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Cox-2 expression, PGE_2 and cytokines production are inhibited by endogenously synthesized *n*-3 PUFAs in inflamed colon of *fat-1* mice

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Abstract

There is great interest in the role of polyunsaturated fatty acids (PUFAs) in promoting (*n*-6 class) or inhibiting (*n*-3 class) inflammation. Mammalian cells are devoid of desaturase that converts *n*-6 to *n*-3 PUFAs. Consequently, essential *n*-3 fatty acids must be supplied with the diet. We have studied the effect of endogenously produced *n*-3 PUFAs on colitis development in *fat-1* transgenic mice carrying the *Caenorhabditis elegans fat-1* gene encoding *n*-3 desaturase. Colonic cell lipid profile was measured by capillary gas chromatography in *fat-1* and wild-type (WT) littermates fed standard diet supplemented with 10% (w/w) safflower oil rich (76%) in *n*-6 polyunsaturated linoleic acid (LA). Experimental colitis was induced by administrating 3% dextran sodium sulphate (DSS). Colitis was scored by histopatological analysis. Cyclooxygenase-2 (*Cox-2*) expression was evaluated by real time polymerase chain reaction. Prostaglandin E_2 (PGE_2) levels and cytokine production were determined by enzyme and microsphere-based immunoassays, respectively. The *n*-6/*n*-3 PUFA ratios in colonic cells of *fat-1* mice were markedly lower (9.83±2.62) compared to WT (54.5±9.24, $P<.001$). Results also showed an attenuation of colonic acute and chronic inflammation in *fat-1* mice with significant decreases in PGE_2 production ($P<.01$) and Cox-2 expression ($P<.01$). High levels of colitis-induced proinflammatory cytokines, interleukin (IL)-18, IL-1α, IL-1β, IL-6, monocytes chemotactic proteins 1, 2 and 3 (MCP 1,2,3), matrix metalloproteinase 9 and tumor necrosis factor α (TNF-α) were down-regulated in DSS acutely and chronically treated *fat-1* mice. The expression of *fat-1* gene in the colon was associated with endogenous *n*-3 PUFAs production, decreased *Cox-2* expression, increased PGE_2 and cytokine production.

Keywords: Cox-2; Fat-1 mice; Colitis; Inflammation

1. Introduction

Experimental observations and epidemiological studies support evidence that dietary long chain *n*-3 PUFAs prevalent in fish oils, protect against the development of inflammation-driven tumorigenesis in the colon [1–4]. In contrast, *n*-6 class dietary PUFAs, abundant in safflower oil and other common seeds have been shown to exert a proinflammatory action [4].

Several mechanisms have been proposed to explain how dietary intake of *n*-3 PUFAs attenuates the inflammatory process. These include eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) competitive inhibition of arachidonic acid (AA), metabolism to proinflammatory eicosanoids, inhibition of inflammatory cytokine production, and metabolism of *n*-3 PUFAs to anti-inflammatory mediators [4–6]. Extensive reviews pertaining to dietary *n*-3 PUFAs and their modulation of the inflammatory process in animal models and in patients with inflammatory bowel disease (IBD) have been published [4,7,8].

Much evidence is available showing that the ratio between dietary *n*-6 and *n*-3 PUFAs, rather than the absolute amount of each class of PUFAs, influences the inflammation process and the associated drive to colonic neoplasia [9]. It is noteworthy that the *n*-6/*n*-3 PUFA ratio in Western diets is 15:1, indicating that these diets are deficient in *n*-3 PUFAs and greatly enriched in *n*-6 PUFAs [10].

Mammalian cells are unable to synthesize *de novo* *n*-6 and *n*-3 PUFAs and fatty acid precursors such linoleic acid (LA) (18:2 *n*-6) and α-linolenic acid (ALA, 18:3 *n*-3) must be supplied to the diet as essential fatty acids. Importantly, *n*-3 and *n*-6 PUFAs are not interconvertible in mammalian cells. Unlike mammals, however, the free-living nematode *Caenorhabditis elegans* expresses a *n*-3 fatty acid desaturase that introduces a double bond into *n*-6-fatty acids at the *n*-3 position of the hydrocarbon chains to form *n*-3 PUFAs [11]. Transgenic mice have been generated that express the *C. elegans fat-1* gene and, consequently, are able to efficiently convert dietary PUFAs of the 6 series to PUFAs of 3-series, such as EPA (20:5 *n*-3) and DHA (22-6 *n*-3) [12]. The desaturase reaction was shown to occur in a number of tissues to varying degrees, ultimately leading to a significant decrease in *n*-6/ *n*-3 PUFA ratios [12]. The *fat-1* mouse

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line allows controlled studies to be performed without multiple diet manipulations and permits elucidation of cellular and molecular events induced by endogenously synthesized *n*-3 PUFAs [13].

Dextran sodium sulphate (DSS)-induced colitis is a widely used murine model of IBD [14]. Following DSS administration, the mouse colon regenerates slowly during several weeks and inflammatory cells infiltrate the colonic mucosa. Melgar et al. [15] described a method to follow the progression of acute colitis to chronicity in C57BL/6 mice by monitoring colonic inflammation during 4 weeks following DSS removal. At day 26, increased body weight, few or absent clinical symptoms, a high inflammatory score, high levels of cytokines and histopathological changes characterized the chronic phase.

Eicosanoids are key mediators and regulators of inflammation and are generated from 20 carbon PUFAs [16,17]. Inflammatory cells typically contain a high proportion of *n*-6 PUFA AA and low proportions of other 20-carbon PUFAs. AA is the major substrate of cyclooxygenase-2 (Cox-2) for eicosanoid synthesis. Eicosanoids, which include prostaglandins, are involved in modulating the intensity and duration of inflammatory responses [16,17]. Induction of colitis in laboratory animals results in the appearance of inflammatory eicosanoids such as prostaglandin E_2 (PGE_2) and LTB_4 in the colonic mucosa.

The major aim of this study was to determine in *fat*-1 mice the effects of endogenously synthesized *n*-3 PUFAs on the development of DSS-induced colitis from the acute phase to the chronic recovery phase. To monitor the development of the inflammatory process, we assessed histopathological changes, *Cox-2* expression, PGE_2 levels and cytokines production in colons of wild-type (WT) and *fat-1* mice.

2. Material and methods

2.1. Animals and diet

Breeder transgenic mice expressing *C. elegans fat-1* gene encoding a *n*-3 desaturase were provided by Dr. J.X. Kang, Department of Medicine and Dermatology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. The WT C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed at the Rockefeller University Animal Facility at a 12 h light/dark cycle with controlled temperature and humidity (20°C, humidity above 60%) and were given water *ad libitum*. Studies were conducted in strict compliance with animal protocols approved by the Rockefeller University Institutional Animal Care and Use Committee. The *fat-1* mice originally received were fourth-generation offspring of mixed (C57Bl/6J×C3H) genetic background. Backcrossing with inbred WT C57 BL/6J to a syngenic background was performed using the accelerated backcrossing/speed congenic procedure. The *fat-1* mouse line used in the present study is on a ~99.8% homogenous C57BL/6J background. After weaning, mice were fed standard AIN76A diet for 7 weeks supplemented with 10% safflower oil (safflower diet); safflower oil contains 77% *n*-6 linoleic acid (Research Diets, New Brunswick, NJ, USA). This diet containing ~0.1% *n*-3 fatty acids of total fat supplied has been previously used to efficiently test the conversion of *n*-6 to *n*-3 PUFAs in tissues of *fat-1* mice [12].

2.2. RNA isolation, DNA synthesis, reverse transcriptase-polymerase chain reaction analysis

Colonic tissues were dissected from 9-week-old mice fed safflower diet for 7 weeks. Following euthanasia by carbon dioxide asphyxiation, the entire length of the colon was removed from the ileal-cecal junction. The colon was then gently washed with cold saline, weighted and rapidly frozen at −80°C. Total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) including DNase I treatment to remove contaminant genomic DNA. First strand cDNA was generated from 1 μg RNA by using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carisbad, CA, USA) in presence of random primers (300 ng) and 10 mM dNTP Mix (Promega), following the instructions provided by the manufacturer. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in 1 μL of cDNA reaction mixture with forward primer 5′-TGTTCATGCCTTCTTCTTTTTCC-3′ and reverse primer 5′-GCGACCATACCTCAAACTTGGA-3′ for *fat-1*; forward primer 5′-ATCTCAGCACTGCATCCTGC-3′ and 5′-CACCATAGAATCCAGTCCGG-3′ reverse for *Cox-2* using PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) at the following cycling program: 94°C for 3 min (1 cycle); 94°C for 30 s, 60°C for 30 s, 68°C for 1 min 30 s (40 cycles); and 72°C for 7 min (1 cycle). The linearity of the dissociation curve was analyzed using the Opticon Monitor 2 software (Biorad, Hercules, CA, USA), and the mean cycle time of the linear part of the curve was designated Ct. Each sample was analyzed in triplicate and normalized to β-actin using the following equation: $2^{(\Delta Ct)}$ where $\Delta Ct = \text{mean } Ct_{Cox\text{-}2} - \text{mean } Ct_{\beta\text{-}actin}$.

2.3. Conversion of n-6 PUFAs to n-3 PUFAs in mouse colonic tissues

Analysis of fatty acid composition was performed using whole colons ($n=20$, for each experimental group) by gas chromatography using a simplified method previously described in detail [18]. Briefly, an aliquot of tissue homogenate (<50 μl) in a glass methylation tube was mixed with 1 ml of hexane and 1 ml of 14% BF_3/MeOH reagent. After blanketed with nitrogen, the mixture was heated at 100°C for 1 h, cooled to room temperature and methyl esters extracted in the hexane phase following addition of 1 ml H_2O. The samples were centrifuged for 1 min, and the upper hexane layer was removed and concentrated under nitrogen. Fatty acid methyl esters were quantified with gas chromatography/ mass spectrometry by using HP-5890 Series II gas chromatograph equipped with Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA, USA) attached to an HP-5971 mass spectrometer. The injector and detector were maintained at 260°C and 280°C, respectively. The oven program were initially kept at 150°C for 2 min, then ramped to 200°C at 10°C/min and held for 4 min, ramped again at 5°C/min to 240°C, held for 3 min and finally ramped to 270°C at 10°C/min and maintained for 5-min carrier gas-flow rate was maintained at a constant 0.8 ml/min throughout. Total ion monitoring was performed encompassing mass ranges from 50 to 550 atomic mass units. Fatty acid mass was determined by comparing areas of fatty acids to that of fixed concentration of internal standards.

2.4. Induction of colitis by DSS administration

Acute and chronic colitis was induced by administration of 3% DSS (36,000–50,000 MW; MP Biomedicals, Solon, OH, USA) in drinking water. The DSS solution was prepared daily. Mice fed 10% safflower diet for 7 weeks were treated with DSS for 5 consecutive days and sacrificed 2 days after DSS removal for assessment of acute colitis and at 26 days to follow the progression from acute to chronic inflammation according to Melgar et al. [15]. Control mice received drinking water only. Clinical symptoms including stool consistency and fecal bleeding (Hemoccult, Beckam Coulter, Fullerton, CA, USA) were recorded in control and DSS-treated mice. To avoid a confounding effect of individual variations among mice studied, we examined a large number of transgenic and control mice with acute colitis ($n=40$) and chronic colitis ($n=52$). Mice were weighed once a week and particular attention was given to abnormal signs of distress and malaise (e.g., decreased roaming, dullness, loss of weight, roughened hair coat).

2.5. Tissue collection and histopathological analysis of colitis

Following euthanasia by carbon dioxide asphyxiation, whole colon was removed from the ileal-cecal junction to the rectum, carefully rinsed with cold saline and colon length was measured. The whole colon was injected with 10% neutral buffered formalin and rolled up in a tissue cassette. Tissue sections (5 μm) were prepared and stained with hematoxylin and eosin for histological examination. Colitis stages were scored and recorded by histopathological analysis, according to morphological criteria described in detail by Cooper et al. [19]. These include: grade 0, normal colonic mucosa; grade 1, shortening and loss of the basal-one third of the actual crypts with mild inflammation and edema in the mucosa; grade 3, loss of all crypts with severe inflammation of the mucosa with surface of epithelium intact and grade 4, loss of crypts and of surface epithelium with severe inflammation in the mucosa, muscolaris propria and submucosa.

2.6. Measurement of PGE_2 levels in colonic tissue

Whole mouse colons ($n=12$) after careful removal of adherent fat were washed with cold phosphate-buffered saline, immediately snap-frozen in liquid nitrogen and stored at −80°C until use. Colonic tissue was homogenized using a glass-Teflon tissue homogenizer in ice-cold 500-μl lysis buffer containing (pH=7.4) 10 mM Tris HCl, 1 mM EDTA, 10 μM indomethacin and 1% protease and phosphatase inhibitors cocktails (Sigma, Saint Louis, MO, USA). Tissue homogenates were transferred to microcentrifuge tubes vortexed, and centrifuged at 4° for 10 min at 10,000×*g*. Five microliters of supernantant from each sample were withdrawn to determine protein concentration using a *DC* Protein Assay Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. Determination of PGE_2 levels was carried out using a PGE_2 Monoclonal Enzyme Immunoassay Kit (Cayman Chemical Ann Arbor, MI, USA). After dilution of 1:100 with EIA buffer, samples were successively diluted to 1:50 and 1:25 in EIA buffer and run in triplicate concomitantly with a standard curve. Absorbance at 405 nm was measured in a microplate reader (PerkinElmer, Woodbridge, Canada).

2.7. Multi-analyte profiles of inflammatory proteins in colonic tissue

Multi-Analyte profiles (MAPs) were collected measuring several tissue antigens as biomarkers of inflammation. MAPs analysis was performed using Luminex xMAP technology developed by Rules-Based Medicine (RBM, Austin, TX, USA). The Luminex Technology is a microsphere-based assay in a single reaction assigning each inflammation antigen-specific assay a microsphere set labeled with a unique fluorescence signature. Lysates from normal/inflamed colonic tissues ($n=8$ for each

group) were prepared according to Carothers et al. [20]. Briefly, mucosal tissue was removed by gentle scraping with the edge of a microscope slide. The material harvested by scraping was collected in standard lysis buffer supplemented with a complete protease inhibitor cocktail, homogenized and centrifuged at 12,000×*g*. The resulting clear supernatant was frozen at −80°C until use.

2.8. Statistical analysis

Data are expressed as mean values±S.E. or S.E.M. Student's *t* test (independent two population *t* performed with Origin 6.0) was applied to determine statistically significant differences between two mean values. $P<.05$ was considered significantly different.

3. Results

3.1. n-3 fatty acid desaturase activity in colonic cells

Incorporation of *C. elegans n*-3 fatty acid desaturase into the mouse genome of colonic cells was assayed by semiquantitative RT-PCR (data not shown). Having determined the presence of *C. elegans n*-3 desaturase mRNA in mouse colonic cells, we next assayed *n*-3 fatty acid desaturase activity by determining the fatty acid profile and the *n*-6/ *n*-3 PUFA ratios in colonic cells of *fat-1* and WT littermates using total lipid methylation and capillary gas chromatography. The concentration of fatty acids in the colonic tissue is presented as percentage of total fat. The results show a marked difference in fatty acid profiles with increased amounts of *n*-3 PUFAs (ALA 18:3, EPA 20:5, docopentaneonic acid [DPA] 22:5) and lowered amounts of *n*-6 PUFAs (LA 18:2, AA 20:4) in colonic tissues of transgenic mice compared to WT mice (Table 1). Consequently, the *n*-6/*n*-3 PUFA ratios in colonic cells of *fat-1* mice were markedly reduced (9.83± 2.62) compared to control mice (54.5±9.24, $P<.001$, Fig. 1).

3.2. n-3 PUFA modulation of acute and chronic ulcerative colitis

Acute and chronic experimental ulcerative colitis models were produced in mice by administering 3% DSS in drinking water. Clinical symptoms of acute colitis were observed during the 5-day administration of DSS; mice that developed acute colitis showed diarrhea and marked rectal bleeding. Peak levels of diarrhea and visible fecal blood were found on Day 5. The fecal bleeding was aggravated in WT compared to *fat-1* animal. Body weight loss was not consistently observed in the both mouse groups during DSS administration (data not shown). Histopathological analysis of acute ulcerative colitis was carried out using hematoxylin and eosin stained sections of the whole colon by selecting in multifocal area of ulcerative colitis, the area having the highest level of inflammation. The inflammation score of *fat-1* mice (2.67±0.2) was significantly reduced compared to WT (3.57±0.1; $P<.01$, Fig. 2A). The control mice showed more pronounced histopathological changes during acute ulcerative colitis, characterized by partial loss of mucosal and complete loss of crypt epithelium with collapse of lamina propria, mild to moderate mucosal to submucosal neutrophilic inflammation, mild submucosal edema, multifocal lymphatic ectasia and multifocal crypt epithelial hyperplasia at margins of ulceration. No evidences for re-epithelialization or colon length was found. Fecal bleeding was more sustained in WT compared to transgenic animals (data not shown). Acute colitis in C57BL/6J mice does not resolve after DSS removal and it progresses to a severe, chronic colitis [15]. At 26 days, the inflammation score in colons of transgenic mice (1.92±0.1) was significantly reduced compared to control mice (2.7±0.16; $P<.01$), Fig. 2B. Chronic inflammatory process was characterized by having areas with acute ulcerative colitis signs with partial epithelial re-epithelialization. No differences in colon length were found. Fecal bleeding was more sustained in WT compared to transgenic animals (data not shown).

Table 1
PUFA composition of total cellular lipids from colons of WT and transgenic *fat*-1 mice

% PUFAs	WT	*fat-1*
ALA 18:3 *n*-3	0	0.06±0.01
EPA 20:5 *n*-3	0	3.4±0.60
DPA 22:5 *n*-3	0	1.4±0.52
DHA 22:6 *n*-3	0.93±0.1	3.11±0.73
LA 18:2 *n*-6	25.78±1.5	17.80±0.98
AA 20:4 *n*-6	15.78±0.9	11.90 ±0.7
Total *n*-3 FA (%)	0.93±0.1	7.97±0.60
Total *n*-6 FA (%)	41.56±1.2	29.7±1.4

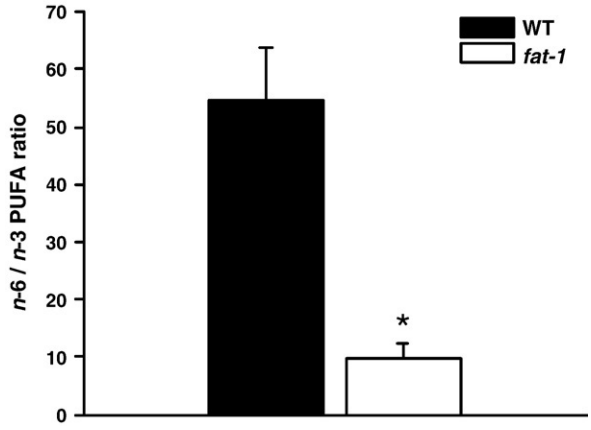

Fig. 1. *N*-3 desaturase enzymatic activity in colonic cells from *fat-1* and control mice. *N*-6/ *n*-3 PUFA ratios for each group ($n=20$). *$P<.001$ vs. WT.

3.3. Cox-2 expression

Cox-2 transcript levels were evaluated in RNA extracted from colons of untreated and DSS acute/chronic inflamed colons of WT and *fat-1* mice. Results (Fig. 3) show comparable low levels of colonic *Cox-2* expression in both untreated WT and transgenic mice. *Cox-2* expression was significantly increased in WT mice during DSS-induced acute and chronic stages of inflammation; in contrast, notwithstanding DSS administration, *Cox-2* mRNA levels were significantly down regulated ($P<.01$) in colons of *fat-1* mice.

3.4. PGE_2 production by normal and inflamed colons of WT and fat-1 and mice

Analysis of *ex vivo* PGE_2 production in control, acute and chronic inflamed colonic tissues was performed on WT and *fat-1* mice. A quantitative enzyme immunoassay (ELISA) for PGE_2 demonstrated no significant differences between basal colonic PGE_2 levels (Fig. 4) of WT (90.76±7.5 pg/ml) and transgenic mice (59.5±4.2 pg/ml; $P<.01$). In contrast, the PGE_2 levels assayed during the DSS-induced acute inflammation were significantly higher in colonic cells of WT animals (1084±30.8 pg/ml) compared to *fat-1* mice (587±35.3 pg/ml; $P<.01$). Similarly, in DSS-induced chronic colitis the PGE_2 amounts were significantly lower in colons of transgenic mice (784±47.3 pg/ml; $P<.01$) compared to WT littermates.

3.5. Analysis profile of cytokine production

The cytokine profile was assessed on whole colon of WT and transgenic *fat-1* mice following acute and chronic DSS treatment. The values, expressed as fold increase over control values of WT mice, are reported in Table 2. Proinflammatory interleukins IL-1β, IL-7, IL-18 and tumor necrosis factor α (TNF-α) levels were reduced in both

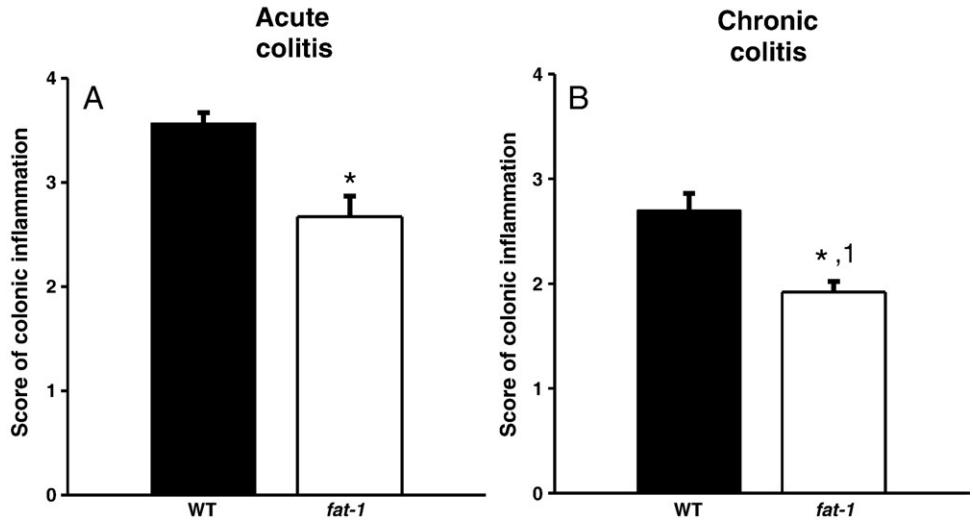

Fig. 2. Inflammation scoring of DSS induced acute (A, n=40) and chronic ulcerative colitis in *fat*-1 and control mice (B, n=52). Data are mean±S.D. *P<.01 vs. WT.

acute and chronic inflamed *fat-1* colons compared to WT controls. By contrast, IL-1α, IL-4 and IL-6 were elevated only during acute colitis and anti-inflammatory IL-10 levels were predominantly high during chronic colitis. High levels of the monocytes chemotactic proteins (MCP)-1, 3 and 5 in colons of WT mice were decreased in *fat-1* mice during both the acute and chronic phase. Similarly, the macrophage inflammatory proteins, MIP 1α, 1β and 1χ, were reduced in acute inflamed *fat-1* colons; and the high content of MIP-3β in DSS-treated WT mice was markedly lower in *fat-1* mice following DSS-induced inflammation. Matrix metalloproteinase (MMP)-9 and T plasma haptoglobin levels, a marker of the acute phase of inflammation, were higher in *fat-1* mice compared to WT controls (data not shown).

4. Discussion

The results reported herein show that transgenic *fat-1* mice expressing *C. elegans* *n*-3 fatty acid desaturase were able to convert constitutively *n*-6/*n*-3 PUFAs and consequently markedly lowered the *n*-6/ *n*-3 PUFAs ratio in colonic cells. The endogenous long chain *n*-3 PUFAs production was associated with significant attenuation of DSS-induced histopathological changes during acute and chronic colitis. These results, first published in abstract form [21], strengthen the view that *n*-3 PUFAs exert an anti-inflammatory action, and are in agreement with studies reported by other investigators [22]. Another significant finding of the present study was that colonic PGE_2 levels in DSS-treated *fat-1* mice were markedly reduced compared to WT control mice. In contrast with a previous study [22] a decrease in PGE_2 content was observed in both acute and chronic colitis.

A number of plausible mechanisms have been proposed to explain the biological effectiveness of *n*-3 PUFAs in inhibiting PGE_2 formation. It has been suggested that increased amounts of EPA and DHA, the main *n*-3 PUFAs synthesized in colonic *fat-1* cells, competitively inhibit the incorporation of AA into membrane phospholipids, ultimately resulting in blunted activity of the biosynthetic route leading from AA to the formation of eicosanoids, including PGE_2 [4].

Several observations indicate that the suppressive effect of *n*-3 PUFAs on PGE_2 formation might also result from their action on the

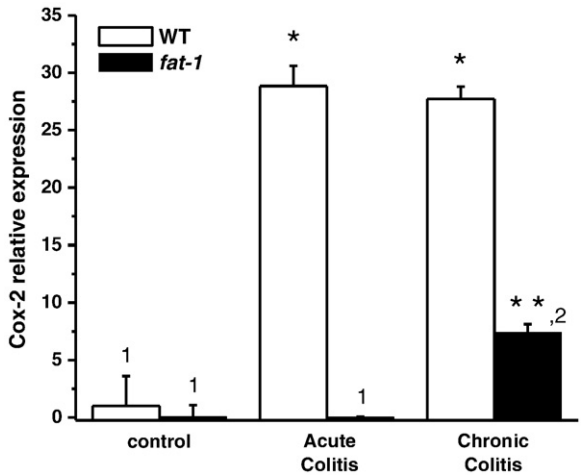

Fig. 3. Cox-2 expression in mouse colonic cells. TaqMan real-time PCR shows basal levels of Cox-2 expression in untreated WT and *fat-1* mice. In both DSS induced acute and chronic colitis increased levels of Cox-2 in WT mice were markedly decreased in *fat-1* mice. Data are represented as mean±S.E.M. of relative values of expression in 3 individual experiments of triplicate samples. **P<.01, *P<.001 vs. control.

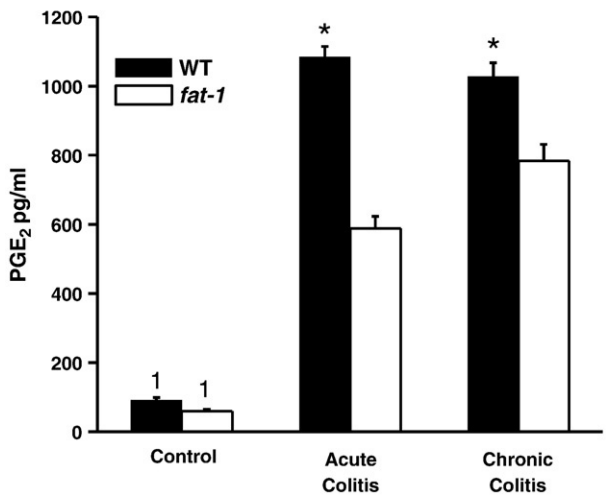

Fig. 4. PGE_2 quantitative enzyme immunoassay in control, acute and chronic inflamed WT and *fat-1* colons. Values are mean±S.D. (n=10 for each group). *P<.001 vs. control.

Table 2
Cytokine fold induction of DSS treated colonic tissue of WT and *fat-1* mice. Values are expressed as fold increase±S.E.M. vs. untreated WT animals [1]

	Acute colitis		Chronic colitis	
	WT	*fat-1*	WT	*fat-1*
Interleukins				
Proinflammatory				
IL-1α	13±0.5**,†††	7.7±0.4†††	1±0.02	1±0.02
IL-1β	9.4±0.05***	1.6±0.02	8.6±1.2***	2±0.6
IL-2	1.3±0.1	1.4±0.1†††	0.7±0.1	0.6±0.02†††
IL-3	2.7±0.3	1.9±1.2	-	-
IL-4	15±2.1**	8.4±0.9††	6.1±0.3	6.3±0.5
IL-5	2±1.6	0.7±0.5	6.1±0.1†††	6.3±0.3†††
IL-6	55±2.1***	41±1.5†††	1.8±0.5	1.7±0.02
IL-7	4±0.6**	1.1±0.4	2.3±0.02*,†	1±0.04
IL-18	6.8±0.4***,†††	0.9±0.1†	26.4±0.1***	0.2±0.04†
Anti-inflammatory				
IL-10	1.3±0.01	1.5±0.03	0.8±0.03*,††	1.4±0.09
IL-11	14.5±0.5***	6.5±0.2	1.5±0.8	1.1±0.01
Monocyte chemotactic protein				
MCP-1	11.5±1.2***,†††	2.7±0.5	2.0±0.7*	1±0.3
MCP-3	9.4±1.1***,†††	1.8±0.3	2.1±0.3*	0.8±0.1
MCP-5	7.6±0.6***,†††	2.4±0.3	2.3±0.6*	0.8±0.2
Macrophage inflammatory protein				
MIP-1α	3±0.1**,†	1.4±0.2	2±0.3**	1±0.03
MIP-1β	9±0.03***,†††	1.3±0.02	1.4±0.04	0.6±0.02
MIP-1χ	20±2.1***,†††	5.6±0.7	1.3±0.05*	0.43±0.005
MIP-3β	8±0.9***,†††	2±0.3	5±0.8***	3±0.023
Others				
MMP-9	18.5±1.2***,†††	5±0.3	9±0.7	2.8±0.47
MPO	375±15.6**,†††	300±9.3	9±0.2	8±0.8
TNF-α	13±0.05***,†††	7±0.2	3±0.04*	2±0.01

[1] Values from treated *fat-1* animals were compared to the untreated WT animals. Paired *t* test between WT and *fat-1* mouse groups during acute and chronic DSS-treatment respectively. *P<.05; **P<.01; ***P<.001 (see Methods). One-way analysis of variance test between acute and chronic DSS treated mouse groups: $^{\dagger}P$<.05; $^{\dagger\dagger}P$<.01; $^{\dagger\dagger\dagger}P$<.001.

PGE_2 biosynthetic pathway downstream AA incorporation into cell membranes, i.e., at the level of phosholipase A_2 (PLA_2), an enzyme pivotal in the hydrolysis of membrane-bound AA, and at the level of COX-2 activity and expression. Thus, PLA_2 activity was significantly decreased in colonic mucosa of rats fed a diet rich in *n*-3 PUFAs as compared to rats maintained on a diet rich in *n*-6 PUFAs [23]. PUFA of the *n*-3 series competed with AA for binding to the COX-2 catalytic site and thereby inhibited prostaglandin formation [24]. DHA and EPA were shown to reduce COX-2 mRNA levels and PGE_2 synthesis in human breast cancer cells and to down-regulate COX-2 mRNA expression in human brain-metastatic melanoma cells [25]. Treatment of a human cholangiocarcinoma cell line with DHA inhibited COX-2 promoter activity in a time- and dose-dependent time [26].

DSS is a potent inducer of *Cox-2* expression in colonic cells (our results, see also Ref. [27]), and therefore, *n*-3 PUFA inhibition of *Cox-2* expression affords an attractive mechanistic explanation for the inhibitory action of *n*-3 PUFAs on colonic PGE_2 synthesis in DSS-treated *fat-1* mice. To the best of our knowledge, this is the first report of an inhibitory action of endogenously synthesized *n*-3 PUFAs on *Cox-2* gene expression in vivo using a genetically-modified mouse without the confounding effects of multiple dietary interventions.

Evidence is available showing that Toll-like receptors (TLR), particularly TLR-4, are regulated by *n*-3 PUFAs [28]. This is an interesting finding since TLR-4 is a potent inducer of *Cox-2* expression in a variety of human and mouse cells, including intestinal cells [28]; of note, after DSS injury, *Cox-2* expression increased only in WT mice but not in TLR-4 null mice [28]. In colonic cells of DSS-treated *fat-1* mice mRNA levels of the Toll-interacting protein (Tollip), a cytoplasmic inhibitor of TLR signaling, were higher compared to control animals, suggesting that *n*-3 PUFAs may differentially act at several sites of the TLR-signaling pathway [22].

While the inhibitory action on PGE_2 formation identifies an important molecular site of action of newly formed *n*-3 PUFA in colonic cells of *fat-1* mice and highlights their relevance in impeding PGE_2 pro-carcinogenic action in many tissues, including the colon [17,29], the role of PGE_2 as an anti-inflammatory agent remains a matter of considerable debate. Thus, a number of studies indicate that in the DSS-induced murine colitis model PGE_2 exerts a protective role in regulating the epithelial response to injury by maintaining mucosal homeostasis through EP4 receptor activation [30,31]. In contrast, a proinflammatory role of PGE_2 in IBD has been recently proposed involving activation of the IL-23/IL-17 axis [32]. Interestingly, the degree of colonic injury caused by dinitrobenzene sulfonic acid administration to rats was markedly reduced following systemic administration of 15-deoxy PGJ_2 [33], indicating that cyclopentenone prostaglandins may also exert a protective effect against inflammation.

Cumulatively, the results indicate that PGE_2 has both anti and proinflammatory actions depending on local concentration and target cells. Changes in the inflammatory action of PGE_2 and other bioactive lipids during stages of acute inflammation known as "lipid-mediator-class switching" have been recently reviewed in detail by Levy et al. [34].

The murine DSS-model of human colitis has been extensively used for studying the production of cytokines and chemokines by inflammatory cells [15]. Our results, derived from a large cytokine and chemokine panel assayed in colons of DSS treated WT and *fat-1* mice indicate that during acute colitis phase mice showed severe clinical symptoms associated with high levels of proinflammatory cytokine production. Interestingly, when the inflammation progressed to a recovery phase following DSS removal, colonic epithelial damage was still evident but cytokine production appeared to be blunted.

The protection afforded against DSS-induced colitis in *fat-1* mice was correlated with a marked decrease in the production of proinflammatory proteins. This inhibitory action was most pronounced during acute colitis. Our results show a marked reduction in proinflammatory interleukins such as IL-1 IL-α, IL-β, IL-6 and IL-18. Similarly, both levels of MCP-1, 3 and 5 proteins that play a key role in the recruitment of monocytes to the sites of injury and infection, and of MIP 1α, 1β and 1χ, proinflammatory molecules endowed with chemotactic activity for granulocytes were significantly decreased in the colons of *fat-1* mice.

MMPs are implicated in many stages of angiogenesis, including matrix degradation, endothelial migration and proliferation, and the release of angiogenic growth factors from the extracellular matrix. The present findings show a marked reduction of MMP-9 content in DSS-treated *fat-1* colon preparations compared to WT control specimens. Susuki et al. [35] reported that inhibition of lung metastasis of colon cancer cell line by EPA and DHA was associated with reduced activity of MMP-2 and 9. It would seem, therefore, that the wide spectrum of anti-inflammatory effects imposed by *n*-3 PUFAs on colons of *fat-1* mice includes reduction of MMP-2 and 9 levels.

In the present study, levels of TNF-α and IL-1α, key components of the nuclear factor κB (NF-κB) pathway, were markedly reduced in DSS-treated colonic *fat-1* cells compared to WT mice (Table 2). This finding together with the previous observation that in DSS- treated mice colonic NF-κB expression was down-regulated [22] indicates that a key inhibitory effect of endogenously synthesized *n*-3 PUFAs is exerted on NF-κB proinflammatory pathway.

An additional level of complexity in the anti-inflammatory effects of *n*-3 PUFAs is provided by the observation that a novel series of DHA and EPA- derived bioactive lipids termed resolvins and protectins exhibit anti-inflammatory activity and play a key role in the resolution of acute inflammation [4]. These lipid mediators have been shown to decrease formation of inflammatory cytokines such as IL-1α and TNF-α. Remarkably, in DSS-treated *fat-1* mice — but not in

WT control animals — levels of resolvins such us RvE1 and resolvin D3 were high during acute colitis [22], signifying that part of the anti-inflammatory action of *n*-3 PUFAs resides on their metabolic conversion to potent bioactive lipid molecules.

In summary, the major findings of the present study are: (i) enhanced intracellular production of *n*-3 PUFA*s* in colonic cells of transgenic *fat-1* mice was associated with a significant reduction of DSS- induced acute and chronic inflammation compared to WT littermates, (ii) notwithstanding sustained feeding of a diet rich in *n*-6, linoleic acid (LA), an AA precursor, the production of PGE_2 was significantly reduced in *fat-1* mice, and this inhibitory effect was associated with marked down-regulation of *Cox-2* gene expression and (iii) elevated *n*-3 PUFA levels in colonic cells of *fat-1* mice were associated with decreased production of proinflammatory cytokines during acute and chronic colitis.

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