and a dramatic increase of proliferative and migratory SMCs that form a prominent neointima layer. At the day 14 time point, cellular activities decline, while the flow-obstructing neointimal lesion is fully developed. Here, we will discuss the trends of three main lipid groups that previously little was known about in restenosis: diacylglycerols (DAGs), lysophosphatidylcholine (LysoPC), and sphingomyelins (SMs) (Table 1).

Our study is the first to directly identify the dynamic changes of DAG, LysoPC, and SM species during the pathogenesis of restenosis. The previous knowledge on their contribution to cardiovascular diseases was largely limited to either in vitro cell culture studies or measurements in atherosclerotic plaques.^{24,25} For example: (1) altered LysoPC production led to dysfunction of cultured SMCs;²⁶ (2) deletion of a phospholipase (iPLA2 β , responsible for synthesis of arachidonic acid and LysoPC) ameliorated restenosis in a transgenic mouse model;²⁷ (3) while DAG is a known activator of certain Protein Kinase C (PKC) family members, aberrant Protein Kinase C activation was closely linked to SMC pathology and restenosis;²⁸ (4) SM is correlated with SMC apoptosis, and its level was elevated in whole-tissue homogenates of atherosclerotic plaques.²⁹ Taken together, these studies indirectly linked the three lipid groups to restenosis.

MALDI-MSI Indicates Up-Regulation of Diacylglycerols in the Injured Arterial Wall

We first analyzed the temporal dynamics of DAGs for a possible relevance to restenosis. To sort out the lipids that showed prominent temporal dynamics with a close relevance to restenosis, we analyzed diacylglycerols (DAGs), which in the literature have been linked to pathogenic pathways in restenosis.³⁰ The data shows that at postangioplasty day 3, there was a statistically significant increase of DAGs in the injured artery compared to the uninjured control. The signal was localized in the middle of the artery wall, correlating with the medial layer; the neointima was not yet formed at this time point. At day 7, coincident with fast growth of neointima in the injured artery, all measured DAG lipid species showed a statistically significant increase with p -values <0.0001 . Additionally, most DAG lipids had AUC values >0.75 at day 7, indicating that the changes were great enough for discriminating these DAG species between injured and uninjured tissues. DAG signal in the injured artery was substantially attenuated at day 14, while remaining upregulated compared to the day-14 uninjured control. Figure 2B shows the relative intensity of DAG (36:1), m/z 627.5381, a representative DAG species illustrating the dynamic trends of this lipid class. Briefly, each figure shows injured carotid arteries compared side-by-side with their respective

contralateral uninjured arteries from same animal ($n = 3$, data shown in Figure S1). At day 3, day 7, and day 14, m/z 627.5381 is significantly upregulated compared to uninjured control, with the increase peaking at day 7. Statistics in Figure S1 confirm that this trend is conserved for most DAG lipid species identified. To reveal the temporal changes of different DAG lipid classes, the day 3, day 7, and day 14 injured tissues were statistically compared. DAG (36:2), m/z 625.5219 is shown in Figure 3A as an example of this lipid class. From day 3 to day 7, DAG (36:2) signal increased, and then declined at day 14 but remained above the day 3 level. Statistics is provided for all DAG lipid species (Table S1), demonstrating a temporal regulation in injured arteries.

DAGs are one of the most widely studied secondary signaling messengers. They are indispensable for maintaining the normal physiology of various organs and tissues, mainly through activating multiple classes of protein kinase C (PKC).^{30,31} Their aberrant abundance and/or distribution are associated with pathogenic conditions.³² The activation of various isoforms of PKC, in particular, PKC delta and PKC beta, has been reported to contribute to the development of restenosis.^{33–35} However, how DAGs change during this process has not been clearly revealed.^{36,37} Herein, for the first time, our MALDI-MSI study characterized restenosis-associated temporal-spatial DAG profiles that closely correlate to previously reported dysregulated PKC activities. As such, these data also serve as a positive control validating our MALDI-MSI methodology for profiling the changes of other lipids correlated with various stages of restenosis.

MALDI-MSI Indicates Up-Regulation of Lysophosphatidcholines in the Injured Arterial Wall

We next analyzed other prominently regulated lipids, including LysoPCs. Multiple LysoPCs were found to be greatly accumulated following arterial injury, predominantly in the neointima/medial area where SMCs undergo a phenotypic switching that is primarily responsible for the generation of the neointimal lesion. Figure 2C shows the relative intensity of LysoPC (18:1), m/z 522.3578, as an example of this lipid class. At day 3, day 7, and day 14, m/z 522.3578 was significantly upregulated compared to the control, with the greatest increase in intensity occurring at day 7. Statistics in Figure S2 confirm that this trend is conserved for other LysoPC lipids. Therefore, the general pattern of changes in LysoPCs is very similar to that of DAGs. Compared to uninjured control, most of the LysoPC species significantly increased in injured arteries at all 3 time points. The ROC curves indicate that the highest AUC values are present at day 7, likely due to the dramatic injury-induced LysoPC lipid upregulation in the neointimal lesions at this time point. To understand the temporal dynamic regulation of the LysoPC lipid classes, day 3, day 7, and day 14 injured tissues were statistically compared. As an example, the LysoPC (16:1), m/z 494.3265 signal (Figure 3B) increased from day 3 to day 7, and then slightly decreased at day 14 compared to day 7, likely due to a resolution of the injury-related processes. To further confirm the identification of lipids, representative on-tissue fragmentation of a LysoPC lipid ion is provided in the supplemental section (Figure S5), demonstrating the presence of m/z 184.0727 as the diagnostic ion of the phospholipid headgroup of LysoPC.

LysoPCs are a group of highly active phospholipids that are involved in a wide range of physiological and pathological events.³⁸ Recently, the functions of LysoPCs in cardiovascular disease have been extensively studied,^{39,40} and a positive correlation between increased circulating LysoPCs and risks of coronary diseases has been revealed.^{41,42} However, while in vitro studies showed that SMC pro-restenotic phenotypes were stimulated by LysoPCs, the responses of different vascular cell types were highly variable,^{43,44} and the restenosis-related dynamics of LysoPCs in the arterial wall has not been reported. Our study provides the first temporal dynamic profiling of LysoPCs closely correlated to the disease context of restenosis, thus contributing novel information for further mechanistic studies.

MALDI MSI Indicates Down-Regulation of Sphingomyelins in the Injured Arterial Wall

Aside from upregulated lipids, MALDI-MSI also identified several down-regulated lipids, such as sphingomyelins (SMs) during the development of restenosis. Figure 2D shows the relative intensity boxplots of SM (38:1), m/z 781.6229, as an example, to demonstrate the trends of intensity changes of SM species. Compared to uninjured control, SM (38:1) is significantly down-regulated in injured arteries at day 3, day 7, and day 14. Statistics in Figure S3 confirm that this trend is conserved for most of the SM species identified. As we have seen with the DAGs and LysoPCs, SMs also exhibit the most dramatic changes at day 7 post injury, as indicated by the highest AUC values at day 7. To illustrate the temporal changes of SM lipids, we use SM (40:1), m/z 787.6760, as an example in Figure 3C. SM (40:1) is significantly upregulated at day 14, compared with day 3 and day 7. Additional dynamic statistics are available for all SM species in Table S1, which confirm that SM at day 14 is significantly upregulated compared to day 3 and day 7. This dynamic profile of SM is distinct from those of DAGs and LysoPCs, further confirming the specificity and effectiveness of our MALDI-MSI methodology.

As the most abundant sphingolipids stored in plasma membranes, sphingomyelins can be converted into ceramides, which ultimately lead to the synthesis of sphingosine and sphingosine-1-phosphate (S1P). S1P is a well-established mitogenic stimulus implicated in tumorigenesis and metastasis. Based on studies with genetic deletion of S1P receptors,^{45,46} it has been postulated that S1P could accumulate in injured arteries and contribute to the signaling events that lead to restenosis. Consistently, the observed postinjury decrease of SMs, the primary substrates for the synthesis of S1P, might account for an increase of S1P. While our study provides an interesting correlation with literature evidence, further studies are needed to define the functional roles of SMs in restenosis.

Histology Reveals Distinct Stages of Restenotic Development in Injured Rat Carotid Arteries

Following MALDI MSI analysis, artery cross sections were subjected to VVG staining (Figure 2A). VVG stains collagen as red and elastin as black, which can be used to distinguish the adventitial layer (shown in red collagenous regions) and medial layer (shown in black elastin region). While neointima was not seen on day 3 injured artery sections, it developed into a layer of significant thickness at day 7 and progressed to the maximum thickness at day 14 in injured tissues (denoted with the presence of a blue arrow in Figure 2A). This neointimal growth at day 7 and day 14 exhibited in the histology is consistent with

previous reports,^{19,47} thus validating the accuracy of the angioplasty model used. Furthermore, since the same artery section was used first for MALDI-MSI and then VVG staining, results from these two can be readily coregistered and correlated (Figure 4) on the stained section, providing spatial information and relative lipid abundance within the distinct layers of the artery.

MALDI-MSI Is Combined with Histology to Reveal Neointima Specific Dynamic Lipid Changes

As indicated by histology, the neointima was not seen in injured day 3 tissues but developed into a visibly thick inner layer of the artery in injured tissues collected at day 7 and day 14. We thus compared lipid signal intensities specifically in the neointima layer to provide new information in more detail. Statistical analysis of annotated neointima lesions in injured arteries of day 7 and day 14 indicates that the abundance of DAGs and LysoPCs was higher at day 7 compared with day 14 (Figure 5). As shown in Figure 5A,B, example DAG (34:2), m/z 597.4889, and example LysoPC (18:2), m/z 502.3420 both exhibit the same trend of decreasing from day 7 to day 14. In contrast, example SM (40:1), m/z 809.6544, shown in Figure 5C, is higher at day 14 compared with day 7. Further statistical analysis of the intensity of each layer of the carotid artery (the neointima, the media, and the adventitia) are provided in Table S3 for injured day 7 arteries and Table S4 for injured day 14 arteries. Statistics provided in Table S3 confirm that SMs were overall more abundant in the day 14 neointima compared with the day 7 neointima. Additional spatial statistics comparing the adventitia, media, and neointima regions are provided in the supplemental sections for day 7 and day 14 injured arteries (Table S3 and Table S4, respectively). The data indicates that the neointimal levels of DAGs (day 7), LysoPCs (day 7 and day 14), and SMs (day 14) are statistically different from both the adventitia and the media. It should be noted that there is technical limitation in spatially resolving all three layers: the adventitia, media and neointima, given the small size of a rat artery (~1 mm) and the minimum spatial resolution of 75 μm on the MALDI-Orbitrap LTQ XL instrument without oversampling from neighboring pixels. Moreover, many of the lipids investigated here are of relatively low abundance in the tissue, potentially compromising detectability from increasing spatial resolution with smaller raster steps. Further MSI analysis with improved spatial resolution using TOF-SIMS was necessary to fully resolve all three layers with minimal overlapping between neighboring pixels that are shared by the outlined regions of interest. Nevertheless, we are able to use MALDI-MSI to visualize statistically significant temporal level changes of DAGs, LysoPCs, and SMs in the neointima, from postinjury day 7 to day 14, time points when substantial neointima has developed.

TOF-SIMS Tandem MS Imaging for Identification and High-Resolution Imaging

TOF-SIMS tandem MS imaging was employed to observe the neointima lesions developed in day 7 injured tissue sections and to obtain MS/MS fragmentation for some low abundance lipid species. In Figure 6A we have produced an ion image of a 2.0 mm $\times$ 2.0 mm large-area mosaic tile map encompassing an entire tissue section; Figure 6B,C represents successive enlargement of the indicated regions of 1.0 mm $\times$ 1.0 mm and 500 μm $\times$ 500 μm areas, respectively.

The set of images was collected using an ion fluence of $<1.50 \times 10^{11} \text{ Bi}_3^+/\text{cm}^2$, which is 2 orders of magnitude below the static limit, i.e., the threshold for molecular damage. In each ion image the lateral resolution was between 1.01 and 1.85 μm . At this high spatial resolution, we are able to clearly observe the collagen (adventitia), elastin (media), and neointima layers in the cross-section of an injured artery showing the distribution of $\text{C}_3\text{H}_8\text{N}^+$ ion. High resolution MS imaging enabled us to fully resolve the different regions of the artery. Additionally, the identification of specific DAG and SM moieties was also obtained from the TOF-SIMS tandem MS imaging data (Figures S6 and S7) to confirm tentative lipid identifications.

CONCLUSIONS

In this study, we performed the first MALDI-based lipidomic imaging on cross sections of artery from a small animal model of restenosis, which revealed distinct temporal-spatial dynamics of lipids that are functionally related to restenosis. This imaging methodology exhibited unique strength in mapping dynamic lipid distribution in different layers of the arterial wall. Moreover, since unfixed cryosections were used, in situ display of various lipids could be captured. We were subsequently able to visualize at high resolving power the neointima lesions of the injured artery sections with the complementary MSI technique of TOF-SIMS, and to confirm the identification of DAGs and of SMs, making use of the tandem MS molecular identification. The combined information on the temporal and spatial dynamics of the lipidome is important for discovering potential novel mechanisms underlying restenosis. With the resolution and detection sensitivity continuously improving, the MALDI-MSI and TOF-SIMS tandem MS imaging technologies are becoming powerful tools in vascular studies that will ultimately contribute to the development of next-generation therapeutic methods for vascular diseases and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Workflow for testing spatiotemporal changes of lipids in the development of neointima using a rat model of restenosis. (B) Diagram of the different layers of the artery in uninjured, injured day 3, injured day 7, and injured day 14. The layers are labeled as A: adventitia; M: media; E: endothelial layer; N: neointima layer.

Figure 2.

(A) VVG stains elastin rich regions black and collagen rich regions red in histological analysis to distinguish the media (middle) and adventitia (outer) regions. Neointima (inner) regions are shown with a blue arrow and are seen on day 7 and day 14 in injured arteries following balloon angioplasty. (B) MALDI images of a representative DAG species, m/z 627.5381, demonstrate an increase in DAG (36:1) intensity on day 3, day 7 and day 14, compared with corresponding control arteries of one representative rat. (C) MALDI images of a representative LysoPC, m/z 522.3578, demonstrate an increase in LysoPC (18:1) on day 3, day 7, and day 14, compared with corresponding control arteries. (D) MALDI images of a representative SM species, m/z 781.6229, demonstrate a decrease in SM (38:1) intensity on day 3, day 7, and day 14, compared with corresponding control arteries.

Figure 3.

Temporal dynamics of three representative lipid species in injured arteries. Relative intensity box and whisker plots and MALDI images of (A) m/z 625.5219, DAG (36:2), in injured tissue (B) m/z 494.3265, LysoPC (16:1), in injured tissue (C) m/z 787.6760, SM (40:1), in injured tissue on day 3, day 7, and day 14 for 3 biological replicates to illustrate postinjury temporal dynamics. Scale bar = 1 mm.

Figure 4.

Histology overlay with mass spectrometry imaging reveals neointimal accumulation of diacylglycerol lipids. MALDI images and histology were coregistered for spatial analysis, as shown with m/z 597.489, DAG (34:2), overlaid with the VVG histology. This lipid is enriched in the neointima regions compared with the media and adventitia regions in day-7 and day-14 arteries. Scale bar = 0.5 mm. A = adventitia, M = media, N = neointima.

Figure 5.

Comparison of neointimal abundance of lipids between postinjury day 7 and day 14 artery sections. Relative intensity box and whisker plots and MALDI images of (A) m/z 597.4889, DAG (34:2) (B) m/z 502.3420, LysoPC (18:2) and (C) m/z 809.6544, SM (40:1), shown in the day 7 and day 14 neointimal lesions defined through histology overlays. More statistical information about spatial distribution of each lipid species in the three layers of the artery can be found in the Supporting Information (Table S2 for injured day 7 tissue and Table S3 for injured day 14 tissue). Scale bar = 1 mm.

Figure 6. High resolution MS imaging. High resolution MS images showing the distribution of a nonspecific ion of biological origin, $C_3H_8N^+$ (choline headgroup) in the day 7 injured artery cross section. The image areas are (A) $2.0\text{ mm} \times 2.0\text{ mm}$, (B) $1.0\text{ mm} \times 1.0\text{ mm}$, and (C) $500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$, wherein the layered structure including the neointima lesion is clearly observed.

Table 1.**Identified Lipids Involved in the Development of Restenosis**

(A) List of diacylglycerols (DAGs) involved in the development of restenosis			
m/z	ions	tentative identification	ppm
575.5052/597.4889	[M + H – H ₂ O] ⁺ /[M + Na – H ₂ O] ⁺	DAG (34:2)	2
577.5225/599.5063	[M + H – H ₂ O] ⁺ /[M + Na – H ₂ O] ⁺	DAG (34:1)	5
601.5208/623.5066	[M + H – H ₂ O] ⁺ /[M + Na – H ₂ O] ⁺	DAG (36:3)	2
603.5382/625.5219	[M + H – H ₂ O] ⁺ /[M + Na – H ₂ O] ⁺	DAG (36:2)	4
605.5538/627.5381	[M + H – H ₂ O] ⁺ /[M + Na – H ₂ O] ⁺	DAG (36:1)	4
(B) List of lysophosphatidylcholines (LysoPC) involved in the development of restenosis			
m/z	ions	tentative identification	ppm
476.3158/494.3265	[M + H – H ₂ O] ⁺ /[M + H] ⁺	LysoPC (16:1)	3
478.3292/496.3397	[M + H – H ₂ O] ⁺ /[M + H] ⁺	LysoPC (16:0)	1
502.3314/520.3420	[M + H – H ₂ O] ⁺ /[M + H] ⁺	LysoPC (18:2)	3
504.3471/522.3578	[M + H – H ₂ O] ⁺ /[M + H] ⁺	LysoPC (18:1)	3
(C) List of sphingomyelins (SMs) involved in the development of restenosis			
m/z	ions	tentative identification	ppm
731.6062/753.5921	[M + H] ⁺ /[M + Na] ⁺	SM (36:1)	0
759.641/781.6229	[M + H] ⁺ /[M + Na] ⁺	SM (38:1)	4
787.6706/809.6544	[M + H] ⁺ /[M + Na] ⁺	SM (40:1)	2
801.6844/823.671	[M + H] ⁺ /[M + Na] ⁺	SM (41:1)	0
815.7036/837.6862	[M + H] ⁺ /[M + Na] ⁺	SM (42:1)	4