The stability of the metabolic turnover of arachidonic acid in human unruptured intracranial aneurysmal walls is sustained

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ABSTRACT

Objective: Intracranial aneurysm (IA) is considered a chronic inflammatory condition that affects intracranial arteries. Cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) are considered potential targets of specific medical treatment for IAs. Previous studies have reported the elevated COX2 expression in the IA wall. However, not much has been studied about the upstream regulation of COX2 and PGE2, and the metabolism of arachidonic acid (AA) in human IAs. In this study, we aimed to elucidate the distribution of fatty acids in human IA walls for the first time.
Methods: Samples from 6 ruptured and 5 unruptured human IAs were surgically resected after the aneurysmal clipping and analyzed using desorption electrospray ionization imaging mass spectrometry.
Results: AA and AA-containing phospholipids were not detected in the unruptured IA walls. On the contrast, significantly larger amounts of AA and AA-containing phospholipids were detected in the ruptured IA walls compared to unruptured IA walls.
Conclusions: This study showed for the first time that AA was not detected in unruptured human IA walls. Our findings suggest that the stability of the turnover of AA in human unruptured IA walls is sustained. In contrast, this study showed that larger amounts of AA and AA-containing phospholipids were detected in the ruptured IA walls. More cases and further analysis are necessary to interpret our present results.

1. Introduction

Intracranial aneurysm (IA) is currently considered a chronic inflammatory disease that affects the intracranial arteries [1]. As an underlying mechanism, the prostaglandin E2 (PGE2)-EP2-NF-κB signaling cascade in macrophages was considered by Aoki et al. as a factor regulating such chronic inflammation involved in the progression and rupture of IAs [1]. PGE2 is a lipid mediator and an arachidonic acid (AA) metabolite generated from sequential enzymatic reactions, including cyclooxygenase (COX), and a potential therapeutic target for IAs [1]. Increased levels of free fatty acids (FFAs), including AA, in several brain regions after subarachnoid hemorrhage were reported in a previous

study using a rat model [2]. The association of AA-containing phospholipids in the development and rupture of IA walls in a rat model were also reported in a previous study [3]. However, to date, no report has described the distribution of FFAs in the walls of IA in humans.

Therefore, we investigated the extent of FFA distribution in the walls of IAs in human subjects by using a recently developed imaging mass spectrometry (IMS) technique, desorption electrospray ionization IMS (DESI-IMS).

2. Materials and methods

2.1. Specimen collection and preparation

We included in this study a series of 11 patients with 5 unruptured and 6 ruptured saccular IAs who underwent surgery in the Department of Cerebrovascular Surgery of Saitama Medical University International Medical Center in Hidaka, Japan (supplementary Table 1). The IA samples were obtained by resecting a small part of the aneurysmal wall intraoperatively after the clipping of the aneurysmal neck. The specimens were immediately frozen on dry ice and stored at −80 °C until sectioning. All the specimens were sectioned at a 10 µm thickness using a cryostat for DESI-IMS analysis.

2.2. DESI-IMS data acquisition from the IAs and standard lipids

Sectioned slides were kept at room temperature just before DESI-MS acquisition. Mass spectra were acquired in the negative ion mode. All the experiments were performed with the DESI source attached to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Xevo G2-XS Q-TOF; Waters, Milford, MA, USA). The mass spectra were calibrated externally prior to measurement using a sodium formate solution (500 µM) in 90:10 2-propanol-to-water ratio (v/v). The parameters used for the optimization of DESI and acquisition of data from IA tissues are given in supplementary Table 2. Tandem mass spectrometry (MS/MS) using same instrument was performed to confirm the candidate molecule corresponded to m/z 303.23 applying a collision energy of 10 eV, source temperature 120º C, capillary voltage 4.0 kV, and 98% methanol (98:2; methanol: water) as spray solvent at a flow rate of 2 µL/min. Two standard FFAs (arachidonic acid and oleic acid) and those fatty acids (FAs) containing standard lipids were also measured using the same MS instrument and parameters (supplementary Table 2) to confirm whether FAs were detected in IA walls as FFAs or fragment of lipids. For that purpose, 1 µL solution of standard lipids (10 µg/mL in ethanol) was applied on glass slide and acquired DESI-MS data.

2.3. Fold changes in FAs and lipid contents between ruptured and unruptured IA walls

Fold changes (rupture IA vs unruptured IA walls) of detected FAs and lipids were also analyzed using the average intensity of each FA and lipid. In mass spectrometric measurement, noise is a common fact which can hide MS peaks with small intensities. Therefore, intensities of noise nearby the MS peaks of candidate lipids which were not detected in one group of IA samples were used as their intensities and calculated their fold changes [4].

2.4. Data analyses

The MassLynx 4.1 (Waters) software was used for data acquisition and processing; The HDImaging (Waters) software was used for the image analysis. The MS Excel and SPSS version 16 software were used for the statistical analysis. All the values are expressed as mean ± standard error of mean (SEM). Differences with P values < 0.05 (two-tailed t-test) were considered significant.

2.5. Chemical and reagents

Liquid chromatography/MS-grade methanol, 2-propanol, and ultra-pure water were purchased from Wako Pure Chemical Industries (Osaka, Japan); leucine enkephalin was purchased from Waters (Germany); and sodium formate, AA, oleic acid (OA), phosphatidylcholine (PC; 18:1/18:1), and PC (20:4/20:4) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Ethics committee approval

The institutional review board of Saitama Medical University International Medical Center (No. 16–223) and Hamamatsu University School of Medicine (No. 17–288) approved all aspects of the study. Informed consent was obtained from all the patients.

3. Results

3.1. Distribution of FFAs in the IA walls on DESI-IMS

We first analyzed the DESI-IMS mass spectra acquired from ruptured and unruptured IA walls. Five MS peaks with m/z of 255.23, 279.23, 281.25, 283.26, and 303.23 were identified in the samples, corresponding to palmitic acid (PA; C16:0), linoleic acid (LA; C18:2), OA (C18:2), stearic acid (SA; C18:0), and AA (C20:4), respectively (supplementary Fig. 1 and Supplementary Table 3) according to previous reports [5,6]. AA was further confirmed by MS/MS analysis (Supplementary Fig. 2). Using standard lipids, we have also confirmed that all these FAs were detected as FFAs in this study (Supplementary Fig. 3).

The distribution patterns of PA, LA, OA, SA, and AA were analyzed in both the ruptured and unruptured IA tissues (Fig. 1). AA was detected only in the ruptured but not in the unruptured IA walls (Fig. 1A and B). Moreover, accumulations of LA and AA in the same tissue region as the ruptured IA walls were also observed (Fig. 1A and B). No significant change in the distribution of PA and SA was found between ruptured and unruptured IA walls.

The average intensity of the distribution of FFAs were also analyzed in this study. The distribution of LA and OA were increased in the ruptured IA walls by 3.48-fold (P = 0.003) and 2.85-fold (P = 0.013), respectively, as compared with the unruptured IA walls (Fig. 2A). In the unruptured IAs, the amount of AA in the sample was less than the detection sensitivity of the device, so it was not detected as MS peak corresponding to AA. Compared with the noise levels in the unruptured IA walls, an increase in AA level of approximately 300 times was detected in the ruptured IA walls (Fig. 2B).

3.2. Distribution of AA-containing phospholipids in the IA walls on DESI-IMS

We next analyzed the distribution of AA-containing phospholipids in the IA walls using DESI-IMS. Four MS peaks with m/z of 766.53, 794.55, 810.53, and 885.55 were detected in the ruptured IA walls and assigned to phosphatidylethanolamine (PE; 18:0/20:4), PE (20:0/20:4), phosphatidylserine (PS; 18:0/20:4), and phosphatidylinositol (PI; 18:0/20:4), respectively, on the basis of their mass accuracy (Supplementary Table 3), biological distributions, and data from previous reports [3,7]. Among these phospholipids, PE (20:0/20:4) and PI (18:0/20:4) were observed in abundance especially in the walls of the ruptured IAs (Fig. 3A). In the unruptured IAs, the amounts of AA containing phospholipids in the sample were less than the detection sensitivity of the device, so it was not detected as a peak corresponding to the phospholipids (Fig. 3B). Compared with the noise levels of DESI-IMS data acquired from unruptured IA walls, the intensity level of the distributions of PE (18:0/20:4), PE (20:0/20:4), PS (18:0/20:4), and PI (18:0/20:4) in the ruptured IA walls were increased by approximately 2.0-fold, 3.7-fold, 3.5-fold, and 5.5-fold, respectively (Fig. 3C).

The changes in the distribution of FFAs and phospholipids between ruptured and unruptured IA walls were given by Supplementary Table 4.

3.3. Inspections of the IA walls by the immunohistology

We tried to examine the IA walls by immunohistochemical staining method. The sectioned slides of the samples were stained by hematoxylin-eosin (HE) staining and immunostaining including COX2. It was difficult to interpret the results of the immunostaining because the
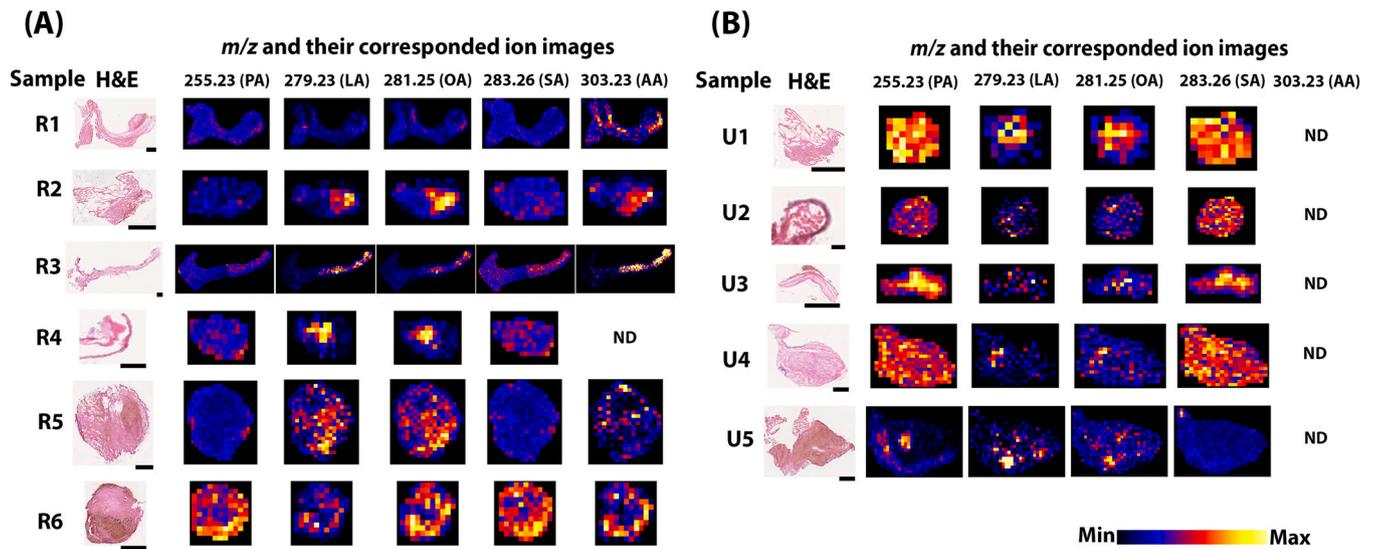
Free fatty acids

Fig. 1. Distribution of free fatty acids (FFAs) in the walls of ruptured (R) and unruptured (U) human intracranial aneurysms (IAs). A, Molecular ion images of FFAs in the ruptured IA human walls. B, Molecular ion images of the FFAs in the unruptured human IA walls. ND indicates not detected. Scale bar: 0.5 mm. H&E: hematoxylin-eosin. Palmitic acid (PA), linoleic acid (LA), oleic acid (OA) and stearic acid (SA) were detected in both ruptured and unruptured human IA walls, but arachidonic acid (AA) was only detected in ruptured human IA walls.
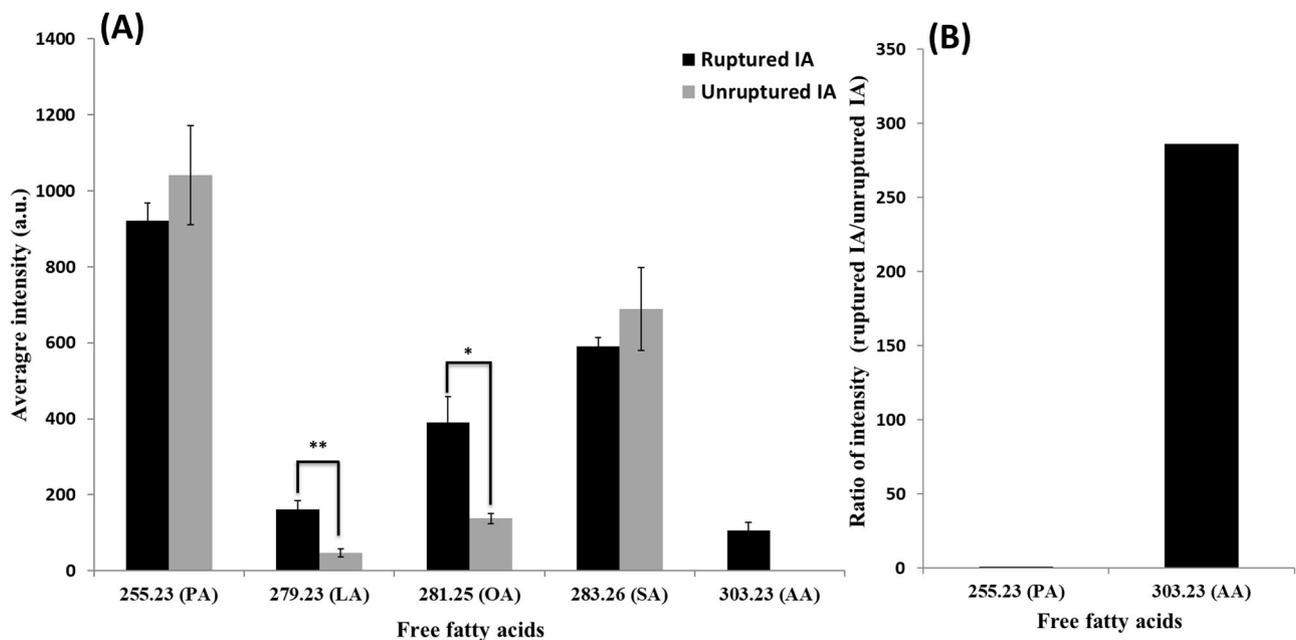

Fig. 2. Differences in the intensity level of the distribution of free fatty acids (FFAs) between the ruptured and unruptured human intracranial aneurysm (IA) walls. A, Average intensity of FFAs in the walls of ruptured and unruptured IAs walls (n = 6 for ruptured IA and n = 5 for unruptured IA). All data are presented as mean ± SEM. * *P = 0.003 and *P = 0.013 (two-tailed *t*-test). Significantly increased distribution of oleic acid (OA) and linoleic acid (LA) in ruptured human IA walls compared to that of unruptured human IA walls was noted in this study. No significance change was found in the distribution of palmitic acid (PA) and stearic acid (SA) between ruptured and unruptured IA walls. B, Ratio of the intensity of palmitic acid (PA) and arachidonic acid (AA) between the ruptured and unruptured IA walls. The noise level in the unruptured IA walls was used as the intensity of AA in the unruptured IA walls. Compared to the unruptured IA walls, about 300-fold increased AA was observed in ruptured IA walls.

condition of the slides was inappropriate for the immunostaining. Under the HE staining, many blood cells were detected on ruptured IA walls different from unruptured IA walls (Fig. 1).

4. Discussion

This study showed for the first time that AA was not detected in the

unruptured human IA walls. Our findings suggest that the stability of the turnover of AA in human unruptured IA walls is sustained. Previous studies have reported the elevated COX2 expression in the IA wall [7,8]. Additionally, IA is considered a chronic inflammatory disease and is conceptually the same as other chronic inflammatory diseases such as cancer, atherosclerosis, and impaired glucose tolerance [1]. Our findings may suggest that the inflammatory responses in human IAs,
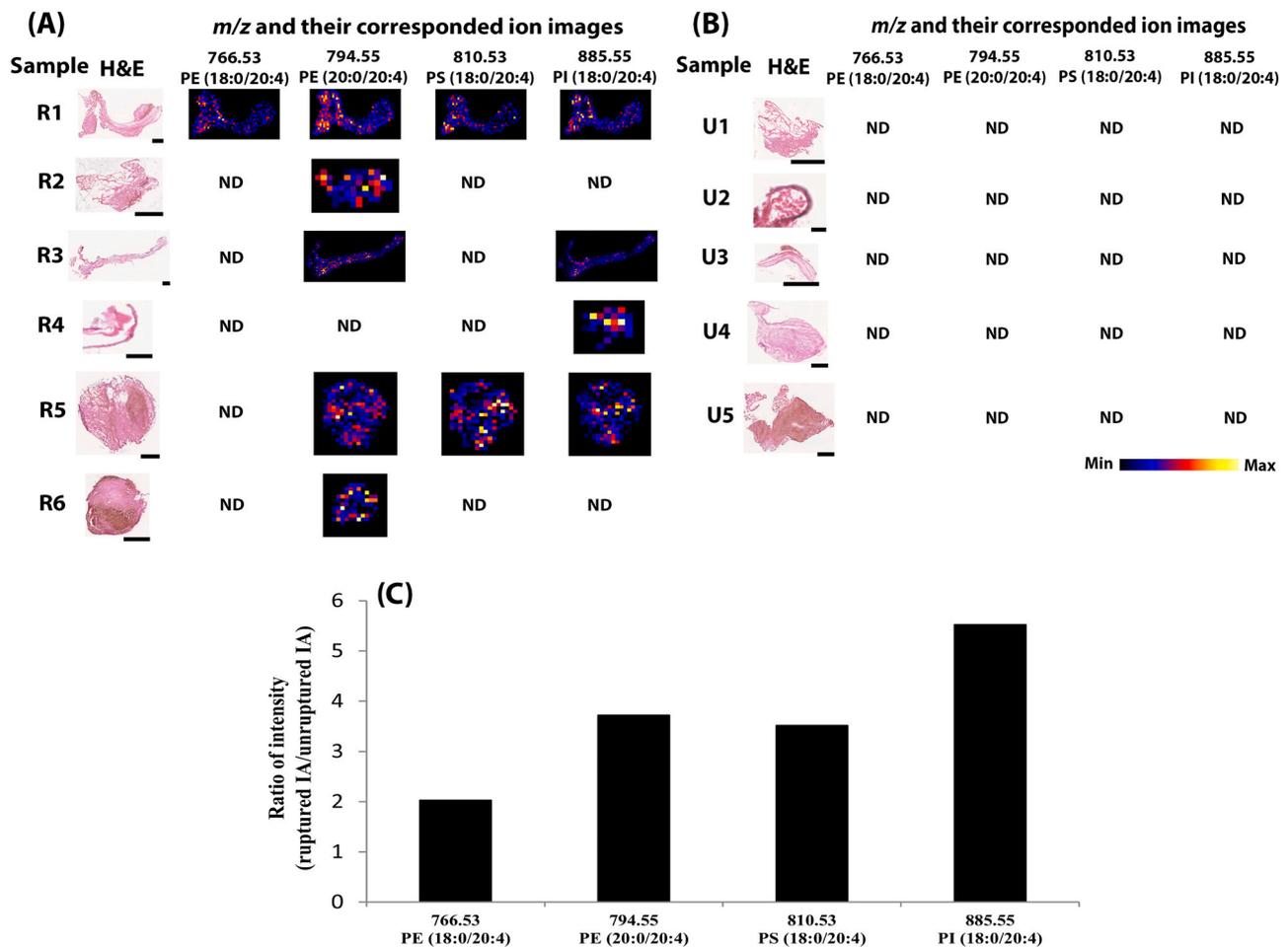
(A)

m/z and their corresponded ion images

(B)

m/z and their corresponded ion images

(C)

Fig. 3. Distribution of arachidonic acid (AA)-containing phospholipids in the walls of human intracranial aneurysm (IA)s. A, Molecular ion images of AA-containing phospholipids in ruptured human IA walls. B, Molecular ion images of AA-containing phospholipids in unruptured human IA walls. ND indicates not detected. AA containing phospholipids were only detected I ruptured IA walls. Scale bar: 0.5 mm. C, Ratio of the intensity of the AA-containing phospholipids between the ruptured and unruptured IA walls. The noise level in the unruptured IA walls was used as the intensity of AA-containing phospholipids in the unruptured IA walls. About 2–6 folds increased AA-containing phospholipids were detected in ruptured IA walls compared to that of unruptured IA walls. PE: Phosphatidylethanolamine; PS: Phosphatidylserine; PI: Phosphatidylinositol.

including the COX2 expression, is not induced from the stimulation that AA regulates independent of its metabolites. Furthermore, considering that AA has been detected enough and suggested to play an important role in other inflammatory disease [9–14], our findings might suggest that unruptured human IAs have a different inflammatory association from other inflammatory diseases regarding the response of AA and COX2.

Unlike the unruptured IA walls, high levels of AA were detected on the ruptured IA walls. We also found high level of LA, precursor in AA synthesis, in the ruptured IA walls, and accumulations of LA and AA in the same tissue region as the ruptured IA walls were also observed. Considering that not only AA but also the precursor of AA increased in the ruptured IA, it may be possible that AA is associated to the progression and rupture of IA walls. However, according to the result that many blood cells were also detected on ruptured IA walls by hematoxylin-eosin staining, it seems reasonable to think that much of the AA in the ruptured IAs is free AA caused by the platelets activated by the rupture of the aneurysms. If that is the case, this finding suggests that DESI-IMS is a useful tool for semi-quantification of the activation of platelets in excised specimens. In this study, we also found high level of OA. We can't have interpreted this result yet. The significance of OA was smaller than that of AA and LA, and more cases are necessary to interpret this result.

We have previously demonstrated that PI (18:0/20.4) accumulated

at high levels in the thickened aneurysmal walls in experimentally induced IA with synthetic dedifferentiated smooth muscle cells [3]. It is uncertain why the current study demonstrated AA-containing phospholipids including PI (18:0/20.4) at the noise levels in the human unruptured IA walls. It might be due to the difference of the wall of the aneurysms in each sample; the difference of the species and experimentally induced or not. On the other hand, high levels of AA-containing phospholipids including PI (18:0/20.4) were detected on ruptured IA walls. PI (18:0/20.4) play key roles in a wide range of cellular processes, including cell migration, invasion, and proliferation [3]. PE also play important roles in the regulation of cell proliferation, metabolism, organelle function, endocytosis, autophagy, stress response, and apoptosis [15]. Therefore, our results might be a clue for exploring the mechanism of rupture and hemostasis in IAs.

The sample size in this study was too small, and the human IA samples, especially those from ruptured IAs, might have been already modified by several factors, which might have affected the results of the present study. Therefore, more cases and further analysis are necessary to interpret our present results. However, our findings might offer new insights into the associations of AA metabolites, including COX2, with IA walls in humans.

5. Conclusion

In this study, DESI-IMS revealed the changes in the distribution of FFAs and lipids in the ruptured and unruptured IA walls in human samples. We found that AA and AA-containing phospholipids were not detected in the unruptured IA while they were detected in a significantly larger amount in the ruptured IA. These findings may offer new insights on the association of AA metabolites, including COX2, with IA walls in humans.

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CRediT authorship contribution statement

Ririko Takeda: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Project administration. **Ariful Islam**: Investigation, Formal analysis, Data curation, Writing – review & editing. **Tomohito Sato**: Validation, Formal analysis, Investigation, Data curation. **Hiroki Kurita:** Resources, Supervision. **Tomoaki Kahyo**: Validation, Investigation. **Tetsumei Urano:** Supervision. **Mitsutoshi Setou:** Methodology, Project administration.

Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.clineuro.2021.106881.

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