Production of lipid mediators across different disease stages of dextran sodium sulfate-induced colitis in mice

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Abstract Although several studies have revealed the role of different lipid mediators in colitis, the comprehensive analysis of their production across different phases of colitis remained unclear. Here, we performed the following analysis in the dextran sodium sulfate (DSS)-induced colitis model using LC-MS/MS. Oral administration of 2% DSS in mice for 4 days resulted in severe intestinal inflammation by day 7, which gradually subsided by day 18. Based on the disease scoring index (assigned on the basis of fecal condition and weight loss), we defined the phases of colitis as induction (days 0–4), acute inflammation (days 4–7), recovery (days 7–9), and late recovery (days 9–18). Across all phases, 58 lipid mediators were detected in the inflamed colon tissue. In the induction phase, the production of n-6 fatty acid-derived prostaglandin E2 and thromboxane B2 increased by ~2-fold. In the acute inflammation phase, the production of n-6 fatty acid-derived leukotrienes increased by >10-fold, while that of n-3 fatty acid-derived hydroxyeicosapentaenoic acids and dihydroxyeicosatetraenoic acids decreased. In the recovery phase, a precursor of protectin D1 (17-hydroxydocosahexaenoic acid) increased over 3-fold. These observations suggested dynamic changes in the production of lipid mediators across different phases of the disease and their potential regulation in healing colitis.—Hamabata, T., T. Nakamura, S. Masuko, S. Maeda, and T. Murata. Production of lipid mediators across different disease stages of dextran sodium sulfate-induced colitis in mice. J. Lipid Res. 2018. 59: 586–595.

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Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic gastrointestinal disease characterized by periods of remission and relapse of symptoms like diarrhea and loss of body weight (1, 2). In UC, inflammation is limited to the colonic mucosa and results in severe ulceration, edema, and hemorrhage. In CD, inflammation can extend throughout the gastrointestinal tract, most commonly affecting the ileocecal region. Currently, in the United States, the number of IBD patients is estimated to be around 1 to 1.3 million (3, 4). Although the pathophysiology of IBD has been investigated previously, its etiology remained elusive.

In order to investigate the pathophysiology of IBD, chemically induced murine IBD models, such as dextran sodium sulfate (DSS)-induced and trinitrobenzene sulfonic acid (TNBS)-induced colitis models, have been widely employed. DSS exerts direct toxicity toward gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. This results in acute colitis, which is characterized by bloody diarrhea, colonic ulceration, and granulocyte (neutrophil) infiltration into the colonic mucosa (5). On the other hand, TNBS hastens colonic autologous or microbiota proteins rendering them immunogenic to the host immune system (5).

Lipid mediators are bioactive substances produced via enzymatic [catalyzed by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP)] or nonenzymatic mechanisms for fatty acid oxidation. Over one hundred lipid mediators have been identified, which differentially regulate the promotion and/or resolution of inflammatory responses (6). They are mainly categorized into two groups on the basis of their molecular structures. The first group comprises the lipid mediators produced from arachidonic acid (AA), an omega-6 (n-6) polyunsaturated fatty acid, which possesses the first double bond between the sixth and seventh carbon atom from the methyl.

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; CD, Crohn’s disease; COX, cyclooxygenase; cPLA2, Ca2+-dependent cytosolic phospholipase A2; CYP, cytochrome p450; DiHETE, dihydroxyeicosatetraenoic acid; DSS, dextran sodium sulfate; EP4, E-type prostanoid receptor 4; HDH, hydroxydehydroarachidonic acid; HEPE, hydroxyeicosapentaenoic acid; HOTET, hydroxyoctadecatrienoic acid; IBD, inflammatory bowel disease; iPLA2, Ca2+-independent phospholipase A2; LT, leukotriene; LOX, lipoxygenase; LXA4, lipoxin A4; PG, prostaglandin; PL, phospholipase A2; TNBS, trinitrobenzene sulfonic acid; TX, thromboxane; UC, ulcerative colitis.

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end of the fatty acid. The second group comprises the lipid mediators produced from the omega-3 (n-3) fatty acids, such as EPA, DHA, and α-linolenic acid (ALA), which possess a double bond at the third carbon atom from the end of the carbon chain.

The n-6 fatty acid-derived lipid mediators have been reported to mediate both the pro- and anti-inflammatory responses in colitis (7). The mucosal biopsies from IBD patients revealed an increase in the production of the COX-derived mediators from n-6 fatty acids, such as prostaglandin (PG)E₂ and thromboxane (TX)B₂ (8). Lack of the PGE₂ receptor gene, namely E-type prostanooid receptor 4 (ep4), aggravated the symptoms of DSS-induced colitis in mice (9). The administration of PGE₂ or an EP4 agonist accelerated mucosal healing in the DSS-induced colitis in rats (10, 11). Inhibition of TXA₂ synthase or administration of a derivative of lipoxin A₄ (LXA₄; a LOX-derived metabolite from n-6 fatty acids) ameliorated the symptoms of TNBS-induced colitis in mice and rats (12, 13). Some n-3 fatty acid-derived lipid mediators have been reported to mediate anti-inflammatory responses during colitis (14). Administration of isomers of the LOX-derived metabolites from n-3 fatty acids, namely protectin D1 or resolvin E1, ameliorated the symptoms of DSS-induced colitis in mice (15, 16).

As described above, several studies have revealed the role of each lipid mediator in colitis. However, these mediators coordinately and intricately modulate the progression and/or resolution of the inflammatory responses during colitis. Thus, it is essential to understand when and how these mediators are produced and metabolized for improving our understanding regarding the etiology of colitis and developing a novel therapy in combating it. Recently, the simultaneous quantification of a number of lipid mediators has become possible using LC-MS/MS (17). Using this technique, in the present study, we performed a comprehensive analysis of the lipid mediators that were produced during the progression and resolution of the inflammatory responses in the DSS-induced model of murine colitis.

MATERIALS AND METHODS

Reagents

Tetranor-PGEM-d₆, 6-keto PGF₁α-d₄, TXB₂-d₄, PGF₂α-d₄, PGD₂-d₄, leukotriene (LT)C₄-d₄, LTB₄-d₄, 5(S) HETE-d₄, 12(S) HETE-d₄, 15(S) HETE-d₄, PAF C₁₆-d₄, and oleoyl ethanolamide-d₄ were from Cayman Chemical; DSS salt reagent grade was from MP Biomedicals; and indomethacin was from Sigma.

Induction of DSS-induced colitis

Eight- to twelve-week-old male C57BL/6 mice were used. All experimental animal care and use committee at the University of Tokyo (P11-576 and P08-258).

Mice were provided 2% DSS containing water ad libitum for 4 days. Control mice were given water. Fecal condition and body weight change were checked every day for assessment of colitis severity. Fecal condition was scored as previously reported (Table 1) (5).

Morphological assessment of DSS-induced colitis

Colon tissue was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The sections (2 μm) were stained with hematoxylin and cosin based on conventional methods. For assessment of colitis severity, histological scoring was performed as shown in Table 2 (18).

Comprehensive analysis of lipid mediators with LC-MS/MS

The colons of the mice were collected and immediately frozen under liquid nitrogen, and then crushed to powder by mixer mill (Retsch, Germany). Five milliliters of methanol were mixed with the powdered tissue and centrifuged at 800 g for 20 min. The supernatant was diluted with 15 ml 0.03% formic acid in water containing 50 μl internal standards (the compositions are shown in Table 3) and loaded onto a preconditioned solid-phase extraction cartridge (Sep-Pak Plus C18; Waters). The cartridge was washed with 3 ml 0.05% formic acid, 3 ml 15% ethanol, and 3 ml hexane. Then, the lipids absorbed on the cartridge were eluted with 50 μl ethyl acetate. This eluent was evaporated by vacuum evaporator for 90 min and reconstituted in 50 μl 90% methanol. Five microliters of sample solution were injected into an LCMS-8030 (Shimadzu, Japan). The gradient program is shown in Table 4. Comprehensive analysis of lipid metabolites was performed by using a software method package for lipid mediators (Shimadzu, Japan) according to the manufacturer’s instructions. Data were analyzed by LabSolutions LCMS version 5.65 (Shimadzu).

Semiquantitative RT-PCR

Total RNA was isolated from colon tissue on each disease stage by using Trizol reagent (Molecular Research Center, Inc.) and reverse-transcribed to cDNA using ReverTra Ace (TOYOBO). Semiquantitative RT-PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and specific primers as follows: cox-1, 5′-ATGAGTCAAGAGGTCTCTCCG-3′ and 5′-GCACCGATATGTAACCAACAGGA-3′; cox-2, 5′-AAGCGGAGCACTTTGAG-3′ and 5′-ATTGATGGTTGCTTTTGTGTA-3′; 5′-lox, 5′-ACCAACCCCC-TGGAGAGAGATGTA-3′ and 5′-GGATACAAACACCCTCAGAC-3′.

| Table 2. Histological score | Score |
|----------------------------|-------|
| Cell infiltration          | 0     |
| Low frequency in the lamina propria | 1     |
| High frequency in the lamina propria | 2     |
| Extending into the submucosa | 3     |
| Transmural extension of the infiltration | 4     |
| Tissue damage              | 5     |
| No mucosal damage          | 6     |
| Lymphoepithelial lesions   | 7     |
| Surface mucosal erosion or focal ulceration | 8     |
| Extensive mucosal damage and extension into deeper structures of the bowel wall | 9     |

| Table 1. Fecal score | Score |
|----------------------|-------|
| Normal               | 0     |
| Soft but still formed| 1     |
| Very soft            | 2     |
| Diarrhea             | 3     |
TABLE 3. Composition of internal standards

| Substance          | Concentration (ng/ml) |
|--------------------|-----------------------|
| Tetranor-PGEM-d₄  | 40                    |
| 6-keto PGF₁₀₋₃₋₄ | 400                   |
| TXB₂-d₄           | 200                   |
| PGE₁₂₋₃₋₄        | 200                   |
| PGD₂-d₄           | 200                   |
| LTC₄₋₃₋₄         | 200                   |
| LTB₄-d₄           | 200                   |
| PGE₂-d₄           | 200                   |
| PGF₂₀₋₃₋₄         | 200                   |
| 6-keto PGF₁₀₋₃₋₄ | 200                   |
| TXB₂-d₄           | 200                   |
| 5(S) HETE-d₈      | 200                   |
| 12(S) HETE-d₈     | 200                   |
| 15(S) HETE-d₈     | 200                   |
| 5(S) HETE-d₈      | 1,000                 |
| 12(S) HETE-d₈     | 500                   |
| 15(S) HETE-d₈     | 200                   |
| PAF C₁₆₋₃₋₄       | 200                   |
| Oleoyl ethanolamide-d₄ | 40            |

Statistical analysis

Results are expressed as the mean ± SEM. LabSolutions (Shimadzu) software was used for the data processing of LCMS-8030, including peak detection and integration. The values were normalized by internal standards and tissue weights. The calculation was performed as below.

(Absolute amounts) = [(Concentration of external standard) × (Injected volume)] × [(Intensity of objective component) / (Intensity of external standard)] × [(Intensity of external standard) / (Intensity of internal standard)]

Other data evaluations were conducted using one-way ANOVA, followed by Bonferroni’s test for comparison between more than two groups. P < 0.05 was regarded as significant.

RESULTS

DSS induces colitis in mice

The mice that received regular drinking water without DSS (vehicle group) did not show any change in their fecal consistency (score 0); moreover, their body weight gradually increased during the course of the experiment (Fig. 1A, B). The mice that received drinking water with DSS passed soft (score 1) feces on day 4. The maximum deterioration in fecal consistency was observed on day 7. Subsequently, the fecal consistency rapidly improved by day 9 and became normal on day 18. Consistent with the above results, the DSS-treated mice lost weight from day 4 to day 7. However, they rapidly gained weight by day 9 and weighed normal on day 18.

It is known that the length of the colon decreases in mice with colitis (19). DSS treatment shortened the colon length of mice from day 4 to day 7. The length of the colon seemed to increase from day 9 to day 18; however, it was significantly shorter compared with the length on day 0 (Fig. 1C, D).

In the DSS-treated mice, morphological examination revealed polymorphonuclear leukocyte infiltration and mucosal injury (including crypt disappearance and goblet cell depletion) on day 7, which partially recovered on day 18 (Fig. 1E). The score for the histological assessment of inflammation increased upon DSS treatment, was observed to be maximum on day 7, and subsequently decreased by day 18 (Fig. 1F).

Based on these observations, we defined day 0 as baseline, day 4 as induction, day 7 as acute inflammation, day 9 as recovery, and day 18 as late recovery phase (Fig. 1G).

We also assessed the role of COX in the progression of murine colitis (days 0–7). As shown in supplemental Fig. S1, oral administration of a nonselective COX inhibitor, indomethacin (0.625–1.25 mg/ml for 4 days in drinking water) aggravated the colitis and lost weight further in the DSS-treated mice.

Production of n-6 and n-3 fatty acid-derived lipid mediators

We performed a comprehensive analysis of the lipid mediators produced in the inflamed colon tissue at each phase of the disease using LC-MS/MS. Across all the phases of colitis, a total of 35 n-6 fatty acid (AA)-derived and 23 n-3 fatty acid (EPA, DHA, and ALA)-derived lipid mediators were detected.

We have summarized the number of n-6 and n-3 fatty acid-derived lipid mediators in Fig. 2A and showed the relative change in production of each lipid mediator in Fig. 2B. The number of n-6 fatty acid-derived lipid mediators that increased during the inflammatory response was 34 on day 4, 28 on day 7, 30 on day 9, and 8 on day 18. Thus, the number of lipid mediators was the highest on day 4 (induction phase), and gradually decreased by day 18 (late recovery phase). On the other hand, the number of n-3 fatty acid-derived lipid mediators that increased during the inflammatory response was 12 on day 4, 11 on day 7, 16 on day 9, and 8 on day 18. Thus, the number of lipid mediators was the lowest on day 7 (acute inflammation phase) and the highest on day 9 (recovery phase). These results suggested that the major type of fatty acid used for the

TABLE 4. Gradient program

| Step | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
|------|------------|--------------------|--------------------|
| 0    | 0          | 90                 | 10                 |
| 1    | 5          | 75                 | 25                 |
| 2    | 10         | 65                 | 35                 |
| 3    | 20         | 25                 | 75                 |
| 4    | 25         | 5                  | 95                 |
| 5    | 27         | 90                 | 10                 |
production of lipid mediators changed from n-6 to n-3 as the disease progressed.

Production of COX- and LOX-derived lipid mediators

We next categorized the lipid mediators on the basis of their biosynthetic pathway, as shown in Fig. 3A, and showed the relative change in production of each lipid mediator in Fig. 2B. Across all the phases of the disease, the production of several lipid mediators (especially the LOX-derived lipid mediators) changed dramatically. The number of LOX-derived lipid mediators produced was 21 on day 4, 19 on day 7, 23 on day 9, and 5 on day 18. Thus, the number of mediators produced on day 4 (induction phase) was high; however, it further increased on day 9 (recovery phase). On the other hand, the number of COX-derived lipid mediators was 19 on day 4, 15 on day 7, 14 on day 9, and 7 on day 18. Thus, the number of mediators produced was the highest on day 4 (induction phase) and subsequently decreased by day 18 (late recovery phase). These results demonstrated that the active synthase responsible for the production of these lipid mediators also changed from COX to LOX as the disease progressed.

Production of each lipid mediator

Production of prostanoids, the COX-derived lipid mediators from AA (an n-6 fatty acid), including PGE₂ and TXB₂, increased about 2-fold on day 4 (induction phase) and decreased subsequently by day 18 (late recovery phase) (Fig. 4A). The production of almost all LOX-derived LTs (such as LTB₄) and HETEs (such as 5-HETE) increased on day 7 and day 9, respectively. The production of LTB₄ increased more than 10-fold on day 7 (acute inflammation phase), while that of 5-HETE increased about 3-fold on day 9 (recovery phase).

The production of almost all n-3 fatty acid (EPA, DHA, or ALA)-derived lipid mediators, including hydroxyeicosapentaenoic acids (HEPEs; such as 18-HEPE) and dihydroxyeicosatetraenoic acids (DiHETEs; such as 5,6-DiHETE) decreased on day 7 (acute inflammation phase). While the production of 18-HEPE was reduced to about 25%, that of 5,6-DiHETE was reduced to about 12.5% of the original level (Fig. 4B). The production of HEPEs (such as 5-HEPE) and hydroxydocosahexaenoic acids (HDoHEs; such as 17-HDoHE) increased on day 9 (recovery phase). While the production of 5-HEPE increased about 4-fold, that of
17-HDoHE (a precursor of protectin D1) increased over 3-fold. In addition, the production of 13-hydroxyoctadecatetraenoic acid (HOTrE) increased over 4-fold on day 9 (recovery phase).

We also investigated how COX modulates production of each lipid mediator in murine colitis (supplemental Fig. S2A, B; on day 7). Oral administration of indomethacin (0.625–1.25 mg/ml for 4 days in drinking water) decreased the production of not only COX metabolites but also other lipid mediators, including LOX and CYP metabolites in the DSS-treated mice.

Relative mRNA expression of each enzyme

Next, we assessed the mRNA expression of each enzyme that produced lipid mediators in the inflamed colon tissue (Fig. 5). The expression of cpla2, which digests fatty acids from cell membranes, significantly increased (16.6 ± 10.9-fold) on day 4. The expression of cox-2 also increased (7.0 ± 2.8-fold) on day 4. The expressions of 15-lox and 5-lox tended to increase (but not significantly) on day 4 and day 7, respectively (15-lox, 33.5 ± 28.6-fold on day 4; 5-lox, 6.7 ± 4.1-fold on day 7). The expression levels of cox-1 and cyp4a12a/b did not change through all the phases.

DISCUSSION

DSS-induced colitis in mice mimics the innate immune responses, such as infiltration of granulocytes, observed in patients with IBD (especially in patients with UC) (5). In the present study, using this disease model, we revealed the dynamic changes taking place in the production of 58 lipid mediators during the progression of colitis.

Our analysis revealed that the n-6 fatty acid-derived lipid mediators were mainly produced during the induction phase, while the n-3 fatty acid-derived ones were mainly produced during the recovery phase. Two potential reasons may be responsible for this change in the fatty acid used for the derivation of the lipid mediators. The first reason may be that the lack of n-6 fatty acids may lead to metabolization of n-3 fatty acids. Supporting this hypothesis, the amount of AA (an n-6 fatty acid) drastically decreased after the induction of colitis, while that of EPA and DHA (n-3 fatty acids) did not change. The second reason may be that the isoform of active PLA2, which digests fatty acids from cell membranes, changes as the disease progresses. Several isoforms of

Fig. 2. Production of n-6 and n-3 fatty acid-derived lipid mediators. A: Each bar shows the relative amount of each lipid mediator present on the respective day compared with that present on day 0. Each of them is numbered based on B. The horizontal axis indicates the days (4, 7, 9, and 18), while the vertical axis indicates the relative amount (log2) of each lipid mediator. The bars in the front row depict the relative amounts of the n-6 fatty acid-derived lipid mediators, while the bars in the rear row depict the relative amounts of the n-3 fatty acid-derived lipid mediators. B: The heat map shows the relative amount of each lipid mediator present on the respective day compared with that present on day 0. The red columns indicate percent increases and the blue columns indicate percent decreases of the indicated lipid mediators compared with day 0.
PLA$_2$, such as cPLA$_2$ and Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$), are present. A previous study demonstrated that the inhibition of cPLA$_2$ reduced the production of AA (an n-6 fatty acid), while the inhibition of iPLA$_2$ reduced the production of DHA (an n-3 fatty acid) (20) in the rat brain astrocytes. Indeed, we found a transient increase in the expression of cpla$_2$ only in the induction phase of colitis.

An epidemiological study showed that continuous intake of food containing a high amount of n-6 fatty acids increased the risk of developing UC in humans (21). Inhibition of the synthesis of LTB$_4$, an n-6 fatty acid-derived lipid mediator, ameliorated the symptoms of TNBS-induced colitis in rats (22). In contrast, continuous intake of food containing a high amount of n-3 fatty acids has been demonstrated to ameliorate the symptoms of colitis in mice and IBD in humans (23, 24). Treatment with the n-3 fatty acid-derived lipid mediator, resolvin D$_1$/D$_2$, decreased neutrophil activity and resulted in the amelioration of the symptoms of DSS- and TNBS-induced colitis in mice (25). These observations suggested that changing the fatty acid used for deriving lipid mediators was crucial for healing colitis.

Our analysis related to the metabolic pathways for biosynthesis of lipid mediators showed that the COX-derived lipid mediators were mainly produced during the induction phase, while the LOX-derived ones were mainly produced during the recovery phase. Consistently, the expression of COX-2 increased in the induction phase, while expression of 5-LOX tended to increase in the later acute inflammation phase. Previous studies have reported that the expression of COX was observed to be upregulated in the colonic epithelial cells and lamina propria of UC patients (26). In contrast, several reports demonstrated a considerably high expression of 5-LOX in the infiltrating neutrophils (27). Bannenberg et al. (28) suggested the relationship between infiltrating cell events and production of lipid mediator with progression of peritonitis in mice. Here, we showed that the intestinal morphology changed drastically according to the phase of disease progression, epithelial detachment, and neutrophil/monocyte infiltration. These changes in gene expression and morphology were likely to influence the involvement of different enzymes as well as substrates.

Although several studies have focused on the role of COX in colitis, its actual role remains unclear. Long et al. (29) reported that the regular intake (greater than five doses per month) of COX inhibitors (such as nonsteroidal anti-inflammatory drugs) increased the risk...
of developing IBD, while El Miedany et al. (30) reported that the selective inhibition of COX-2 was beneficial for the IBD patients. PGE₂ is a major COX-derived lipid mediator. A previous study demonstrated that the pharmacological stimulation of EP4, the receptor for PGE₂, aggravated the symptoms of TNBS-induced colitis in
mice (31). Additionally, deletion of the ep4 gene aggravated the symptoms of DSS-induced colitis in mice (9). There have been several contradicting reports regarding the role of LOX. The pharmacological inhibition of 5-LOX or the production of its metabolite (LTB₄) ameliorated the symptoms of TNBS- and DSS-induced colitis in mice (22, 32). In contrast, other studies reported that the pharmacological inhibition of 15-LOX aggravated the symptoms of DSS-induced colitis in mice (33). In the present study, we revealed that nonselective COX inhibition aggravated the DSS-induced colitis accompanied by drastic changes in production of LOX/CYP metabolites as well as COX metabolites. Thus, a detailed investigation of the precise function of each class of lipid mediator, the timing and localization of its biosynthetic pathway, quantity produced, and mechanism of activation would be indispensable for the management of IBD in the future.

We also detected some lipid mediators with unknown functions, such as 13-HoTrE [that was derived from ALA by the action of LOX (17) and increased more than 10-fold during the recovery phase] and 5,6-DiHETE [that was derived from EPA by the action of CYP (17) and decreased to 12.5% of its original level during the acute inflammation phase]. These mediators may play important roles in the progression of colitis.

In this study, we demonstrated using the DSS-induced colitis model in mice that the production of diverse classes of lipid mediators altered dramatically with progression of the disease. These findings may help in revealing the pathophysiology of IBD. Multidimensional regulation of various lipid mediators, along with their substrates and enzymes (synthases) required for their production, would be needed for adopting better therapeutic tactics in combating IBD. 

Fig. 4. Continued.
Fig. 5. Relative mRNA expression of each enzyme. The horizontal axis indicates the days (0, 4, 7, 9, and 18), while the vertical axis indicates the relative mRNA expression of each enzyme compared with that present on day 0. *P < 0.05, compared with day 0.

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