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NOTE

Physiology

The profile of urinary lipid metabolites in healthy dogs

Taiki KIDA[1)], Arisa YAMAZAKI[1)], Koji KOBAYASHI[1)], Tatsuro NAKAMURA[1)], Takayuki NAKAGAWA[2)], Ryohei NISHIMURA[2)] and Takahisa MURATA[1)]*

[1)]Department of Animal Radiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

[2)]Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

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ABSTRACT. Polyunsaturated fatty acids, including arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), are converted to hundreds of lipid mediators by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP), or through non-enzymatic processes, and they reflect inflammatory states of the body. We comprehensively analyzed lipid metabolites in dog urine using a liquid chromatograph-mass spectrometry (LC-MS/MS) to describe their metabolic characteristics. We detected 31 AA-derived metabolites, four EPA-derived metabolites, and a DHA-derived metabolite in all urine samples. Among AA-derived metabolites, 15, 5, 3, and 8 were generated by COX, LOX, CYP, and non-enzymatic oxidation respectively. This study will be the first step to use profiles of urinary lipid metabolites for better understanding and diagnosis of canine diseases.

KEYWORDS: dog, lipid metabolite, urine

Polyunsaturated fatty acids (PUFAs) are an essential component of mammalian bodies. Besides serving as building blocks of plasma and other membranes, PUFAs and their metabolites play physiological roles as bioactive molecules. Omega-6 (n-6) and 3 (n-3) PUFAs, such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), are converted to hundreds of lipid mediators by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) or through non-enzymatic processes. Prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) derived from AA have been shown to play crucial roles in human and animal diseases [4, 5, 7, 10, 16, 17].

PUFA-derived lipid metabolites are excreted in urine. Previous studies have shown that the quantity of such urinary lipid metabolites significantly changes according to physiological states and diseases. For instance, a metabolite of prostaglandin E_2 (PGE_2), a major proinflammatory mediator, is increased in urine of smokers [15]. It is also associated with a risk of many types of cancer in human, including colorectal, gastric, and prostate cancer [9, 11, 18]. Our group also demonstrated that the urinary level of a prostaglandin D_2 (PGD_2) metabolite reflects the severity of the food allergic symptoms in human and mice [8, 13]. As urine can be collected in a non-invasive and simple way, it is a convenient tool to quantify these lipid metabolites that reflect the metabolic changes of the whole organism associated with canine diseases. As the first step to such application, we performed a liquid chromatograph-mass spectrometry (LC-MS/MS)-based analysis of lipid metabolites in urine of healthy dogs to describe their metabolic characteristics.

We collected and utilized urine samples from 12 healthy dogs without any abnormality in medical checks including physical examinations, complete blood count, blood serum chemistry, urinary test, chest radiography, and ultrasound in Anim Pet Clinic (Tokyo, Japan) under the owners' consents. We also confirmed that these dogs did not have a history of allergy. General information on each subject is shown in the Table 1. The samples were handled and analyzed as previously described [12]. Urine samples were stored until used at −28°C for 3–7 months. Informed written consent was obtained from all the owners at admission. The collected urines were centrifuged (20,000 × *g*, 5 min) and the supernatant were mixed with internal standards (Table 2). After the solid phase extraction (Oasis HLB, Waters, MA, USA), the sample solutions containing lipids fractions were eluted with methanol. The sample solution (5 µl) was injected to liquid chromatography (Nexera 2, Shimadzu, Kyoto, Japan) equipped with mass spectrometer (LCMS-8060, Shimadzu).

Metabolites of AA, EPA, and DHA, totaling 117 types in each urine sample were measured with LC-MS/MS Method Package for Lipid Mediators Ver. 2 with LabSolutions software (Shimadzu), following instruction of the manufacture. Each metabolite was

*Correspondence to: Murata, T.: amurata@mail.ecc.u-tokyo.ac.jp, Department of Animal Radiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Table 1. Characteristics of individual healthy dogs

ID	Sex	Age (year, month)	Breed	Urine collection method
001	MC	10, 4	Toy Poodle	Spontaneous urination
002	M	4, 2	Maltese	Urinary catheter
003	FS	6, 11	Pekinese	Cystocentesis
004	FS	1, 4	Toy Poodle	Spontaneous urination
005	FS	4, 8	Toy Poodle	Cystocentesis
006	F	3, 9	Corgi	Spontaneous urination
007	FS	13, 9	Shiba	Spontaneous urination
008	FS	8, 2	Toy Poodle	Cystocentesis
009	FS	11, 2	Miniature Dachshund	Cystocentesis
010	F	12, 1	Miniature Dachshund	Cystocentesis
011	M	4, 8	Toy Poodle	Urinary catheter
012	FS	8, 2	Mix	Cystocentesis

M, male; F, female; C, castrated; S, spayed.

Table 2. The list of internal standards (IS)

	Name	Concentration (ng/ml)
1	Tetranor-Prostaglandin E Metabolite-d_6 (Tetranor-PGEM-d_6)	25.0
2	6-keto-Prostaglandin $F_{1\alpha}$-d_4	25.0
3	Thromboxane B_2-d_4 (TXB_2-d_4)	25.0
4	Prostaglandin $F_{2\alpha}$-d_4 ($PGF_{2\alpha}$-d_4)	25.0
5	Prostaglandin E_2-d_4 (PGE_2-d_4)	25.0
6	Prostaglandin D_2-d_4 (PGD_2-d_4)	25.0
7	Leukotriene C_4-d_4 (LTC4-d_4)	25.0
8	Leukotriene B_4-d_4 (LTB4-d_4)	25.0
9	5 (S)-Hydroxyeicosatetraenoic Acid-d_8 (5 (S)-HETE-d_8)	25.0
10	12 (S)-Hydroxyeicosatetraenoic Acid-d_8 (12 (S) HETE-d_8)	25.0
11	15 (S)-Hydroxyeicosatetraenoic Acid-d_8 (15 (S) HETE-d_8)	25.0
12	Oleoyl Ethanolamide-d_4 (OEA-d_4)	0.5
13	Eicosapentaenoic Acid-d_5 (EPA-d_5)	500.0
14	Docosahexaenoic Acid-d_5 (DHA-d_5)	50.0
15	Arachidonic Acid-d_8 (AA-d_8)	500.0

identified by retention time and selected reaction monitoring ion transition. The urine samples were preserved with different period. Each lipid metabolite containing in the urine has a different stability. Therefore, a metabolite was defined as "detected" only when it was found in the all samples.

Figure 1 shows metabolic pathway maps of AA, EPA, and DHA, with each node showing whether the metabolite was detected or not. In the current study, we detected 36 lipid metabolites in total. Supplementary Table 1 showed urinary lipid metabolite profile in each dog. Among them, 31 were categorized in AA-derived metabolites (Fig. 1A through 1D), 4 in EPA-derived metabolite (Fig. 1E), and 1 in DHA-derived metabolite (Fig. 1F).

AA-derived metabolites were further classified according to enzymes responsible for the metabolism, i.e., COX (15, Fig. 1A), LOX (5, Fig. 1B), CYP (3, Fig. 1C), or non-enzymatic oxidation (8, Fig. 1D). Among metabolites detected in the current study, COX-mediated AA metabolites accounted for the largest portion. Various types of PGs and TXs, and their derivatives can be useful biomarkers reflecting inflammatory states of the body. Indeed, for instance, Baltzer *et al.* reported that TXB_2 and metabolites of prostacyclin, PGE_2, and $PGF_{2\alpha}$ were increased in urine of dogs following agility exercise [1].

LT is another major class of AA metabolites produced by LOX. A previous study reported that a major LOX-derived AA metabolite, leukotriene E_4 (LTE_4), was increased in urine of dogs and it could be a useful biomarker that indicates the severity of inflammation in chronic enteropathies [7]. In this study, we identified LTE_4, its precursors (LTA_4 and LTD_4), its metabolites (LTF_4) and many other LOX-meditated metabolites in urine of health dogs. This result suggests that these metabolites are of potential use to describe inflammatory states of the body.

In addition to COX and LOX-mediated metabolites, three types of CYP-mediated metabolites, 5,6-DHET-lactone, 14,15-DHET, and 19-HETE, were detected in all urine samples collected from several canine breeds (Table 1). Isoform composition of CYPs, their expression pattern, and catalytic activities differ between not only species but also canine breeds [2, 14]. Thus, these CYP-mediated metabolites found in this study may be useful biomarkers that describe physiological and disease states of dogs regardless of the species and breeds. Further studies with more breeds and larger sample size for each breed are required to verify this point.

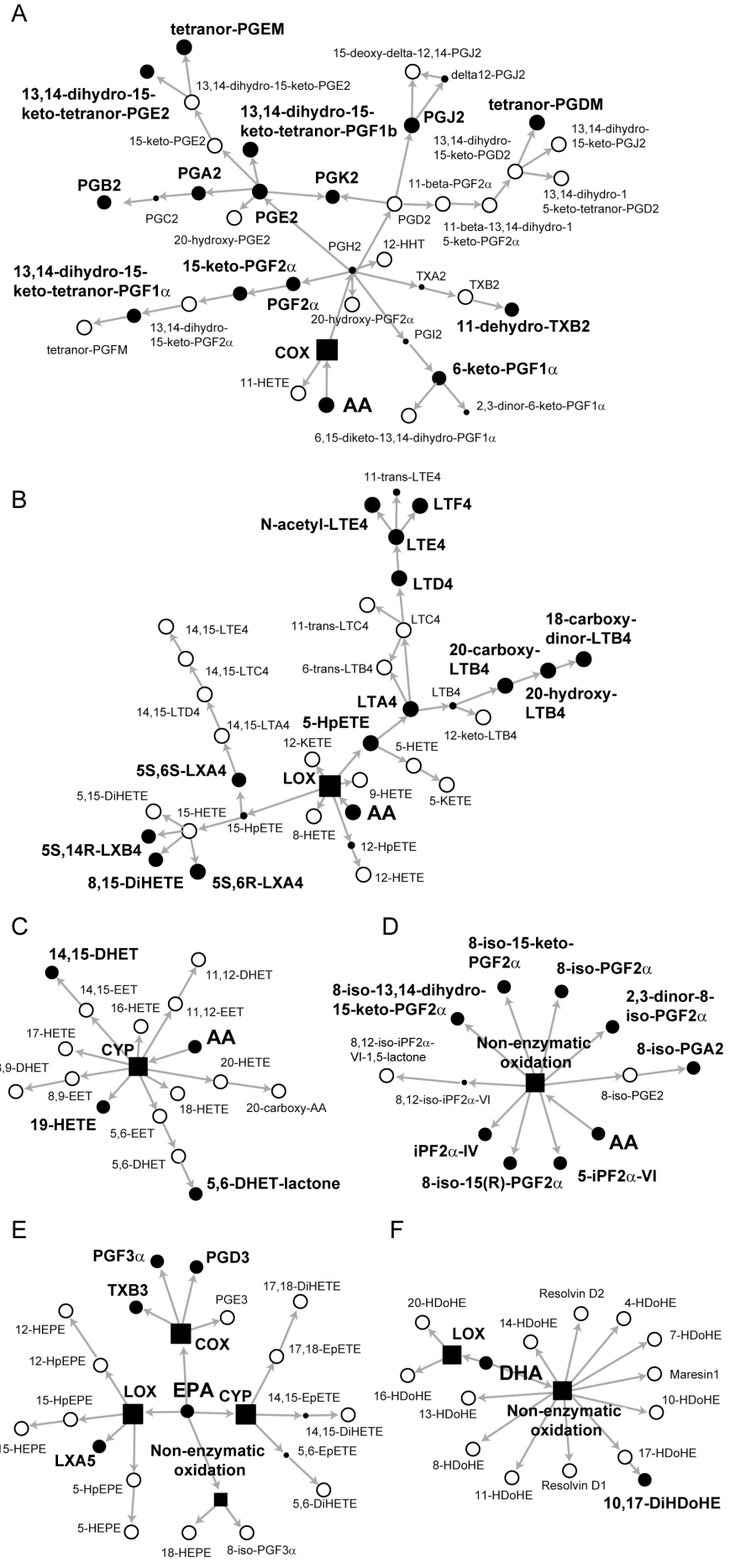

Fig. 1. Metabolic pathway of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AA (**A** through **D**), EPA (**E**) and DHA (**F**) metabolic pathways. AA metabolic pathways were further classified by responsible enzymes: cyclooxygenase (COX, A), lipoxygenase (LOX, B), cytochrome P450 (CYP, C), or enzyme-independent oxidation (D). A black-filled circle represents a lipid metabolite which was detected in the present study, while an open circle represents a metabolite which was measured but not detected. A dot represents a metabolite which was not measured in the present study. A black square represents an enzyme responsible for the oxidation.

Interestingly, we detected as many as eight non-enzymatic oxidation products of AA, called isoprostanes, including 8-*iso*-$PGF_{2\alpha}$, which is frequently used as an index of oxidative stress in human [3, 6]. Increasing levels of isoprostanes detected here may also reflect oxidative stress in dogs.

In this study, we also detected four COX- or LOX-mediated metabolites of EPA: TXB_3, $PGF_{3\alpha}$, PGD_3, and LXA_4, and an enzyme-independent oxidation product of DHA: 10,17-DiHDoHE. As EPA and DHA cannot be synthesized in the body, mammals depend on dietary food source for these n-3 fatty acids. Therefore, amount of these metabolites in urine would be greatly affected by type of food. Additional research with detailed information on food is warranted to investigate the relationship among these EPA- and DHA-derived metabolites in urine, food, and physiological states or diseases.

Besides the lack of information on food mentioned above, there are some limitations in the present study. Firstly, we took a conservative criterion that a metabolite should be found in the all samples to be considered "detected", even though dogs with various baseline characteristics were included in this study. Therefore, we may have missed some metabolites that would be constantly detected in healthy dogs with specific breed, sex, or age range. Secondly, urine samples were obtained by different methods in the present study, namely through a catheter, by cystocentesis, or spontaneous urination. In addition, these samples were stored for different periods before the measurement. These factors may affect profile of lipid metabolites detected.

In summary, we revealed urinary lipid profiles of healthy dogs. Various AA-derived metabolites were detected, which were either COX-, LOX-, or CYP-mediated, or non-enzymatically produced. These profiles can be useful biomarkers that reflect inflammatory states of the body and help us have a better understanding of diseases. It would also be possible to apply such urinary lipid profiles to a screening test or a diagnosis tool.

POTENTIAL CONFLICTS OF INTEREST. All the authors have no conflicts of interest to declare.

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