Original Article

Elevation of the serotonin-derived quinone, tryptamine-4,5-dione, in the intestine of ICR mice with dextran sulfate-induced colitis

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(Received 23 September, 2020; Accepted 20 December, 2020; Published online 3 April, 2021)

Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are chronic inflammatory disorders associated with oxidative stress. The intestines produce 5-hydroxytryptamine that may negatively affect disease state under inflammatory conditions when overproduced. 5-Hydroxytryptamine is a substrate for myeloperoxidase and is converted into reactive tryptamine-4,5-dione. Here, an experimental colitis model was established through oral administration of 5% dextran sulfate sodium to ICR mice for 7 days. Furthermore, the formation of tryptamine-4,5-dione in the colorectal mucosa/submucosa and colorectal tissue was analyzed by chemical and immunochemical methodologies. First, free tryptamine-4,5-dione in the homogenate was chemically trapped by *o*-phenylenediamine and analyzed as the stable phenazine derivative. Tryptamine-4,5-dione localization as adducted proteins in the colorectal tissue was immunohistochemically confirmed, and as demonstrated by both methods, this resulted in the significant increase of tryptamine-4,5-dione in dextran sulfate sodium-challenged mice compared with control mice. Immunohistochemical staining confirmed tryptamine-4,5-dione-positive staining at the myeloperoxidase accumulation site in dextran sulfate sodium-challenged mice colorectal tissue. The tryptamine-4,5-dione locus in the mice was partly matched with that of a specific marker for myeloperoxidase, halogenated tyrosine. Overall, the results possibly indicate that tryptamine-4,5-dione is generated by neutrophil myeloperoxidase in inflammatory tissue and may contribute to the development of inflammatory bowel disease.

***Key Words*: inflammatory bowel disease, serotonin, tryptamine-4,5-dione, myeloperoxidase, halogenated tyrosine**

Oxidative stress is defined as stress provoked by an imbalance between reactive oxygen species (ROS) and antioxidant systems in the body. Elevated oxidative stress results in the modification of various biomolecules, including nucleic acids, proteins, and lipids.[1] The accumulation of these modified biomolecules may be associated with various inflammatory diseases, such as inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis.[2]

IBD represents a group of chronic inflammatory disorders that cause patients to suffer from debilitating conditions including chronic diarrhea, abdominal pain, and melena.[3] In the intestines of patients with IBD, the immune regulatory mechanism is dysfunctional and the infiltration of immune cells into the intestinal mucosa is a key histological feature of the inflammation.[4] Among these immune cells, neutrophils and monocytes secrete a heme myeloperoxidase (MPO) enzyme.[5] It has been demonstrated that MPO protein expression increases in the mucosa of patients with IBD.[6] Previous studies have indicated that MPO reacts with various substrates, such as chloride, bromide, iodide, and thiocyanate, and catalyzes the production of hypochlorous acid (HOCl), hypobromous acid (HOBr), and other molecules.[7,8] These reactive intermediates generate chloramines, halogenated tyrosine residues (3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine, and 3,5-dibromotyrosine), and other products. Indeed, 3-chlorotyrosine was increased in an experimental colitis model.[9,10] Furthermore, MPO also uses other endogenous compounds, such as 5-hydroxytryptamine (5HT), urate, and L-tyrosine, as substrates.

5HT is an important monoamine neurotransmitter in the brain and peripheral nervous system. More than 90% of the 5HT is generated in gastrointestinal enterochromaffin (EC) cells.[11] The density of EC cells is increased in the colonic tissue of patients with IBD compared with that of healthy controls.[12,13] An association with increased 5HT content has also been observed in patients with IBD.[12] These phenomena have also been observed in animal models.[14,15]

As mentioned above, 5HT is a likely substrate for MPO, which results in tryptamine-4,5-dione (TD) formation (Fig. 1).[16,17] TD exhibits high reactivity toward thiol moieties in cellular proteins. Dryhurst's group performed pioneering studies on the characteristics of TD, including its stability and reactivity.[16,18] We previously reported the formation of TD by MPO and the site-specific adduction of TD with glyceraldehyde 3-phosphate dehydrogenase, a model thiol-containing protein.[19] We also showed that some cytoskeletal proteins, such as α- and β-tubulins, vimentin, and neurofilament-L, are preferentially modified by TD in SH-SY5Y neuroblastoma cells. Furthermore, the modulation of β-tubulin self-polymerization by TD *in vitro* was reported.[19] Endogenous TD may trigger some biological events in inflammation. The reaction of TD with a protein, particularly the adduction to a thiol moiety, may affect the activity of transcription factors and phosphorylation events that trigger signal pathways, which are involved in the inflammation of colitis.[6]

To date, reports examining TD *in vivo* are few. A monoclonal antibody specific to TD-modified proteins was generated and

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doi: 10.3164/jcbn.20-161

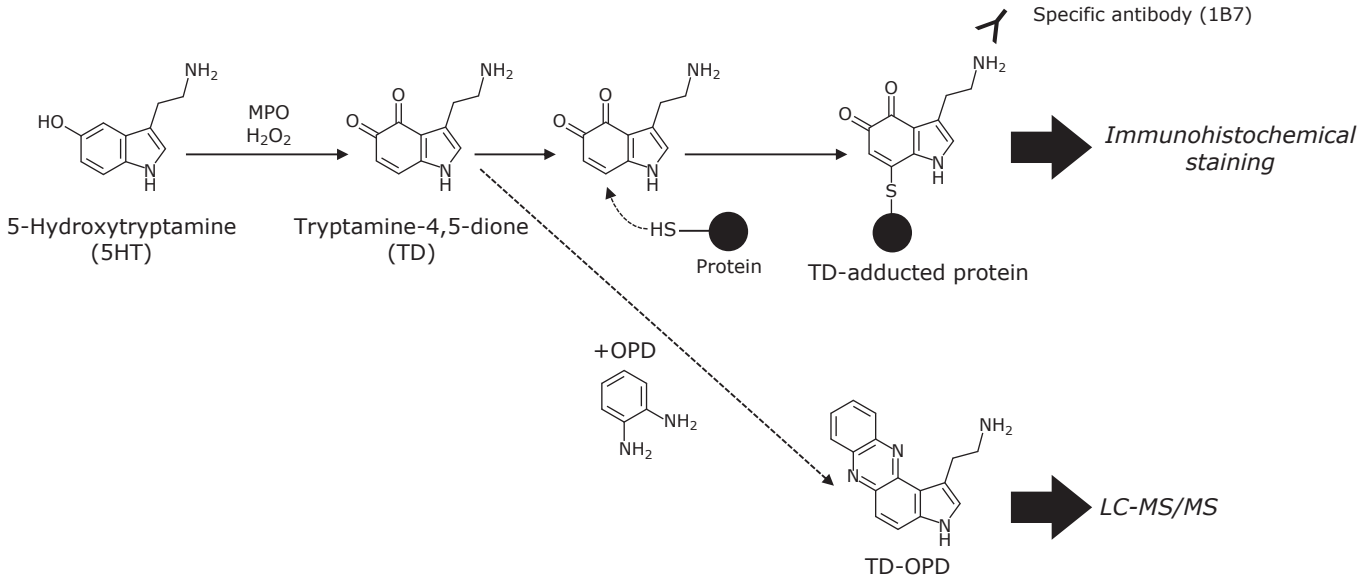

Fig. 1. Generation scheme of tryptamine-4,5-dione (TD) from 5-hydroxytryptamine (5HT) and the formation of a thiol–TD adduct. The formed adduct can be detected by the specific antibody 1B7. Free TD can be trapped and stabilized by reaction with *o*-phenylenediamine (OPD) forming its phenazine derivative (TD-OPD). TD-OPD is quantified by LC-MS/MS.

used for the specific detection of TD as a conjugated form in human atherosclerotic plaques.[20] The production of free TD *in vivo* has only been confirmed in the brain of zebrafish treated with methyl mercury by an electrochemical method.[21] Because TD is quite unstable,[22] a robust method for detecting free TD from biological samples is needed. In this report, to confirm TD formation in an experimental colitis model, both chemical and immunochemical methods were applied to biological specimens from the large intestine of mice.

In the intestine, 5HT is abundant, occurs daily, and is increased in the intestines of patients with IBD.[12,13] Neutrophil MPO is activated primarily at inflammatory sites and is associated with colitis. Taken together, TD may be generated in the inflammatory tissue of colitis patients. To date, however, there have been no reports of TD formation in the gastrointestinal tract. The aim of this study was to detect free TD and TD-conjugated proteins using an experimental colitis model to better understand the 5HT-related events in inflammation.

Material and Methods

Materials. The protein bicinchoninate assay kit, a protease cocktail, and HIKARI (Immunostain Solution B) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Anti-TD-modified protein mouse monoclonal antibody (1B7) and anti-dihalotyrosine mouse monoclonal antibody (3A5) were prepared as previously described.[19,23] Anti-MPO rabbit polyclonal antibody, horseradish peroxidase-labeled goat anti-rabbit immunoglobulin, and the Liquid DAB+ Substrate Chromogen System were obtained from DAKO Japan (Tokyo, Japan). Histofine Mousestain Kit was purchased from Nichirei Biosciences Inc. (Osaka, Japan). Dextran sulfate sodium (DSS) with a molecular weight of 36,000–50,000 was obtained from MP Biomedicals (Santa Ana, CA). Potassium nitrosodisulfonate, 5-hydroxytryptamine hydrochloride, hydrogen peroxide (H_2O_2), *o*-phenylenediamine (OPD), hexadecyltrimethylammonium bromide, ImmunoStar® LD, and isoflurane were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 5-Hydroxytryptamine-d4 (5HT-d4) was obtained from Medical Isotope Inc. (Pelham, NH). *o*-Dianisidine dihydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). Hematoxylin and eosin reagents were obtained from MUTO Pure Chemicals (Tokyo, Japan).

Animals. Overall, 16 female ICR mice (7 weeks old) were obtained from SLC (Shizuoka, Japan) and housed under controlled conditions [21°C and humidity (50 ± 10%) with a 12-h light/dark cycle (lights on from 9:00 to 21:00)]. Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) were followed when maintaining the living conditions and performing the experiments. The study was approved by the Ethics Committee of the University of Hyogo (No. 171, 236).

Induction of colitis. After one week of acclimation, the mice (n = 16) were randomly divided into two groups (n = 8). One group was given *ad libitum* drinking water containing 5% w/v DSS from day 0 to 7. The other group was given distilled water as a control. Each solution was replaced with fresh solution every 2 days. The body weight of each mouse was recorded daily. Disease activity index (DAI) scores, which are based on body weight loss, stool consistency, and stool bleeding, were calculated as previously described.[24] On day 7, the mice were euthanized using isoflurane and blood was collected from the inferior vena cava. The length of the large intestine, which was resected from the colon to the rectum, was measured. The large intestines of three mice from each group were used for immunohistochemical staining. Five large intestines from each group were used for the other experiments. After washing the intestines with ice-cold phosphate-buffered saline (PBS), the colorectal mucosa and submucosa were scraped off and frozen in liquid nitrogen, and the samples were stored at −80°C until use.

Measurement of MPO activity. MPO activity was measured by a previously described method.[25] Briefly, the sample (50 mg/ml) was homogenized in 50 mM hexadecyltrimethylammonium bromide buffer, pH 6.0. The supernatant was mixed with 0.53 mM *o*-dianisidine dihydrochloride plus 0.2 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0. The change in absorbance at 450 nm was measured, and one unit of MPO activity was defined as the amount required to degrade 1 μmol of peroxide per minute at 25°C. Data were expressed as MPO units per mg protein.

Preparation of stable isotopic TD conjugated with *o*-phenylenediamine. Stable isotopic TD-d4-OPD conjugate (internal standard; IS) was prepared by conjugating TD-d4 with

doi: 10.3164/jcbn.20-161

OPD. First, 5HT-d4 (1 mg) was dissolved in 500 µl of water, then added to 6.3 mg of potassium nitrosodisulfonate (Fremy's reagent), and vortexed vigorously.[19,20] The mixture was loaded onto a C_{18} solid-phase extraction (SPE) column (Discovery DSC-18, 500 mg) and washed with 2 ml of water. The purple compound, TD-d4, was then eluted with 1% formic acid in water/CH_3CN (25/75). The eluate containing TD-d4 was mixed with an equal volume of 0.2% OPD solution dissolved in 2% acetic acid–ethanol and incubated for 30 min. After the reaction, the successive isotopic TD-d4-OPD was purified by high-performance liquid chromatography (HPLC) using Combi-RP (20 × 100 mm, Nomura Chemical Co., Ltd., Aichi, Japan) with 0.1% aqueous acetic acid/CH_3CN (7/3) at a flow rate of 5 ml/min. The major peak, exhibiting a characteristic UV-Vis scanning profile of a phenazine derivative,[20] was collected and concentrated.

Quantification of TD trapped by *o*-phenylenediamine. TD was freshly prepared from 5HT as described in the previous section. The isolate from colorectal mucosa and submucosa (20 mg) was mixed with PBS (80 µl) and sonicated on ice with a Branson Ultrasonics Sonifier 150. The samples were centrifuged to remove debris. The supernatant or freshly prepared TD standard (0–1 µM) was mixed with 0.2% OPD solution and incubated for 30 min. To purify the formed TD-OPD (phenazine) from the homogenate, the mixture with IS was applied to a polymer-based reversed-phase SPE column (Phenomenex Strata-X, 30 mg). After washing with 3 ml of 5% methanol in aqueous 0.1% formic acid, the homogenate sample was eluted with 1 ml of 0.1% formic acid in water/CH_3CN (1/1). The samples and standards were analyzed by liquid chromatography quadruple-tandem mass spectrometry (LC-MS/MS, API3000; AB Sciex, Framingham, MA). The separation was done by HPLC (Agilent HP1100) with a Develosil ODS-HG-3 column (2 × 150 mm, Nomura Chemical Co., Ltd.) at a flow rate of 0.2 ml/min using 0.1% acetic acid in water (solvent A) and CH_3CN (solvent B). The gradient program was as follows: initial B0%, 12 min B80%, 13 min B0%, and 30 min B0%. Then, 5 µl of the sample were injected. Positive multiple-reaction monitoring (MRM) transitions were used as follows: TD-OPD 263.4/246.2; IS 267.0/250.2.

***In vitro* model experiments.** Colorectal mucosa/submucosa (25 mg/ml) collected from the DSS-challenged mice was homogenized in CelLytic MT Cell Reagent (Sigma-Aldrich) with a protease inhibitor cocktail. The supernatant was adjusted to 1.7 mg/ml using PBS and incubated with 5HT (100 µM), 4-aminobenzoic acid hydrazide (1 mM), or both at 37°C for 15 min. A bolus of hydrogen peroxide (500 µM) was added three times every 5 min, and catalase (final conc., 0.1 mg/ml) was added to stop the reaction. The samples were mixed with a loading buffer containing Tris(2-carboxyethyl) phosphine hydrochloride and separated using two sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoreses (10% acrylamide). In one gel, the protein was stained with Flamingo™ fluorescent dye (Invitrogen); in the other, the proteins were electro-transferred onto Immobilon-P Transfer Membrane (Merck-Millipore, MA) and blocked with EzBlock Chemi for 1 h at room temperature. After washing the membrane with 0.1% Tris-buffered saline containing 0.05% Tween-20 three times for 10 min each, it was incubated with 1B7 (1 µg/ml) for 2 h at room temperature, rewashed, and incubated with Mouse TrueBlot® ULTRA (1:2,000; anti-mouse IgG horseradish peroxidase) (Rockland Immunochemicals, Pottstown, PA) in Signal Enhancer HIKARI for Immunostain Solution B for 1 h at room temperature. The membranes were washed again, and the protein bands were visualized using ImmunoStar® LD. The image was captured using LAS-1000 Plus Lumino Image Analyzer with Image Gauge software ver. 3.4 (Fujifilm, Tokyo, Japan).

Preparation of tissue sections and hematoxylin and eosin staining. Freshly collected large intestines were washed with PBS and fixed in 10% formalin. The fixed large intestine was made into a "Swiss roll" configuration and embedded in paraffin wax. Tissue sections (3 µm) were mounted onto aminopropyltriethoxysilane-coated glass slides. Hematoxylin and eosin-stained sections were prepared to confirm the histological features.

Immunohistochemistry. The sections were deparaffinized with xylene and rehydrated through a graded series of ethanol solutions. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 30 min at room temperature. For antigen retrieval, sections of 3A5 were activated in a pressure pan (Delicio 6L; T-FAL, Rumily, France) with 0.01 M citrate buffer (pH 6.0) for 10 min. After pressure cooking, the sections were left to cool in the soaking solution for 30 min. Immunostaining for 1B7 and MPO was performed without the antigen retrieval step. For reduced endogenous mouse IgG staining when using mouse primary antibody on mouse tissues, the sections for 1B7 and 3A5 were stained using the NICHIREI-Histofine Mousestain Kit according to the manufacturer's instructions with some modifications. The sections were incubated with anti-TD-modified protein (1B7, 1.0 µg/ml), anti-dihalotyrosine antibody (3A5, 10 µg/ml), or anti-MPO rabbit polyclonal antibody (1:2,000 dilution) overnight at room temperature. The sections were then rinsed with 10 mM PBS (pH 7.2) and incubated with anti-mouse horseradish peroxidase polymer (Histofine Simple Stain MAX-PO) for 1B7 or 3A5. The MPO antibody-treated section was incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin for 30 min at room temperature. Finally, the reaction products were visualized using the Liquid DAB+ Substrate Chromogen System according to the manufacturer's instructions. Nuclei were lightly counterstained with Mayer's hematoxylin.

Statistical analysis. All presented data are representative of two independent experiments. The experiments that assessed significant differences were performed using $n = 5$ samples. The data was analyzed using SPSS ver. 25.0 (IBM, Somers, NY) software. Statistical significance was determined using a Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

Manifestations of colorectal inflammation with DSS model mice. To establish an animal model for colitis, mice were treated with 5% DSS in their drinking water for 7 days. The body weight of DSS-treated mice significantly decreased at day 7 compared with control mice that were given pure water ($p<0.05$) (Supplemental Fig. 1A*). The DAI score was gradually and significantly increased in the DSS group, but not in the control group (Supplemental Fig. 1B*), suggesting that the DSS-treated mice experienced inflammation. At the end of the treatment (day 7), the length of the large intestine in the DSS-treated mice was shorter compared with that in control mice. The histochemical features of the DSS-treated murine intestine revealed a loss of mucosal epithelium, granuloma formation, and inflammation (Supplemental Fig. 1C*). The spleen weight was also increased in the DSS-treated group (Supplemental Fig. 1D*).

Quantitation of free TD in colonic mucosa and submucosa. It is known that TD is highly reactive and unstable.[22] For stabilization, TD in the homogenate of colorectal mucosa and submucosa was derivatized by OPD, and the resulting TD-OPD conjugate as a phenazine derivative was analyzed by a sensitive LC-MS/MS technique as described in Materials and Methods. As shown in Fig. 2A, the standard TD-OPD derivatives, along with their stable isotopic IS TD-d4-OPD, were observed at a retention time of 10.7 min (Fig. 2B). Although endogenous TD-OPD

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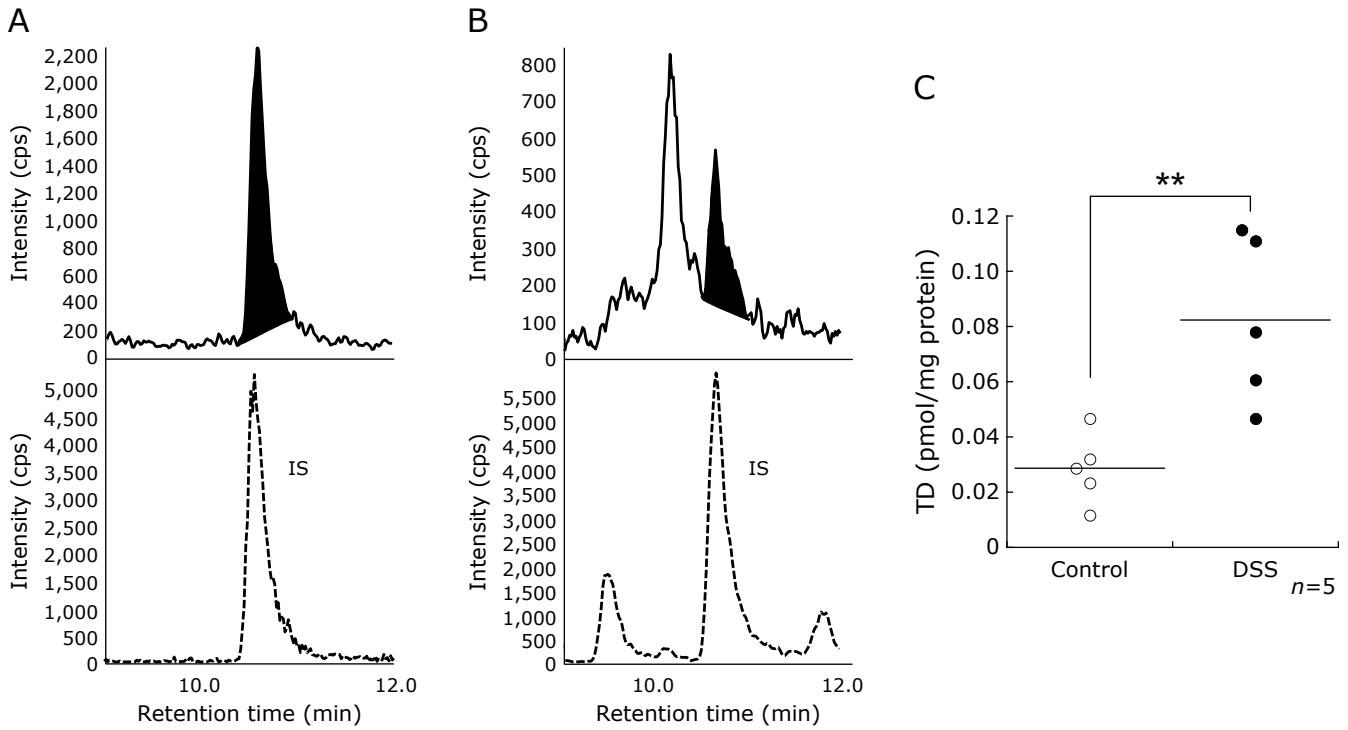

Fig. 2. Chromatograms of multiple-reaction monitoring (MRM) for the TD-OPD derivative and free TD levels in the colorectal mucosa and submucosa from control and DSS-challenged mice. Standard synthetic TD and samples were reacted with OPD reagent, and the resulting TD-OPD was analyzed by LC-MS/MS. (A) The MRM chromatogram of the standard TD-OPD (30 nM of TD) is shown. The dotted line shows stable isotopic TD-d4-OPD used as the internal standard (IS). (B) Representative MRM chromatogram of TD-OPD originating from the homogenate of the colorectal mucosa and submucosa from DSS-treated mice (7 days). Each mouse mucosa was homogenized, and the supernatant was incubated with an OPD solution. TD-OPD, solid line. TD-d4-OPD, dotted line. (C) TD quantitation in the colorectal mucosa and submucosa. TD-OPD from samples were quantified by comparison with the TD-OPD standards. The values were corrected by the protein concentration and expressed as pmol/mg protein. Groups were compared by a Student's *t* test (n = 5, $^{**}p<0.01$). The significance was compared with the control group.

was detected in the homogenates of large intestine (composed of mucosa and submucosa) from control mice (Fig. 2C), the level in the DSS-treated mice was approximately 3.2-fold higher compared with that in the control mice ($p<0.01$, Fig. 2C).

Localization of TD along with other biomarkers in colorectal tissue. As mentioned above, a protein thiol is a predominant target of free TD.[19] TD formation in the colorectal tissue was confirmed as conjugates of TD with tissue proteins using immunohistochemistry with a monoclonal antibody specific to TD-modified protein (Fig. 3).[19] The staining of TD-conjugated proteins was scarcely observed throughout the large intestine of the control mice. In contrast, extensive positive staining was found in the colorectal tissues, particularly in the collapsed mucous membrane, of colitis-induced mice. The co-localization with hypohalous acid-modified proteins was investigated using an antibody specific to 3,5-dihalogenated tyrosine, 3,5-dibromotyrosine and 3,5-dichlorotyrosine.[23] These were formed by the reaction of tyrosine residues with HOCl or HOBr generated from MPO or eosinophil peroxidase. The staining of the hypohalous acid-modified proteins was significantly enhanced in the colitis model, and the localization was mostly consistent with that observed in the staining of TD-modified proteins.

Enzymatic activity of MPO and its localization with TD in colorectal tissue. The activity of MPO, which generates TD from 5HT in the presence of H_2O_2, was also significantly elevated in the colorectal mucosa fraction following DSS administration ($p<0.01$, Fig. 4A). The infiltration of immune cells into the inflamed intestinal mucosa of the DSS-treated mice was observed along with increased levels of MPO (Fig. 4B). MPO accumulation observed in the vicinity of the positively stained TD-modified proteins was confirmed in the colorectal tissue serial sections of DSS-challenged mice (Supplemental Fig. 2*). This indirectly indicated that TD was possibly at least partially generated by MPO. The MPO-positive cells were presumably neutrophils, macrophages, or both expressing MPO. The smeared staining of TD might have been due to MPO secreted by the activated neutrophils in the inflamed tissue. The contribution of endogenous MPO to the formation of TD-modified proteins in the homogenate were examined using the colorectal mucosa/submucosa homogenate from DSS-challenged mice. As shown in Supplemental Fig. 3*, TD-modified proteins were observed in the homogenates incubated with hydrogen peroxide and 5HT. In contrast, the TD level was significantly suppressed in the presence of 4-aminobenzoic acid hydrazide, a known MPO inhibitor, suggesting that endogenous MPO in the homogenate could, to some extent, generate TD from 5HT under higher oxidative stress conditions.

Discussion

To successfully measure TD *in vivo*, an experimental colitis model was prepared by administering DSS to mice, followed by validation of inflammation (Supplemental Fig. 1*). The results indicated that the animals experienced significant colorectal tissue damage induced by DSS exposure. It is known that "fragile" TD can be stabilized by OPD derivatization to form the corresponding phenazine derivative (TD-OPD) in *in vitro* studies.[20] Therefore, we attempted to detect free TD in the form of TD-OPD by LC-MS/MS from the colorectal mucosa

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doi: 10.3164/jcbn.20-161

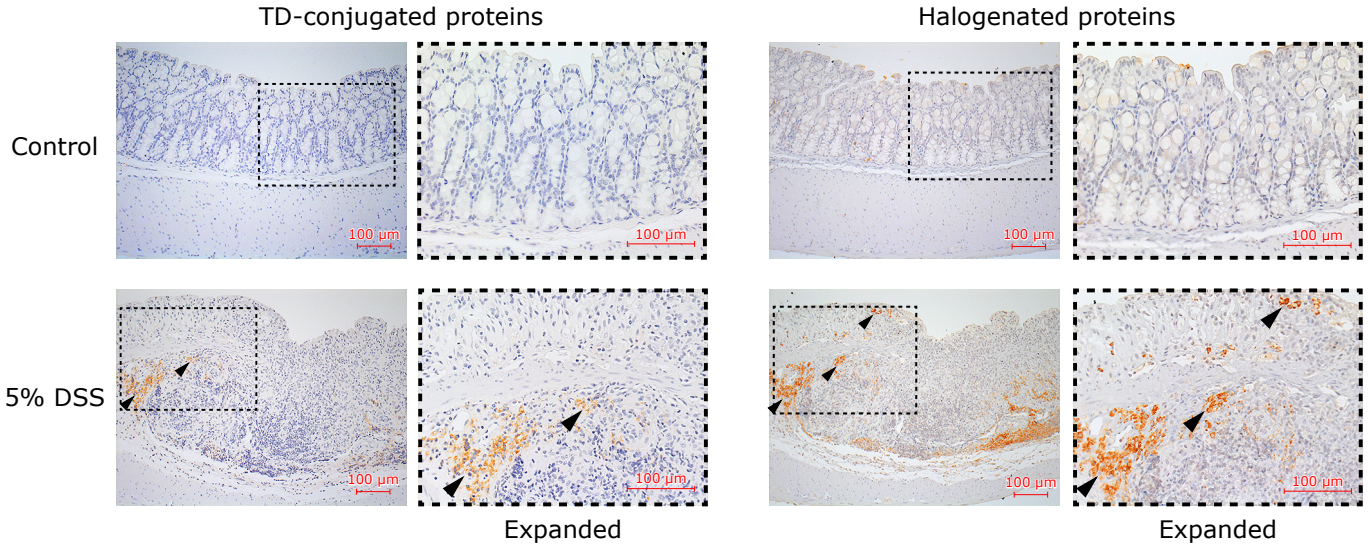

Fig. 3. Immunohistochemical staining of TD-modified and hypohalous acid-modified proteins in the colorectal tissue from control and DSS-challenged mice. On day 7, the colorectal tissues from the control and DSS-treated mice were washed and fixed in 10% formalin. After deparaffinization of the sections, immunohistochemical staining was performed using anti-1B7 or 3A5 antibody as described in Materials and Methods. Representative images of staining of TD-modified or hypohalous acid-modified proteins in colorectal sections of control and DSS-treated mice on day 7. The arrowheads in DSS group indicate the positive staining for TD-modified or hypohalous acid-modified proteins as examples. Scale bars, 100 μm.

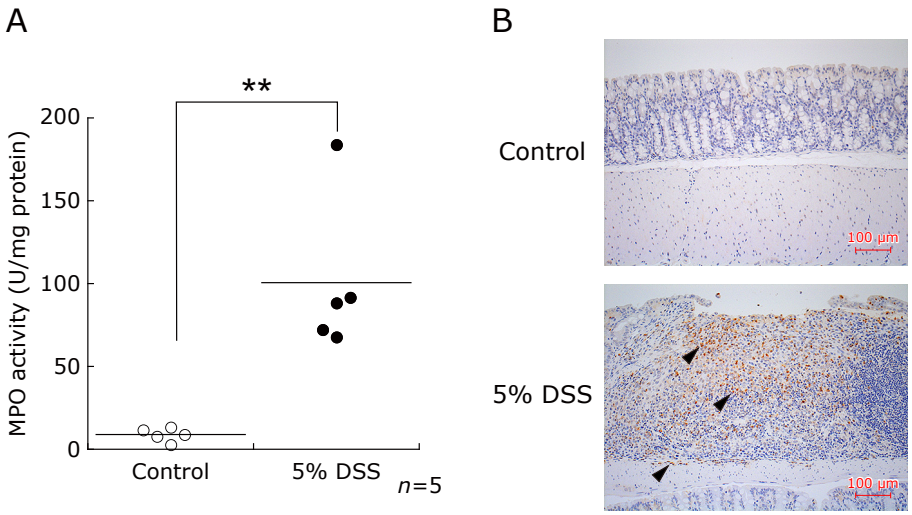

Fig. 4. Myeloperoxidase (MPO) activity and expression in colorectal mucosa and submucosa from control and DSS-challenged mice. (A) MPO activity in colorectal samples from control mice and DSS-treated mice (7 days). The measurement of MPO activity in the homogenate was performed as described in Materials and Methods section. Groups were compared by a Student's *t* test ($n = 5$, $^{**}p<0.01$). The significance was compared with the control group. (B) Representative immunohistochemical staining of MPO in colorectal tissues. On day 7, the colorectal tissues from the control and DSS-treated mice were washed and fixed in 10% formalin. After deparaffinization of the sections, immunohistochemical staining was performed using anti-MPO antibody as described in the Materials and Methods. The arrowheads in the DSS group indicate the positive staining for MPO as examples. Scale bars, 100 μm.

and submucosa from the mice (day 7). TD was significantly increased in the colorectal samples from the colitis-induced mice compared with those of the control mice. Free TD reacts rapidly with glutathione or thiol-containing proteins.[19] Therefore, the observed free TD probably represented a small portion of the total TD formed *in vivo*. To our knowledge, this study is the first to report that chemical identifies endogenous TD from the gastrointestinal tract.

As noted above, TD covalently conjugates with proteins. As an alternative approach for TD detection, immunohistochemical staining with a specific antibody was selected.[19] The staining of TD-adducted proteins was strongly observed in the colorectal tissue from DSS-treated mice (Fig. 3). The existence of free and conjugated TD in tissues suggests that 5HT is oxidized to TD. As mentioned earlier, the production of 5HT in EC cells is often increased under inflammatory conditions in patients with IBD and in experimental models.[12–15] Upon inflammation, neutrophils infiltrate into the colorectal tissue, releasing enzymatically active neutrophil MPO into the tissue, mediating TD conversion from overproduced 5HT,[17] and generating hypohalous acids. MPO enzymatic activity increased significantly in the colorectal mucosa/submucosa homogenate

of DSS-treated mice (Fig. 4A). Positive staining of MPO was observed at the inflamed loci (Fig. 4B). Moreover, as shown in Supplemental Fig. 3*, MPO in the inflamed tissue could generate TD, because the specific MPO inhibitor suppressed the generation of TD-modified proteins. TD and MPO were similarly stained in the colorectal tissue serial sections of DSS-challenged mice; the smeared staining of the TD-adduct was observed at the MPO accumulation site (Supplemental Fig. 2*). These results suggest that MPO is possibly an *in vivo* TD generator. However, TD can also be generated from 5HT and other peroxidase enzymes.[26] Superoxide is also known to generate TD from 5HT, as described by Wrona and Dryhurst.[18] All of these, as well as MPO, might have contributed to the TD produced in the colitis model.

MPO mediates the generation of hypohalous acids that modify protein tyrosine residues into halogenated tyrosine.[27,28] These halogenated tyrosines may increase during inflammation. In fact, increased 3-chlorotyrosine was identified in acid hydrolysates of tissue proteins in an experimental colitis animal model.[10] Moreover, 3-chlorotyrosine was also detected and quantified along with 3-nitrotyrosine and 5-chloro-2'-deoxycytidine in diseased human large intestine samples by LC-MS/MS.[9] We generated a monoclonal antibody specific to dihalogenated tyrosine and used it for immunohistochemical staining of inflamed liver tissue[23] and aged human skin.[29] The antibody was also used for detecting halogenated proteins in rat intestinal mucosa injured by indomethacin,[30] but not used for IBD models. In the present study, halogenated proteins in colorectal tissues were immunohistochemically detected following DSS exposure for the first time (Fig. 3). The localization of the halotyrosine moiety was similar to that of TD-modified proteins.

TD is highly reactive[18] and has been considered to be a neurotoxin.[18,31] In general, a quinone compound covalently forms adducts with thiol moieties; these reactions may be involved in the development of diseases or adaptation to stress.[32] We have reported that the pretreatment of SH-SY5Y cells with TD prevented cell death and suppressed intracellular ROS generation evoked by H_2O_2 exposure.[33] This may occur because cells primed with TD upregulate the expression of the phase-II antioxidant enzymes NAD(P)H: quinone oxidoreductase 1 and heme oxygenase-1. The *in vivo* production of TD in inflammatory tissue was confirmed in this study. Endogenous TD may play a pro- or anti-inflammatory role in the underlying colitis conditions. However, the role of TD in inflammation remains uncertain. Time-dependent TD level changes and the role of TD in colorectal inflammatory tissue in DSS-challenged mice require further investigation.

In conclusion, we successfully detected free endogenous TD as its derivative in the large intestine. TD-conjugated proteins in tissues were immunohistochemically identified in the inflamed large intestine caused by DSS exposure. TD levels, measured by both methods, were significantly increased in tissues from a DSS-induced experimental colitis model. Our finding of *in vivo* TD formation provides new insights to understand the complete picture of inflammation accompanied by the overproduction of 5HT and accumulation of MPO.

Author Contributions

NS planned and performed all experiments and wrote the draft of this paper. AM, HA, ST, and MI advised and supported this study. KS performed the immunohistochemical analysis and discussed the results. YK designed and supervised the study and also wrote the paper.

Acknowledgments

This work is partly supported by grants from the Iijima Memorial Foundation for the Promotion of Food Science and Technology. We are thankful to Ms. Akemi Morita for supporting the experiments. We also thank Dr. Shinsuke Hisaka (Meijyo University) and Dr. Akina Kawara (Okayama University) for their helpful advice on immunohistochemistry.

Conflict of Interest

No potential conflicts of interest were disclosed.

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*See online. https://doi.org/10.3164/jcbn.20-161

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