pISSN: 2093-940X, eISSN: 2233-4718
Journal of Rheumatic Diseases Vol. 24, No. 4, August, 2017
https://doi.org/10.4078/jrd.2017.24.4.192

Original Article

Metabolomics Approach to Explore the Effects of Rebamipide on Inflammatory Arthritis Using Ultra Performance Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry

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Objective. Rebampide is a gastroprotective agent used to treat gastritis. It possesses anti-inflammatory and anti-arthritis effects, but the mechanisms of these effects are not well understood. The objective of this study was to explore mechanisms underlying the therapeutic effects of rebamipide in inflammatory arthritis. **Methods.** Collagen-induced arthritis (CIA) was induced in DBA/1J mice. DBA/1J mice were immunized with chicken type II collagen, then treated intraperitoneally with rebamipide (10 mg/kg or 30 mg/kg) or vehicle (10% carboxymethylcellulose solution) alone. Seven weeks later, plasma samples were collected. Plasma metabolic profiles were analyzed using ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry-based metabolomics study and metabolite biomarkers were identified through multivariate data analysis. **Results.** Low dose rebamipide treatment reduced the clinical arthritis score compared with vehicle treatment, whereas high dose rebamipide in CIA aggravated arthritis severity. Based on multivariate analysis, 17 metabolites were identified. The plasma levels of metabolites associated with fatty acids and phospholipid metabolism were significantly lower with rebamipide treatment than with vehicle. The levels of 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 and thromboxane B3 decreased only in high dose-treated groups. Certain peptide molecules, including enterostatin (VPDPR) enterostatin and bradykinin dramatically increased in rebamipide-treated groups at both doses. Additionally, corticosterone increased in the low dose-treated group and decreased in the high dose-treated group. **Conclusion.** Metabolomics analysis revealed the anti-inflammatory effects of rebamipide and suggested the potential of the drug repositioning in metabolism- and lipid-associated diseases. **(J Rheum Dis 2017;24:192-202)**

Key Words. Rebamipide, Arthritis, Metabolomics, Anti-oxidant

INTRODUCTION

Rebamipide is a gastroprotective agent used for the treatment of gastritis and gastric ulcer. Previous reports have identified that rebamipide acts as an oxygen radical scavenger and exhibits an anti-inflammatory effects [1]. As chronic oxidative stress is considered to underlie the pathophysiological mechanisms of many autoimmune and inflammatory diseases, including rheumatoid arthritis (RA), Sjögren's syndrome, Behçet's disease, and osteoarthritis (OA) [2-5], rebamipide has been verified to reduce inflammation in animal models of above diseases [6] and in limited human trials [7]. Also, we recently demonstrated the therapeutic efficacy of rebamipide in murine models of OA and RA [8,9]. Despite of encouraging results of rebamipide, the underlying mechanisms of the drug that works under inflammatory condition remains mostly unclear.

Increasing evidence in both experimental and clinical studies have suggested that oxidative stress contributes

Received : January 25, 2017, **Revised :** April 21, 2017, **Accepted :** April 22, 2017

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to the pathophysiology of various diseases including cancer [10], and diabetes [11] and the potential benefit of antioxidant in these diseases [12,13]. However, regarding the prevention of cancer there are numerous trials that often provide conflicting conclusions. For example, β-carotene supplements may be associated with a marginal increase in the incidence and mortality from lung cancer in smokers [14]. Actually, previous studies have shown a significant inverted U-shape association between antioxidant dose and their anti-inflammatory properties [15], suggesting the presence of effective dose range in the antioxidant effects regarding their anti-inflammation, anti-aging and chemoprevention.

Metabolomics as an emerging omic science in systems biology, provides a comprehensive and systematic profiling of low molecular weight metabolite from tissue, biofluids, and cells. Metabolomics has demonstrated a powerful tool in the various fields of medical research, such as toxicology, disease diagnosis and natural product discovery, and relies on advanced technology to profile metabolites. Metabolomics has many potential applications and advantages for the research of complex systems, such as herbal medicine, because of their chemical and structural diversity [16].

Despite of its anti-oxidant and anti-inflammatory potential of rebamipide, the mechanisms of action under inflammatory arthritis are not yet well understood. In our previous study, it was shown that rebamipide can induce the activity of NF-E2-related factor 2, a key transcription factor that plays a central role in the protection of cells against oxidative stresses [8], indicating antioxidant capacity of the drug. In that report, the doses of rebamipide per mice were 0.6 and 6 mg/kg/day. Judging by the inverted U-shape association between dose of anti-oxidant and their optimum physiologic function, it could be postulated that excess of dose rebamipide can exacerbate inflammation.

We hypothesized that treatment with excess dose of rebamipide may augment arthritis severity in vivo. In this study, a ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS)-based metabolomics approach was applied to the characterization of altered metabolite by rebamipide treatment in arthritis mice, and the different metabolite profile between the arthritis animals treated with optimal anti-inflammatory dose of rebamipide and those with aggravated arthritis by treatment with relatively higher dose of rebamipide.

MATERIALS AND METHODS

Reagents

Formic acid, leucine enkephalin, sodium formate, caffeine, acetaminophen, reserpine, hippuric acid, glycocholic acid, adipic acid, and methanol (high-performance liquid chromatography grade) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ultrapure water (18.2 MΩ) was obtained using a Milli-Q apparatus from Millipore (Milford, MA, USA).

Animal study and sample collection

1) Animals

Male DBA/1J mice ages 4～6 weeks were purchased from Orient Bio Inc. (Seongnam, Korea). The animals were maintained under specific pathogen–free conditions at the Catholic Research Institute of Medical Science of the Catholic University of Korea and were fed standard mouse chow and water. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea; the procedures conformed to all guidelines of the National Institutes of Health (no. 12-002).

2) Collagen-induced arthritis induction and intraperitoneal administration of rebamipide

Type II collagen (CII) was dissolved overnight at 4°C in 0.1N acetic acid (4 mg/mL), with gentle rotation. Mice were injected intradermally at the base of the tail with 100 μg CII emulsified in Freund's complete adjuvant (1:1 weight/volume; Chondrex, Redmond, WA, USA). To assess the influence of rebamipide on symptom severity in the collagen-induced arthritis (CIA) model, mice were treated with rebamipide (10 or 30 mg/kg) in 10% carboxymethylcellulose (CMC) solution (vehicle) or with vehicle alone by intraperitoneal administration every day after booster immunization for 4 weeks. The arthritis index in these mice was scored twice weekly and expressed as the sum of the scores of 4 limbs. The plasma were collected from each group of mice at 7 weeks after CII immunization.

Clinical assessment of arthritis

The severity of arthritis was determined by three independent observers. The mice were observed three times a week for the onset and severity of joint inflammation for up to 8 weeks after the primary immunization. The severity of arthritis was assessed on a scale of 0 to 4 with the

following criteria, as described previously [17]: 0=no edema or swelling, 1=slight edema and erythema limited to the foot or ankle, 2=slight edema and erythema from the ankle to the tarsal bone, 3=moderate edema and erythema from the ankle to the tarsal bone, and 4=edema and erythema from the ankle to the entire leg. The arthritic score for each mouse was expressed as the sum of the scores of three limbs. The hind paw into which type II collagen+incomplete Freund's adjuvant was injected was excluded. The highest possible arthritis score for a mouse was thus 12. The mean arthritis index was used to compare the data among the control and experimental groups.

Sample preparation

Plasma samples were prepared by cold methanol precipitation. A 50-μL aliquot of each plasma sample was thawed at room temperature, mixed with 150 μL ice-cold methanol, vortexed, and centrifuged at 14,000 rpm for 15 min at 4°C to remove precipitated protein. Subsequently, 100 μL supernatant was diluted with 50 μL water and vortexed. The prepared sample was transferred to autosampler vials and injected into a UPLC-Q-TOF-MS.

Validation of method repeatability

A quality control (QC) sample was prepared by mixing equal amounts of each sample and processed using the same method used for sample preparation [18]. The QC sample was used for column conditioning and method validation [19]. The test mixture comprised commercially available standards added to the QC sample: caffeine (1 μg mL^{-1}), acetaminophen (1 μg mL^{-1}), and reserpine (1 μg mL^{-1}) for the positive ionization mode and hippuric acid (1 μg mL^{-1}), glycocholic acid (1 μg mL^{-1}), and adipic acid (1 μg mL^{-1}) for the negative ionization mode.

UPLC-Q-TOF-MS analysis

The instrument system used in this study was UPLC-Q-TOF-MS. An Acquity UPLC (Waters Corp., Milford, MA, USA) was coupled with a Q-TOF (SYNAPT G2; Waters Corp.). Separations were performed using an Acquity UPLC BEH C18 column (1.7-μm particle size, 2.1-mm inner diameter, 100-mm length; Waters Corp.) at 50°C. Initially, 20-μL QC samples were injected 15 times prior to the main analysis using a short gradient program to condition the column and to optimize the system [20]. Gradient elution was performed using a mixture of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) at a flow rate of 0.4 mL/min. The starting conditions for the short gradient were 90% A and 10% B (v/v) changing to 100% B in a linear gradient over 3 min. This solvent composition was maintained for 2.5 min followed by a return to the starting conditions. Re-equilibration was performed for 2.5 min before the next conditioning injection. For analysis of plasma samples, a longer gradient program was employed using the same solvent system as the conditioning gradient. The starting conditions were 90% A and 10% B (v/v) for 0.5 min, changing to 80% A over 3 min, to 30% A at 5 min, and to 100% B at 13 min. The solvent composition was held at 100% B for 2.5 min before returning to the starting conditions. Re-equilibration of the system for 2 min with 90% A and 10% B (v/v) was conducted prior to the next injection. Ten-microliter samples were injected. All samples were stored at 4°C during the analysis. To eliminate the effect of run order, samples were injected randomly.

Mass spectrometry was performed in both positive and negative ionization modes with an electrospray ionization source interface. The following parameters were employed. The capillary voltage was set to 3,200 V and 2,500 V for positive and negative ionization modes, respectively, and the cone voltage was 40 V for both ionization modes. The desolvation and cone gas was nitrogen at a flow rate of 600 L/h and 100 L/h, respectively. The source temperature was 120°C, and the dissolution temperature was 350°C. Leucine-enkephalin (0.2 μg/L in 50% methanol) was utilized as the lock mass (mass-to-charge ratio [m/z] 556.2771 for positive mode and 554.2615 for negative ionization mode) at a flow rate of 20 μL/min. Full scan data were collected at a range of 50~1,200 m/z over a period of 15 min with a scan time of 0.5 s and an inter-scan delay of 0.1 s. m/z in resolution mode. All of the acquired spectra were automatically corrected during acquisition based on the lock mass. The mass spectrometric data were collected into two separate data channels using collision energy alternating between 0 eV (low-energy scans) and 30 eV (high-energy scans) in centroid mode. Before analysis, the mass spectrometer was calibrated with 0.2 mM sodium formate solution.

Data analyses

The raw mass spectrometry data from all of the samples were processed using MarkerLynx XS software version 4.1 (Waters Corp.). This application's manager identifies mass relative retention time pairs (RT-*m/z* pair) and in-

tensities of peaks eluted in at least two of the samples. After being profiled, the area of the peaks representing ion intensities were normalized against the summed total ion intensities of each chromatogram. Sample name, identified RT-*m/z* pair, and the normalized ion intensity were used as fingerprints [21]. Principal component analysis (PCA) identifies inherent group clustering and highlights the markers responsible for the clustering. The sample list, marker list, and PCA results were displayed in the MarkerLynx browser. MarkerLynx software processed chromatographic full-scan data acquired in centroid mode. Multivariate analysis, such as principal component analysis PCA and partial least square-discriminant analysis (PLS-DA) was performed using EZinfo software (Umetrics Inc., Umeå, Sweden). To generate PCA and PLS-DA plots, the Pareto (Par) scale was used for SIMCA-P analysis (Umetrics Inc.). The metabolite candidates were preliminarily selected in the positive and negative ionization modes due to the significant variables in variable importance in the projection (VIP)-value plots as VIP values define the responsibility of each ion for the variations more clearly. The metabolite candidates had VIP values of 1.5 or greater.

Molecular weights and fragment patterns of metabolites obtained from multivariate analyses have been used for molecular identification using database searches. The databases that were search included the Human Metabolome Database (http://www.hmdb.ca/), Chemical Entities of Biological Interest (http://www.ebi.ac.uk/Databases/), MassBlank.jp-High Resolution Mass Spectral Database (http://www.massbank.jp/index.html), Scripps Center for Mass Spectrometry (http://masspec.scripps.edu/index.php), LIPID MAPS-LIPID Metabolites, Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/), and Pathways Strategy (http://www.lipidmaps.org/).

RESULTS

Rebamipide attenuates inflammatory arthritis at low-dose (10 mg/kg), but not at high-dose (30 mg/kg)

We investigated whether rebamipide would suppress inflammation in an experimental murine model of inflammatory arthritis (CIA). The results showed that the dose of low-dose rebamipide (10 mg/kg) administered intraperitoneally from day 14 after primary immunization with CII emulsified in Freund's complete adjuvant reduced the clinical arthritis score as compared with mice with CIA treated with vehicle (10% CMC solution), whereas high-dose rebamipide (30 mg/kg) did aggravate arthritis severity (Figure 1).

Multivariate analyses of metabolic profiles in plasma

To assess the differential effects of rebamipide on inflammatory arthritis depending on administered dose, metabolite profiles in the plasma of mice treated with rebamipide at low- and high-dose were investigated. In the multivariate statistical analysis, PCA and PLS-DA was applied to analyzing the characteristics in the chromatographic data of each group. PCA was examined for the overview of the metabolomic data set and PLS-DA model was established to confirm the metabolic differences among the groups. In PCA sore plot, the total variance expressed from the two components in positive ionization mode was 61.7% (PC1: 47.2% and PC2: 14.5%) and that from three components in negative ionization mode was 55.2% (PC1: 23.3%, PC2: 18.7%, and PC3: 13.2%). In PLS-DA, the score plot in positive ionization mode was expressed by 43.2% (PC1) and 30.5% (PC2) variance and that in negative ionization mode was by 53.7% (PC1), 38.6% (PC2), and 23.3% (PC3). In PLS-DA score plots, four groups were separated from one another, indicating that the groups had distinct metabolic phenotype fea-

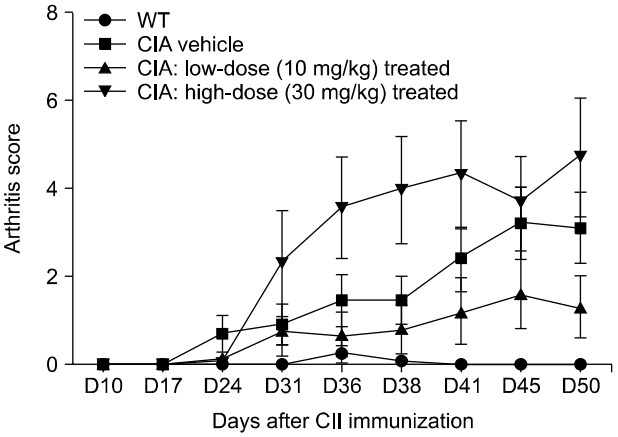

Figure 1. Treatment with rebamipide (10 mg/kg) suppresses inflammatory arthritis in mice with collagen-induced arthritis (CIA). CIA was induced in DBA/1J mice by immunization with type II collagen (CII) in adjuvant. Changes in arthritis score in rebamipide-treated mice compared with vehicle-treated mice. Rebamipide dissolved in 10% carboxymethylcellulose solution (vehicle) was given intraperitoneally to 2 different groups (each receiving 10 or 30 mg/kg; n = 10 mice per group) daily for 4 weeks, starting after booster immunization. A third group (n = 10) received vehicle alone. WT: wild-type, D: day.

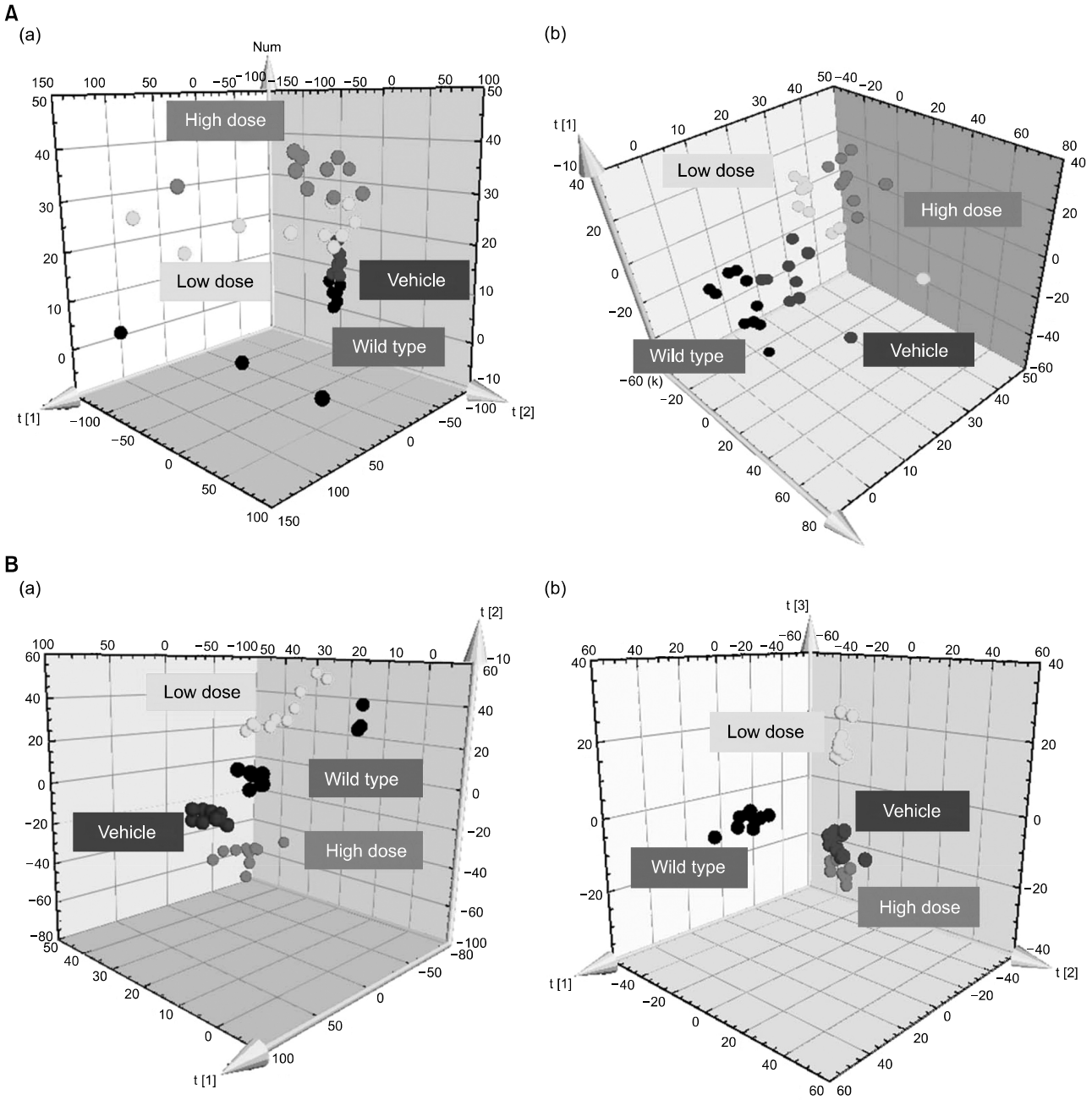

Figure 2. (A) Principal component analysis (PCA) score plots and (B) partial least square-discriminant analysis (PLS-DA) score plots based on plasma metabolic profiling. (a) Positive ionization mode. (b) Negative ionization mode. In PCA, the score plot was obtained with the two PCs presenting 47.2% (PC1) and 14.5% (PC2) variance in positive ionization mode and that was with three PCs presenting 23.3% (PC1), 18.7% (PC2), and 13.2% (PC3) in negative ionization mode. In PLS-DA, the score plot was obtained with the two PCs presenting 43.2% (PC1) and 30.5% (PC2) variance in positive ionization mode and that was with three PCs presenting 53.7% (PC1), 38.6% (PC2), and 23.3% (PC3) in negative ionization mode.

tures (Figure 2). In addition, the distinction between high-dose treated group and low-dose treated group was clear.

Identification of potential metabolites

Based on multivariate analysis, 128 out of 516 variables (total variables determined in both positive and negative ionization modes) were related to treatment of rebamipide and 17 metabolites were identified through the

Table 1. Identification of potential biomarkers

rRT_m/z	Molecular formula	Fragmentation	Identified metabolite	Related metabolism	Change trend			
					W vs. V	L vs. V	H vs. V	H vs. L
0.34_317.18 [M+H]	$C_{23}H_{40}N_8O_6$	$C_{17}H_{17}N_3O_2$ ($-C_{16}H_{24}N_5O_4$)	Enterostatin (VPDPR)	Peptide	–	↑ *	↑ *	–
1.04_354.19 [M+H]	$C_{50}H_{73}N_{15}O_{11}$	$C_{32}H_{50}N_{10}O_7$ ($-C_{18}H_{24}N_5O_4$)	Bradykinin		–	↑ *	↑ *	–
1.94_305.25 [M+H]	$C_{20}H_{32}O_2$	$C_{17}H_{25}$ ($-C_3H_6O_2$)	Arachidonic acid	Fatty acid	–	↓	↓	–
2.03_257.25 [M+H]	$C_{16}H_{32}O_2$	$C_{16}H_{33}O$ ($-O$)	Palmitic acid		–	↓ *	↓ *	–
2.07_283.27 [M+H]	$C_{18}H_{34}O_2$	$C_{17}H_{35}$ ($-CO_2$)	Oleic acid		–	↓	↓	–
1.51_333.20 [M+Na-2H]	$C_{18}H_{34}O_5$	$C_{17}H_{35}O_3$ ($-CO_2$)	HpODE		–	↓	↓	–
1.56_468.31 [M+H]	$C_{22}H_{46}NO_7P$	$C_5H_{13}O_5P$ ($-C_{17}H_{34}NO_2$)	LysoPC (14:0)	Phospholipid	–	↓ *	↓ *	–
1.80_496.34 [M+H]	$C_{24}H_{50}NO_7P$	$C_5H_{13}O_5P$ ($-C_{19}H_{38}NO_2$)	LysoPC (16:0)		–	↓ *	↓ *	–
1.99_524.37 [M+H]	$C_{26}H_{54}NO_7P$	$C_5H_{13}O_5P$ ($-C_{21}H_{42}NO_2$)	LysoPC (18:0)		–	↓ *	↓ *	–
2.77_787.67 [M+H]	$C_{44}H_{86}NO_8P$	$C_5H_{13}O_5P$ ($-C_{39}H_{74}NO_3$)	PC (18:0/18:1)		–	↓	–	–
1.25-370.30 [M+H]	$C_{21}H_{39}NO_4$	$C_8H_{14}O_3$ ($-C_{13}H_{26}NO$)	Tetradecenoylcarnitine	Acylcarnitine (β-oxidation)	–	↓ *	↓ *	–
1.34_372.31 [M+H]	$C_{21}H_{41}NO_4$	$C_8H_{14}O_3$ ($-C_{13}H_{28}NO$)	Tetradecanoylcarnitine		–	↓ *	↓ *	–
1.40_398.33 [M+H]	$C_{23}H_{43}NO_4$	$C_8H_{14}O_3$ ($-C_{15}H_{30}NO$)	Hexadecenoylcarnitine		–	↓ *	↓ *	–
1.53_400.43 [M+H]	$C_{23}H_{45}NO_4$	$C_8H_{14}O_3$ ($-C_{15}H_{32}NO$)	Palmitoylcarnitine		–	↓ *	↓ *	↓ *
1.84_317.18 [M+H]	$C_{20}H_{30}O_4$	$C_{12}H_{16}O_3$ ($-C_8H_{15}O$)	15-deoxy-$\Delta^{12,14}$PGJ2	Prostaglandin	–	–	↓	↓ *
2.21_369.23 [M+H]	$C_{20}H_{32}O_6$	$C_{12}H_{17}O_5$ ($-C_8H_{15}O$)	Thromboxane B3		–	–	↓ *	↓
2.07_381.17 [M+Cl]	$C_{21}H_{30}O_4$	$C_{21}H_{28}O_3$ ($-H_2O$)	Corticosterone	Corticosteroid hormone	–	↑ *	↓	↓ *

rRT = retention time of metabolite/retention time of internal standard (IS) (reserpine). ↓ : indicates decrease, ↑ : indicates increase, –: indicates no significant changes, W: wild type, V: vehicle-treated CIA mice, L: low-dose rebamipide (10 mg/kg)-treated CIA mice, H: high-dose rebamipide (30 mg/kg)-treated CIA mice, HpODE: hydroxyoctadecadienoic acid, LysoPC: lysophosphatidylcholine, PGJ2: prostaglandin J2. *Indicates significant change ($p < 0.01$), the other indicates significant change ($p < 0.05$).

metabolite identification process (Table 1). The significance of differences in the identified metabolites among the groups was determined based on ANOVA t-tests. Alteration in lipid metabolism was notable.

The plasma levels of 15 out of total 17 metabolite was decreased by rebamipide, either low-dose, high-dose or both. In contrast, some peptide molecules, like enterostatin (VPDPR) and bradykinin dramatically increased in rebamipide-treated groups at both doses. The plasma levels of fatty acids, including arachidonic acid, palmitic acid, oleic acid, and hydroxyoctadecadienoic acid (HpODE) as well as those of phospholipid, including lysophosphatidylcholine (LysoPC[14:0]), LysoPC (16:0), and LysoPC (18:0) were significantly lower in rebamipide-treated groups at low and high dose than those of vehicle-treated CIA mice. The plasma level of PC (18:0/18:1), another class of phospholipid was lower only in low-dose treated mice compared with vehicle-treated

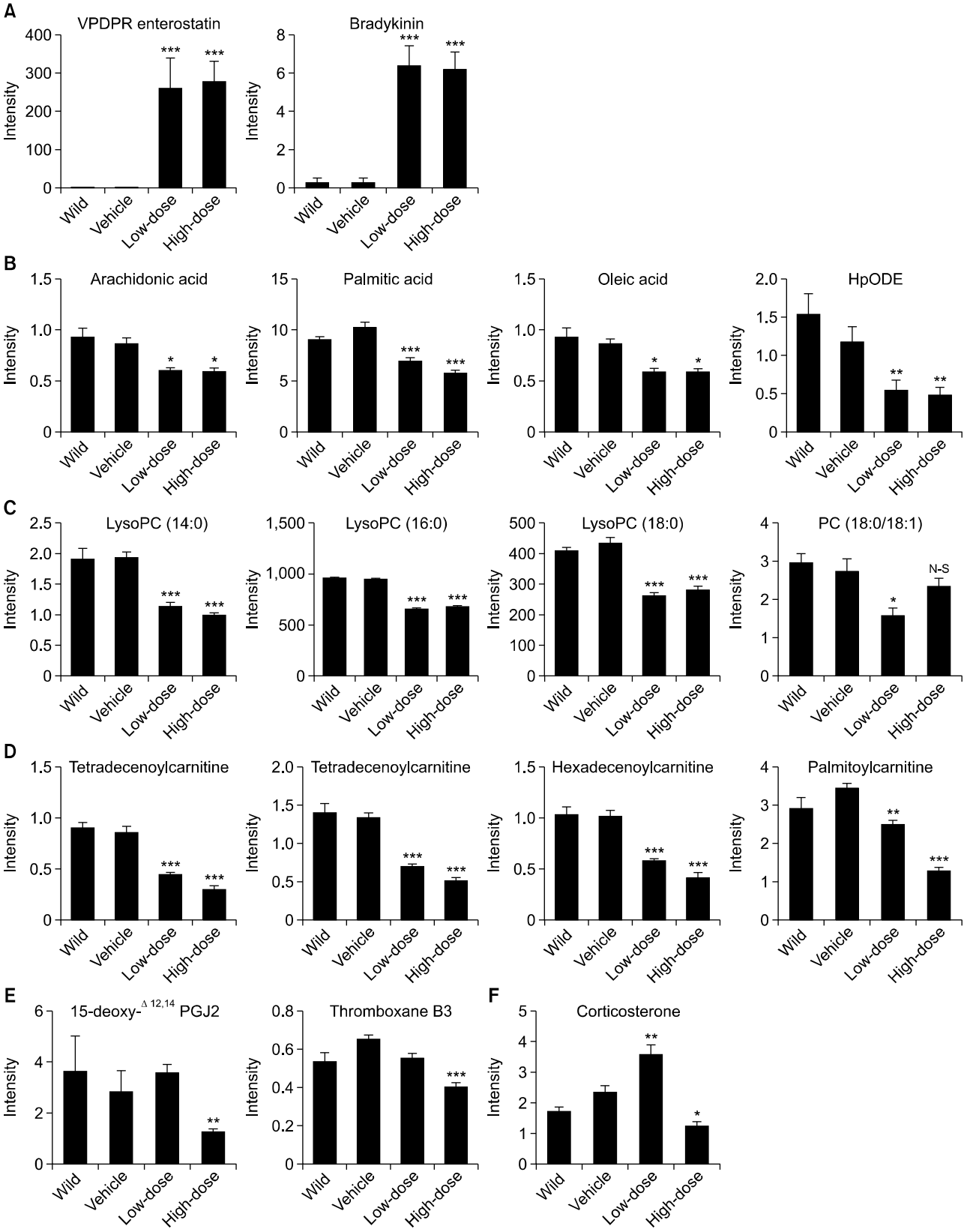

Figure 3. Comparison of metabolites with significant changes involved in (A) peptide metabolism, (B) fatty acid metabolism, (C) phospholipid metabolism, (D) acylcarnitine (β-oxidation) metabolism, (E) prostaglandin metabolism, and (F) corticosteroid hormone metabolism. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with vehicle control. HpODE: hydroxyoctadecadienoic acid, LysoPC: lysophosphatidylcholine, PGJ2: prostaglandin J2.

mice. The plasma level of PC (18:0/18:1) between wild type mice and vehicle-treated group did not differ. Thus, it could be hypothesized that PC (18:0/18:1) can reflect the attenuated inflammation by rebamipide treatment, at least, under the condition of inflammatory arthritis. In line with our results, several report demonstrated that decrease of PC (18:0/18:1) may be involved in anti-inflammatory properties of non-steroidal anti-inflammatory drugs [22]. Interestingly, the CIA mice treated with high-dose rebamipide (30 mg/kg) did not show any alteration in plasma level of PC (18:0/18:1). Regarding acylcarnitine metabolism that indicates fatty acid oxidation, all the arthritis animals that were treated with rebamipide, at both low-dose and high-dose of the drug, the plasma levels of four metabolites involved in acylcarnitine metabolism significantly showed lower level, when compared to vehicle-treated group. Among the four metabolites, the suppression pattern of plsma palmitoylcarnitine level by rebamipide treatment in arthritis mice showed dose-dependent manner, whereas others did not.

Because the arthritis severity was higher in mice treated with high-dose of rebamipide (30 mg/kg) than those treated with vehicle, we sought to investigate the different metabolite between low-dose and high-dose group. We found that the two metabolite, 15-deoxy-$\Delta^{12,14}$ Prostaglandin J2 (PGJ2) and thromboxane B3, showed significant lower plasma level in high-dose group as compared with arthritis mice treated with vehicle (Table 1, Figure 3). Of our interest, plasma levels of the 15-deoxy-$\Delta^{12,14}$ PGJ2 and thromboxane B3 in low-dose treatment CIA mice did not differ with those of vehicle-treated group. Regarding corticosteroid hormone metabolism, the plasma level of corticosterone was significantly increased by low dose of rebamipide treatment in CIA mice compared with vehicle-treated group, whereas the plasma level of corticosterone was significantly decreased by high-dose rebamipide treatment (Table 1, Figure 3). These opposing results on corticosteroid hormone according to different doses may suggest that low-dose of rebamipide in inflammatory milieu can stimulate intrinsic corticosteroid hormone, therefore inducing anti-inflammatory properties. On the other hand, relatively high-dose of rebampide can work in a contrary direction regarding the effects on corticosteroid hormone. As shown Figure 3, high-dose rebamipide treatment in inflammatory milieu significantly inhibited the corticosterone, the metabolite involved in corticosteroid hormone. That result corresponds to augmented inflammatory severity by 30 mg/kg dose rebamipide treatment, because corticosteroid possesses strong anti-inflammatory property through regulation of gene transcription and cell signaling [23,24]. It can be postulated that the different effects on 15-deoxy-$\Delta^{12,14}$ PGJ2, thromboxane B3 and corticosterone might explain the opposing effects of different dose of rebamipide on arthritis severity.

Taken together, rebamipide treatment in mice with inflammatory arthritis showed concentration-dependent the anti-oxidant activity. However, as our results showed, it does not mean that superior antioxidant effects of the drug always lead to more prominent anti-inflammatory effects. Although 30 mg/kg dose of rebamipide was not lethal, relatively high-dose of rebamipide treatment resulted in deteriorated arthritis severity compared with vehicle-treated CIA mice.

DISCUSSION

This is the first study to identify the metabolite alteration by systemic treatment with rebamipide in a murine model of inflammatory arthritis, through an UPLC-Q-TOF-MS-based method to reveal underlying mechanisms of its anti-inflammatory property. The metabolomics results revealed that 17 metabolites could be used as potential biomarker. In this study, rebamipide showed effective anti-oxidant properties and the effects was dose-dependent manner, although the differences were marginal. Here, we showed that high-dose (30 mg/kg) rebamipide treatment in CIA mice caused exacerbated arthritis severity compared with vehicle-treated mice, whereas low-dose (10 mg/kg) of the drug significantly attenuated arthritis severity. The opposing in vivo effects by different doses of rebamipide might correspond with augmented plasma level of corticosterone in low-dose rebamipide-treated CIA mice and reciprocal inhibition of corticosterone by treatment with high-dose of rebamipide. Interestinlgy, only high-dose of rebamipide significantly suppressed plasma concentrations of 15-deoxy-$\Delta^{12,14}$ PGJ2 and thromboxane B3 compared with vehicle- and low-dose-treated group.

In contrast to conventional prostaglandins, PGJ2 and its derivatives possesses a cyclopentenone ring, therefore permitting them to ligate nuclear receptors and to modify intracellular signaling [25]. 15-deoxy-$\Delta^{12,14}$ PGJ2 is a final metabolite derived from prostaglandin D, and had been known to be a major endogeneous ligand of peroxisome proliferator-activated receptor-γ (PPAR-γ) [26]. PPAR-γ

is a transcriptional factor that is predominantly expressed in adipose tissue. It controls the storage and release of fat and regulates insulin resistance and blood glucose levels [27]. Recent accumulating evidences have suggested that PPAR-γ is a major regulator in adipogenesis [28]. In line with that, recent study showed that 15-deoxy-$\Delta^{12,14}$ PGJ2 induces pre-adipocyte 3T3-L1 cell adipogenesis [29]. Interestingly, thiazoledinediones are a group of PPAR-γ agonist that is widely used in the treatment of type 2 diabetes for recent two decades. Despite of its beneficial effects on blood glucose level and plasma free fatty acids, the drug has adverse effects such as body fat gain. It could be postulated that these 'adipogenic' effects of the drug may arise from the stimulating effects on PPAR-γ. Here, the UPLC-Q-TOF-MS-based metabolomics analysis revealed that plasma level of 15-deoxy-$\Delta^{12,14}$ PGJ2 was significantly lower in high-dose rebamipide-treated arthritis mice, compared with vehicle- and low-dose rebamipide-treated group. These dose-specific effects of rebamipide might suggest the its potential at clinical application of the drug in diseases associated with lipid metabolim, such as obesity and atherosclerosis.

Our study showed that rebamipide treatment at a therapeutic dose (10 mg/kg) can increase level of circulating corticosterone. Corticosteroids are potent endogenous anti-inflammatory and immunosuppressive agents. Corticosterone is released early in the course of inflammation, through the stimulation of hypothalamic-pituitary-adrenal axis by inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α. Previous studies have shown that decreased adrenocortical function exists in patients with rheumatoid arthritis (RA) [30]. Recent two reports by Straub et al. [31,32] unveiled the relatively low levels of steroid hormones in relation to inflammation in patients with RA, although absolute serum levels of cortisol, IL-6 and TNF-α were higher in the patients. In healthy subjects, there was a dose-response relationship between serum concentrations of inflammatory cytokine and cortisol level [33]. On the other hand, circulating cortisol levels in RA patients are inadequately low in relation to serum levels of IL-6 and TNF [31]. The disproportion phenomenon was also shown in experimental arthritis model [34]. Thus, it is reasonable to assume that relatively low levels of corticosterone to inflammatory condition are involved in maintaining and progression of inflammatory process in RA. Unfortunately, the mechanisms underlying increment of corticosterone by rebamipide treatment in arthritis mice were not investigated in the present study. Next study to investigate the influence of rebamipide to adrenocortical system is needed.

Thromboxane B3 is a stable hydrolysis product of thromboxane A3. It is synthesized from eicosapentaenoic acid by cyclooxygenase and thromboxane synthase. As the name indicates, thromboxane is a vasoconstrictor and facilitates platelet aggregation by binding to thromboxane receptor. Previous in vivo studies have suggested that to target thromboxane receptor can attenuated renal injury in experimental model of lupus [35]. Also, to block thromboxane receptor and thromboxane synthesis through inhibiting thromboxane synthase can play protective roles in cyclosporine-induced nephrotoxicity in experimental model [36] and in patients with deteriorated renal allograft function [37]. Taken together, relatively high-dose of rebamipide might be translated in renal injury of which the pathogenesis of which is involved in enhanced thromboxane signal, while low-dose of rebamipide might work as anti-inflammatory and anti-oxidant drug in chronic inflammatory and degenerative diseases.

Here, we showed that the plasma level of enterostatin and in CIA mice significantly increased by more than 3,000% ($>$300 fold) following systemic administration of rebamipide compared with vehicle-treated arthritis group. Enterostatin is recognized as a satiety peptide that selectively suppresses fat intake [38]. Enteorstatin is produced in the pancreas, gastrointestinal tract and in specific regions of the brain. In addition to its effects on feeding, enterostatin directly regulates energy expenditure [39], and inhibits insulin secretion [40]. As rebamipide is a gastroprotective agent, we carefully presumed that mucosal protection by rebamipide treatment resulted in increasing level of enterostatin. Remarkable induction of enterostain by rebamipide treatment might suggest the potential of 'drug repositioning' of rebamipide in metabolic diseases such as diabetes and eating disorders such as bulimia.

UPLC-Q-TOF-MS-based metabolomics provided useful information for understanding of the metabolic changes after systemic administration of rebamipide in plasma of mice with inflammatory arthritis. Seventeen potential biomarkers were identified to be involved in peptide, fatty acid, phospholipid, oxidation, prostaglandin, and corticosteroid hormone pathways. These potential biomarkers and their corresponding pathways can help further in understanding the underlying mechanisms of rebamipide and potential application of the drug in other in-

flammatory and degenerative diseases. Especially, dramatic inducing effects on enterostatin and bradykinin may suggest the potential of the drug, especially in the aspect of obesity and vascular diseases such as atherosclerosis.

CONCLUSION

By using UPLC-Q-TOF-MS-based metabolomics analysis, we identified the altered plasma level of 17 metabolites that are associated with peptide, fatty acid, phospholipide, oxidation, prostaglandin, and corticosteroid hormone pathways, followed by rebamipide treatment in mice with inflammatory arthritis. The changes of metabolite level in plasma indicated the anti-oxidant effects of rebamipide. Augmented arthritis severity by high-dose rebamipide treatment in arthritis mice may correspond to suppressed corticosterone concentration. On the other hand, low-dose rebamipide treatment reciprocally increased the plasma level of corticosterone. Further studies to verify our results may provide insight into the immunoregulatory mechanism of rebamipide.

ACKNOWLEDGMENTS

This study was supported by the research fund (BCMC14AA05) of Institute of Clinical Medicine Research and Basic Research Program through the National Reseach Foundation of Korea (NRF) founded by the Ministry of Science, ICT & Future Planning (2014R1A1 A3051143).

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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