MAG-EPA reduces severity of DSS-induced colitis in rats

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Morin C, Blier PU, Fortin S. MAG-EPA reduces severity of DSS-induced colitis in rats. Am J Physiol Gastrointest Liver Physiol 310: G808–G821, 2016. First published March 24, 2016; doi:10.1152/ajpgi.00136.2015.—Ulcerative colitis (UC) is a chronic disease characterized by diffuse inflammation of the intestinal mucosa of the large bowel. Omega-3 (ω3) fatty acid supplementation has been associated with a decreased production of inflammatory cytokines involved in UC pathogenesis. The aim of this study was to determine the preventive and therapeutic potential of eicosapentaenoic acid monoglyceride (MAG-EPA) in an in vivo rats model of UC induced by dextran sulfate sodium (DSS). DSS rats were untreated or treated per os with MAG-EPA. Morphological, histological, and biochemical analyses were performed following MAG-EPA administrations. Morphological and histological analyses revealed that MAG-EPA pre-treatment (12 days pre-DSS) and treatment (6 days post-DSS) exhibited strong activity in reducing severity of disease in DSS rats. Following MAG-EPA administrations, tissue levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 were markedly lower compared with rats treated only with DSS. MAG-EPA per os administration decrease neutrophil infiltration in colon tissues, as depicted by myeloperoxidase activity. Results also revealed a reduced activation of NF-κB pathways correlated with a decreased expression of COX-2 in colon homogenates derived from MAG-EPA-pretreated and treated rats. Tension measurements performed on colon tissues revealed that contractile responses to methacholine and relaxing effects induced by sodium nitroprusside were largely increased following MAG-EPA treatment. The combined treatment of MAG-EPA and vitamin E displayed an antagonistic effect on anti-inflammatory properties of MAG-EPA in DSS rats.

eicosapentaenoic acid; colitis; inflammation; TNF-α

THE INFLAMMATORY BOWEL DISEASES (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal (GI) tract (1). Both are chronic diseases characterized by diffuse inflammation of the intestinal mucosa; UC mainly affects the colon, whereas CD can occur in any part of the GI tract (1). The precise causative agent of IBD is unknown; however, multiple hereditary, environmental, microbiological, and immunological factors are believed to contribute to the pathogenesis (12, 28, 53). In IBD the inappropriate activation of the immune system by intestinal bacteria contributes to increase the intestinal permeability and the breakdown of epithelial tight junctions (1). This leads to increased infiltration of neutrophils and T cells and enhanced levels of proinflammatory cytokines including TNF-α, IL-6, and IL-1β (50).

Immunomodulating agents and corticosteroids have been used to treat IBD, although not always with positive outcomes (29, 39). Thus new therapeutic approaches are needed to decrease GI inflammation and to prevent the recurrence of IBD disease. Current evidence from experimental animal studies, epidemiological studies, and clinical trials indicated that dietary supplementation of omega-3 (ω3) polynsaturated fatty acids (PUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) could be efficient in the treatment of IBDs, which would decrease the need for steroids and prevent the recurrence of UC and CD diseases (23, 58). Prospective cohort studies suggest that ω3 PUFA intake is associated with a decrease and higher ω6 intake with an increased risk of developing UC (19, 23). The protective effects of ω3 PUFA may be due to the modulation of inflammatory events that take place within the GI environment (63). Several mechanisms to explain the benefits of ω3 PUFAs are widely held, including 1) via substrate competition, preventing conversion of arachidonic acid into proinflammatory eicosanoids such as prostaglandins (PGs) and leukotrienes (LTs); and 2) serving as an alternative substrate to produce less potent 5-series LTs and 3-series PGs and thromboxanes (54). ω3 PUFAs were found to competitively inhibit the production of PGE2 and LTB4, which in turn inhibit the activation of NF-κB and thus the release of inflammatory cytokines such as IL-1β and TNF-α (37, 58). ω3 PUFA-derived mediators, including E-series resolvins such as resolvin E1 from EPA as well as D-series resolvins and protectins such as protectin D1 from DHA, exert potent anti-inflammatory, inflammation-resolving, and immunomodulatory actions both in vitro and in vivo (54).

Dietary ω3 PUFA in experimental rodent models of IBD, including DSS colitis, has shown beneficial effects. In a UC experimental model, rats supplemented with cod liver oil had reduced colonic damage and inflammation compared with the sunflower oil group (63). Fish oil supplementation also resulted in lower colonic inflammation and a decrease in LTB4 in colonic tissue (70) in UC rat model. Recently, it has been shown that systemic treatment with aspirin-triggered resolvin D1 or resolvins D2 resulted in an improved disease activity score, reduced colonic damage, and a decrease in neutrophil infiltration in two mouse models of colitis (5). Many human supplementation studies in IBD have assessed the ability of ω3 PUFA on clinical outcomes. Several studies reported decreased gut mucosal disease activity and improved histological scores following ω3 PUFA intake (2, 20, 59). Interestingly, although there appear to be some benefits of ω3 PUFA in IBD, meta-analyses have failed to confirm this on relative risk of relapse in UC and reduction in requirement for corticosteroids (32, 62). It is therefore of interest to investigate whether eicosapentaenoic acid monoglyceride (MAG-EPA) can have beneficial effects on intestinal inflammation. Indeed, MAG-EPA compounds are nontoxic and well absorbed by the GI tract, and their metabolites are found in blood circulation and tissues (16,
Fatty acids in monoglyceride form confer increased bioavailability of ω3 and are generally recognized as safe and are widely used as emulsifying agent in the food industry. Moreover, ω3 PUFA monoglycerides have been demonstrated to increase the oral bioavailability of ω3 PUFA compared with commercially available marine oil (13, 16, 36, 37). Preclinical and clinical studies showed that the intestinal absorption of DHA and EPA given as ethyl ester (EE) was lower than seen in the case of TG or free acid (14, 31). Moreover, Dyerberg et al. (14) and Cruz-Hernandez et al. (13) have shown that EPA in the form of monoaoylglyceride alone or in reesterified triglycerides increases the oral bioavailability of EPA compared with natural triglyceride form made from fish oil. Moreover, Banno et al. (4) demonstrated that DHA monoglycerides and diglyceride are absorbed and transported more effectively than DHA-TG and EE in rats under a water-restricted condition. In a preclinical model, Cruz-Hernandez et al. have shown that malabsorption due to enzyme insufficiency may lead to decreased circulating and tissue levels of EPA and such a deficiency can be reversed by using MAG provided as sn-1(3)-MAG or protected sn-2-MAG (13). Hence, Philippoussis et al. (41) demonstrated that lipids in monoglyceride form display better induction of apoptosis in T cells compared with corresponding free fatty acid. According to these data, we thought that the MAG-ω3 might be favorable in terms of absorption and utilization efficiency. The aim of this study was to evaluate possible anti-inflammatory and resolving effects of MAG-EPA in DSS-induced colitis in rats on disease severity, selected markers of inflammation, and mechanical properties of colon tissue samples. Results indicate that MAG-EPA exerts potent anti-inflammatory and resolving effects in DSS-induced colitis in rats, a finding consistent with the inhibition of NF-κB pathway.

**MATERIALS AND METHODS**

**Synthesis of EPA monoaoylglyceride.** MAG-EPA was synthesized as previously described with highly purified EPA EE as starting material. In the resulting molecule, EPA is attached at the sn-1 position of glycerol (16).  

**DSS rat model of colitis.** Dextran sulfate sodium (DSS, molecular weight: 36 ± 50 kDa; MP Biomedicals, Cleveland, OH) was used to initiate induction of UC. DSS-induced colitis causes to the rodents to exhibit many symptoms characteristic of human UC, including bloody feces, diarrhea, and weight loss, as well as histopathological lesions characteristic of human UC, including mucosal ulceration and shortening of the large intestine. Wistar rats weighing 180–200 g were obtained from Charles River Laboratories (Montreal, QC, Canada). Rats were housed in our animal facilities in a 12:12-h light-dark cycle, at 22 ± 2°C ambient temperature, and maintained on normal rodent chow and tap water ad libitum. Rats were acclimated 7 days before starting the experiments. All studies involving animals were approved by the institutional animal care committee of the Université du Québec à Rimouski (protocol no. CPA-53-13-121). Rats received 4% DSS (molecular weight: 36 ± 50 kDa; MP Biomedicals) in their drinking water for 6 days until loose stools, diarrhea, and macroscopic hematochezia appeared. At day 6 when the mentioned symptoms appeared DSS was reduced to 0.4% in their drinking water for 6 days. Rats were randomly assigned to group control, DSS, DSS + MAG-EPA pretreated, DSS + MAG-EPA treated, and DSS + MAG-EPA + vitamin E treated (6 day post-DSS administration); n = 6 rats per group.

MAG-EPA (318 mg/kg) and vitamin E (53 mg/kg) were given orally directly to the back of the mouth with a pipette tip. MAG-EPA treatments were administrated daily. The oral dose of 318 mg/kg was chosen according to Health Canada Draft Guidelines to obtain a human equivalent dose of 3.0 g/day (60% of the maximum daily dose allowed by Health Canada). Moreover, the dose of vitamin E 53 mg/kg was also based on the recommended dose allowed by Health Canada (500 mg/day) for human using formula for dose translation described by Reagan-Shaw et al. (44). Animals were observed daily for fluid intake, weight changes, and significant symptoms (e.g., loose stools, diarrhea). Body weight and stool consistency were determined daily for all animals. The disease activity index (DAI) was determined daily as a composite score where weight loss, stool consistency, and rectal bleeding are rated from 0 to 4 as defined in Table 1.

At day 12, the colonic tissue was examined to confirm the presence of experimental UC, and blood and tissue samples were collected for further analyses.

For the oxidative EPA metabolism experiment, rats were randomly assigned to group DSS 0 h, DSS + MAG-EPA 3 h, DSS + MAG-EPA 6 h, and DSS + MAG-EPA + vitamin E 6 h (6 days post-DSS administration); n = 3 rats per group. MAG-EPA (318 mg/kg) and vitamin E (53 mg/kg) were given as a single dose orally directly to the back of the mouth with a pipette tip. At 3 and 6 h posttreatment, the rats were euthanized by cardiac puncture and exsanguinations, which were performed under deep anesthesia by intraperitoneal administration of pentobarbital. Blood and tissue samples were collected for further analyses.

**Histological analysis and MUC2 detection level.** Rats were fixed in 10% buffered formalin and paraffin embedded, after which thin sections (3 μm thick) were stained with hematoxylin and eosin (HE) and Alcian blue according to standard protocols (35, 36). Images were acquired with a Hamamatsu ORCA-ER digital camera attached to a Nikon Eclipse TE-2000 inverted microscope (Nikon-Canada, Mississauga, ON, Canada). Images were obtained (objective ×20 and ×100), from colon thin sections derived from control and DSS as well as from DSS + MAG-EPA-pretreated and treated rats. MUC2 level was determined in colon homogenates by specific ELISA (Cloud-Clone, Houston, TX).

**Cytokine analysis.** TNF-α, IL-1β, and IL-6 levels were determined in colon homogenates by use of specific ELISA (eBioscience, San Diego, CA). The intestine-resident cells and the activated leukocytes in colon produce many cytokines. Therefore, measuring cytokines in colon tissue homogenates represents the best way to determine the level of mediators and the extent of inflammation in colon for studying the efficacy of MAG-EPA in our in vivo model.

**Western blot analysis.** Western blots using specific antibodies against the phosphorylated form of p65 NF-κB (P-p65NF-κB), NF-κB, COX2, and β-actin proteins were performed on colon homogenate fractions. Immunostaining of the blots were digitized and analyzed with Lab-Image software 2.7.

**Isometric tension measurements.** The isometric force was measured on unopened ring segment (2–3 mm long, including mucosa, circular layer, and neuronal plexus) mounted between two stirrups in a 10-ml double-jacketed organ bath containing Krebs solution at 37°C, bubbled continually with the 95% O₂-5% CO₂ mixture, to which an initial load of 1 g was applied. The mechanical effects induced by specific agonists were measured as previously described (36). Tissues were allowed to equilibrate for 1 h in Krebs solution and washed out every 15 min until stable tension was reached.
15 min. Passive and active tensions were assessed by using transducer systems (Radnoti Glass Technology, Monrovia, CA) coupled to LabChart 8 software (Grass-Astro-Med, West Warwick, RI) for facilitating data acquisition and analysis.

**Online SPE-LC-MS analysis of EPA oxidative metabolites.** EPA oxidative metabolites were detected in colon homogenates by using the method described by Kortz et al. (27). A 1100 series HPLC system coupled with a tripleQuad 6410 mass spectrometer (Agilent) with electrospray ionization in negative ionization mode. Single ion monitoring m/z 317.22 (EPA+1[O]), m/z 333.21 (EPA+2[O]) and m/z 349.21 (EPA+3[O]) was recorded.

**Smooth muscle and epithelial cell isolation and culture.** Smooth muscles were isolated as described in Morin et al. (38). Minced colon sections (1 mm or less) were incubated 1 h in a shaking water bath at 37°C, 5% CO₂ into a Hank’s balanced salt solution (HBSS) supplemented with 1% antibiotics, 0.2% type IV collagenase, and 0.05% type IV dlastase. The cell suspension was filtered through a sterile 100-μm mesh to remove the undigested tissue. Then, the cell suspension was centrifuged at 400 g for 10 min and resuspended in RPMI culture medium supplemented with 10% fetal bovine serum and 0.3% antibiotics. The cells were plated in culture flasks and treated with culture medium supplemented with 10% fetal bovine serum and 0.3% antibiotics. The cells were positive for this marker. Epithelial cells were isolated by the method of Rusu et al. (47). Intestinal fragments of rat colon were washed in warm (37°C) HBSS supplemented with 1% antibiotic. After several washes, fragments were immersed in the digesting solution consisting in HBSS supplemented with 0.1% collagenase and 0.01% dispase, for 20 min at 37°C in a shaking bath. Then, intestinal segments were longitudinally wide opened and the predigested epithelium was scraped with a sterile cotton swab. The resulting material was incubated in HBSS to stop the enzymatic reaction. The cell suspension was filtered through a sterile 100-μm mesh and pelleted by centrifugation at 400 g for 10 min. The pellet was suspended in RPMI medium supplemented with 100 nM hydrocortisone, 20 nM triiodothyronine, 1 ng/ml epidermal growth factor, 1 μg/ml insulin, 10 μg/ml acid linoelic/albumin, 1% Glutamax, and 1% nonessential amino acids, with 1% antibiotic solution and 2% FBS, and the cells were maintained at 37°C in a humidified incubator (5% CO₂). To validate the quality of our smooth muscle cell preparation we have performed α-actin smooth muscle staining on cells isolated from rat colon. We have obtained that 95% of cells were positive for this marker. Epithelial cells were isolated by the method of Rusu et al. (47). Intestinal fragments of rat colon were washed in warm (37°C) HBSS supplemented with 1% antibiotic. The animals in the MAG-EPA-pretreated group showed significant protection against the change in DAI score compared with nontreated rats (Tables 1 and 2).

**RESULTS**

**Effect of MAG-EPA on disease severity.** We aimed to investigate the potential beneficial effects of MAG-EPA given as pretreatment or treatment in experimental colitis models. We used the well-established DSS chemical model of intestinal inflammation. The DAI represents the grade of colitis that was used to assess the efficacy of MAG-EPA. Animals received a solution of filtered water containing 4% DSS ad libitum during a 6-day period. Following this 6-day period, DSS was replaced by water containing 0.4% DSS in drinking water for 6 days, and at day 12 the animals were euthanized. DSS administration to rat (4% in drinking water) for 6 days induced a severe illness characterized by bloody diarrhea, culminating in a significant increase in DAI score (Fig. 1A), and was maintained until day 12. In the first protocol (MAG-EPA pretreatment protocol) daily oral administration of MAG-EPA (318 mg/kg) was initiated from day 0 and continued until study termination (day 12). The animals in the MAG-EPA-pretreated group showed significant protection against the change in DAI score compared with nontreated rats (Tables 1 and 2).
pared with DSS animals (Fig. 1A). In the second protocol (MAG-EPA treatment protocol) daily oral administration of MAG-EPA (318 mg/kg) was initiated 6 days post-DSS induction and continued until study termination (day 12). As shown in Fig. 1, MAG-EPA-treatment results in a rapid decrease in DAI score from day 7 to 12 associated with a significant improvement of animals conditions. It is generally accepted that colon length is inversely associated with the severity of DSS-induced colitis. Morphological examination of the colonic tissue 12 days after the beginning of the DSS regimen revealed a significant shortening of colon length in the DSS-treated group (10.56 ± 0.48 cm) compared with control rats (14.58 ± 0.13 cm, Fig. 1B). Interestingly, MAG-EPA pretreatment markedly prevented colon length reduction (13.93 ± 0.50 cm, Fig. 1B). Furthermore, our results showed that MAG-EPA treatment 6 days post-DSS administration decreased colon length reduction (13.65 ± 0.35 cm) compared with the DSS group.

Effect of MAG-EPA treatments on DSS-induced leukocyte infiltration in colon tissue. To determine the colonic damage and extent of leukocytes infiltration in the mucosa, HE staining was performed on 3-μm-thick, formalin-fixed, paraffin-embedded colon tissue sections derived from control, DSS, DSS + MAG-EPA-pretreated and DSS + MAG-EPA-treated animals. At day 12, animals in the DSS group display typical histopathological findings characteristic of DSS-induced UC included focal erosions of the epithelium, crypt dilatation, and heavy inflammatory cell infiltration in the mucosa (Fig. 2, A and B). Moreover, hyperemia, surface blood, ulcers, and areas of tissue erosion were observed primarily in the distal colon and rectal mucosa of DSS group, whereas in tissues derived from MAG-EPA-pretreated rats significant histopathological improvements were observed. Thus MAG-EPA pretreatments prevent the progression of DSS-associated lesions and leukocyte infiltration in the mucosa (Fig. 2C). Results also demonstrated that, in the MAG-EPA-treated group, the administration of MAG-EPA 6 days post-DSS ameliorated epithelial erosion and significantly reduced leukocytes infiltration compared with the DSS-untreated group (Fig. 2D).

Effect of MAG-EPA on mucin production in colon tissues. To determine the effects of per os MAG-EPA treatments on mucin production, Alcian blue staining was performed on colon tissue sections derived from control, DSS, and DSS + MAG-EPA-treated animals. Histological analyses revealed goblet cell hyperplasia and heavy mucus production in rat colon tissues derived from the DSS group compared with tissues derived from the control group (Fig. 3, A and B). MAG-EPA pretreatment (12-day period) prevented mucus overproduction induced by DSS (Fig. 3, B and C). In the MAG-EPA-treated group (6 days post-DSS), results in a reduced goblet cell hyperplasia and mucus production in rat colon tissues compared with DSS animals (Fig. 3, B–D). Image analysis of stained colon sections derived from DSS animals revealed a 2.2-fold increase compared with the levels obtained in sections derived from control rats. However, in colon tissue sections derived from DSS animals pretreated and treated with MAG-EPA, reductions of 53 and 35% were observed compared with DSS tissues sections. Mucin levels were measured by specific ELISA in colon tissue homogenates derived from control, DSS, DSS + MAG-EPA-pretreated, and DSS + MAG-EPA-treated rats. As illustrated in Fig. 3E, mucin level was significantly higher in the colon homogenates of DSS rats compared with the corresponding tissue level in control animals (Fig. 3E). In contrast, the level of mucin was lower in the colon of MAG-EPA-pretreated and treated animals compared with untreated DSS-induced rats (Fig. 3E). Moreover, no significant difference was observed between control and MAG-EPA-treated rats (Fig. 3E).

Effects of MAG-EPA on proinflammatory cytokine levels. Previous reports have suggested that cytokines such as IL-1β, TNF-α, IL-6, and IFN-γ are critically involved in the recruitment and activation of inflammatory cells during the progression of intestinal inflammation (9, 12, 50). To investigate the possible mechanisms by which MAG-EPA decreases colitis severity, levels of key proinflammatory cytokines including TNF-α, IL-1β, and IL-6 were measured by specific ELISA on day 12 in colon tissue derived from control, DSS, DSS + MAG-EPA-pretreated, and DSS + MAG-EPA-treated rats. As illustrated in Fig. 4, TNF-α, IL-1β, and IL-6 levels were significantly higher in the colon homogenates of DSS-induced rats compared with the corresponding tissue levels in control animals. In contrast, the levels of these cytokines were lower in the tissue of MAG-EPA-pretreated and treated animals compared with untreated DSS-induced rats. The effects of preventive and therapeutic MAG-EPA treatment on the extent of neutrophil infiltration into colonic tissue were assessed indirectly by measuring myeloperoxidase (MPO) activity. DSS rats displayed a relevant increase in colonic MPO levels compared with control animals (Fig. 4D). Results revealed that pretreatment with MAG-EPA significantly prevented increases in MPO activity (Fig. 4D). Following therapeutic treatment (6 days post-DSS induction) significantly reduced MPO activity level compared with DSS untreated animals (Fig. 4D).

Effect of MAG-EPA on NF-κB pathway activation. The transcription factor NF-κB controls several genes involved in inflammation such as proinflammatory cytokines, chemokines, and signaling enzymes, and its inhibition is able to prevent experimental colitis (49). To determine whether the above anti-inflammatory effects of MAG-EPA is mediated by this pathway, the activation of NF-κB was investigated by Western blot in colon homogenates derived from control and DSS rats treated or not with MAG-EPA. Western blot and quantitative immunoblot analyses revealed that DSS administration resulted in increased phosphorylated p65 subunit staining in colon fractions compared with preparations derived from control rats (Fig. 5A). However, MAG-EPA treatments decreased the phosphorylation level of p65 NF-κB in colon tissues comparatively to that observed in the DSS-induced group (Fig. 5A). A significant reduction of 82 and 81% were quantified in MAG-EPA pretreated and treated rats following comparative analysis of P-NF-κB-to-total NF-κB ratio following normalization of identical immunoblot membrane areas (Fig. 5B). We thus assessed the expression of COX-2 in tissue homogenates derived from control as well as untreated and MAG-EPA-treated DSS rats. Results revealed a significant increase in COX-2 protein expression in DSS rats compared with expression levels in control animals. Pretreatment and treatment with MAG-EPA, however, reduced COX-2 expression level comparatively to untreated DSS animals (Fig. 5C). Following quantitative analysis of identical immunoblot membrane areas normalized as a function of total β-actin staining in corresponding fractions, significant reductions of 80 and 54% were
quantified in MAG-EPA-pretreated and treated rats compared with the ratio quantified in untreated DSS rats (Fig. 5).

**Effect of MAG-EPA on NF-κB activation and COX-2 protein expression level.** Rat primary epithelial and smooth muscle cells were isolated and treated with TNF-α to determine the anti-inflammatory effect of MAG-EPA on NF-κB activation and COX-2 expression level in both cell types. Western blot and quantitative immunoblot analyses revealed that MAG-EPA treatments decreased the phosphorylation level of p65 NF-κB in epithelial and smooth muscle cells induced by TNF-α comparatively to that observed in the TNF-α-treated cells alone (Fig. 6, A–D). Significant reductions of 67 and 66% were quantified in MAG-EPA-treated epithelial and smooth muscle following comparative analysis of P-NF-κB-to-total NF-κB ratio (Fig. 6, B–E). Moreover, we assessed the expression of COX-2 in cell lysates derived from control as well as TNF-α- and TNF-α + MAG-EPA-treated DSS rats. Results revealed that treatment with MAG-EPA reduced COX-2 expression level comparatively to TNF-α-treated epithelial and smooth muscle cells alone (Fig. 6, C–F).

**Effect of MAG-EPA on colon smooth muscle reactivity.** To determine the impact of preventive and therapeutic treatment with MAG-EPA on colonic tissues in DSS-induced animals, experiments were designed to assess the pharmacomechanical properties of distal colonic smooth muscle in response to muscarinic agonist and relaxing agent. Rat distal colon rings

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Fig. 2. MAG-EPA reduced inflammatory cell infiltration in DSS-induced colitis in rats. Hematoxylin and eosin (HE) staining of colon thin sections derived from control (A), DSS (B), DSS + MAG-EPA-pretreated (C), and DSS + MAG-EPA-treated rats (D). The degree of inflammation was assessed by determining the extent of inflammatory infiltrates present in colon sections stained with HE. Scale bar, 50 μm; 6 images were used per group. E: bar graph showing the total leukocytes enumerated per mm² on colon thin section derived from control, DSS-induced, DSS-induced plus MAG-EPA-pretreated, and DSS-induced plus MAG-EPA-treated animals; *P < 0.05.
derived from control, DSS, DSS+MAG-EPA-pretreated, and DSS+MAG-EPA-treated animals were mounted in organ bath and thereafter challenged with methacholine (MCh) and sodium nitroprusside (SNP). Figure 7A shows typical recordings induced by cumulative concentration of MCh on colon rings from control, DSS, DSS + MAG-EPA-pretreated, and DSS + MAG-EPA-treated animals. As can be seen, inflamed tissue derived from DSS colitis rats displayed a marked inhibitory effect on MCh-induced tension compared with tension developed by tissues derived from control rats. In contrast, DSS rats pretreated or treated with MAG-EPA displayed increased MCh-induced contraction (Fig. 7A). Figure 7B depicts the cumulative concentration response curve to MCh in colon tissues derived from control and three series of DSS-induced animals. Whereas DSS consistently induced a decrease reactivity to MCh with an apparent EC50 value of 0.98 μM, treatment with MAG-EPA 6 days post-DSS significantly increased reactivity, with an EC50 value of 0.61 μM. Results also revealed that MAG-EPA pretreatment (EC50 = 0.58 μM) increased MCh-induced contraction compared with the tension developed by tissues derived from DSS rats. Figure 7C demonstrates the relaxing effects induced by 1 μM SNP on 1 μM MCh-precontracted colon rings. In DSS-inflamed tissue, the SNP-induced relaxation was significantly reduced (39.5 ± 3.4%) compared with the responses quantified in control tissues (97.6 ± 2.4%, Fig. 6C). However, MAG-EPA treatment resulted in an increased SNP-induced relaxation (87.5 ± 4.0%) compared with DSS untreated tissues. MAG-EPA pretreatment

Fig. 3. MAG-EPA reduced mucin production in DSS-induced rats. Alcian blue staining of colon thin sections derived from control (A), DSS (B), DSS + MAG-EPA-pretreated (C), and DSS + MAG-EPA-treated rats (D). Images are representative of n = 6 per group. E: mucin level was assessed in colon homogenates by using specific ELISA as described in MATERIALS AND METHODS. Experiment was repeated 6 times, with 6 replicates per group; *P ≤ 0.05.
prevents DSS-induced reduction of SNP relaxing effect in colonic smooth muscle.

Effect of vitamin E on MAG-EPA-mediated anti-inflammatory properties. Fish oil and \( \omega-3 \) supplements often contain small amounts of vitamin E used as an antioxidant to prevent spoilage. To date it was generally accepted that addition of vitamin E could protect and stabilize the \( \omega-3 \) to preserve their benefic effects on inflammation and cell proliferation. However, recently some studies demonstrated that vitamin E in \( \omega-3 \) formulation displays antagonistic effects resulting in an inhibition of beneficial effects of \( \omega-3 \) (10, 17, 30). Thus experiments were performed to determine whether vitamin E exerts synergistic or antagonistic effects on anti-inflammatory properties of MAG-EPA in DSS-induced colitis in rats. Combined treatment of vitamin E (53 mg/kg) with MAG-EPA (318 mg/kg) was given daily 6 days post-DSS administration, after which its effects on DAI, proinflammatory cytokine profiles, and metabolites produced were investigated. Figure 8A demonstrates that combined treatment of MAG-EPA and vitamin E in DSS rats incurred an increase in DAI similar to that of DSS animals. However, treatments with MAG-EPA alone reduced progression of colitis resulting in significant reduction of DAI compared with DSS rats (Fig. 8A). Morphological examination of the colonic tissue revealed a significant shortening of colon length in the MAG-EPA + vitamin E-treated group (11.03 ± 0.29 cm) compared with MAG-EPA-treated rats (13.65 ± 0.35 cm, \( P = 0.031 \), Fig. 8B). Moreover no significant difference was observed between DSS + MAG-EPA + vitamin E-treated and DSS rats (Fig. 8B). The levels of the proinflammatory cytokines TNF-\( \alpha \), IL-1\( \beta \), and IL-6 were determined in plasma derived from control, DSS, and DSS + MAG-EPA-treated rats in the absence and presence of vitamin E (Fig. 8C). Data demonstrate that combined MAG-EPA and vitamin E treatment also abolished the effect induced by MAG-EPA on proinflammatory cytokine levels in DSS rats. EPA oxidative metabolites was determined by SPE-LC-MS (solid-phase extraction liquid chromatography tandem mass spectrometry) in colon homogenate derived from DSS 0 h, DSS + MAG-EPA-treated for 3 and 6 h, and DSS + MAG-EPA + vitamin E-treated rats for 6 h (Fig. 8D). After 3 h, no significant change in EPA\( +1[O] \), EPA\( +2[O] \) or EPA\( +3[O] \) was observed compared with 0 h. At 6 h posttreatment, EPA\( +1[O] \) but not EPA\( +2[O] \) or EPA\( +3[O] \) was significantly increased in the MAG-EPA group but not in MAG-EPA + vitamin E group (Fig. 8D). The levels of TNF-\( \alpha \) were measured by specific ELISA in plasma derived from control and three series of DSS-treated rats. As shown in Fig. 8E, TNF-\( \alpha \) levels were significantly higher in the plasma of MAG-EPA + vitamin
E-treated rats compared with the corresponding plasma levels in MAG-EPA-treated animals. Moreover, no significant difference was quantified between DSS untreated and MAG-EPA + vitamin E-treated rats. Taken together, these results indicate that vitamin E displays antagonistic effects on MAG-EPA anti-inflammatory properties in our rat model of UC.

DISCUSSION

ω3 fatty acids have anti-inflammatory properties and can be beneficial in the treatment of inflammatory diseases, such as UC (58). DSS-induced UC in rats is a well-established model of IBD, which is clinically and histologically similar to human UC (15, 60). It is induced by TNF-α and IL-1β activation and is characterized by neutrophil mucosal infiltration (49). In the present study, daily oral administration of MAG-EPA as preventative and therapeutic treatment in rats exhibited a statistically significant histopathological and clinical improvement. MAG-EPA pretreatment or 6 days post-DSS treatment (human equivalent of 3 g/day) were found to decrease epithelial erosion, neutrophilic infiltration, and proinflammatory marker levels (TNF-α, IL-1β, IL-6, and COX-2) as well as disease severity. We thus propose that MAG-EPA is able to reduce UC severity in a DSS rat model.

Anti-inflammatory effects of MAG-EPA treatments in DSS model of UC. Clinical intervention studies and animal experiments showed that ω3 PUFA have anti-inflammatory properties (58) that are based on their ability to antagonize the activity of arachidonic acid, thereby reducing the production of inflammatory and chemotactic derivatives and suppressing cell-mediated immune responses (8, 11, 46, 58). In the present study, we assessed the ability of preventative and therapeutic treatment with MAG-EPA to resolve inflammation in an in vivo model of UC induced by DSS. Our data revealed that MAG-EPA pretreatment prevented DSS-induced histological injury and resulted in a lower colitis activity score. MAG-EPA 6 days post-DSS displayed resolving properties and reduced colitis severity in our preclinical model. Moreover, histopathological analysis also correlated with the reduction in clinical scores, showing an overall reduction in both inflammation and tissue injury in colon of MAG-EPA-treated animals. Pearl et al. (40) demonstrated that higher levels of arachidonic acid (AA), AA-to-EPA ratio, docosapentaenoic acid (DPA), and DHA and lower level of linoleic acid (LA), α-linolenic acid (ω-LNA), and EPA are seen in inflamed mucosa in UC and correlate with severity of inflammation. This suggests an alteration in fatty acid metabolism in the inflamed gut mucosa, which may offer novel targets for intervention and should be considered if nutritional strategies are used. Prospective studies have shown that total dietary n-3 PUFAs, EPA, and DHA, were associated with protection from UC in a cohort aged over 45 yr (23). Small open-label or pilot studies reported clinical benefits of fish oil supplementation in UC (33, 48). A meta-analysis
identified 13 studies of fish oil supplementation in IBDs reporting outcomes related to clinical score, sigmoidoscope score, gut mucosal histology score, induced remission, and relapse (32). Several studies have indicated that NF-κB is activated in many inflammatory disorders of the human colon in addition to infection, including CD and UC (3, 39, 52, 65, 68). Moreover, we and others have recently demonstrated that the anti-inflammatory actions of ω3 PUFA and MAG-ω3 compounds may partially be explained by inhibition of NF-κB-mediated COX-2 induction and activity (36, 58). In this study, we further demonstrate that MAG-EPA treatment is able to reduce NF-κB activation pathways, resulting in decreased levels of proinflammatory mediators such as COX-2, TNF-α, IL-6, and IL-1β. Treatment of UC with corticosteroids, though improving symptoms, may lead to side effects such as weight gain, cushingoid appearance, osteoporosis, diabetes mellitus, increased blood pressure, increased risk of heart failure, and increased cardiovascular risk (29, 39). As a result, these adverse effects have led to the restricted use of these agents for the treatment of UC. Several lines of evidence indicated that ω3-PUFA could be efficient in the treatment of IBDs, which would decrease the need for steroids and prevent the recurrence of UC and CD (29). It is therefore of key clinical interest to find an

![Fig. 6. MAG-EPA decreased NF-κB activation and COX-2 expression in epithelial and smooth muscle cells. Typical Western blot (A) and subsequent quantitative analysis (B and C) in epithelial cell lysates derived from control, TNF-α, and TNF-α + MAG-EPA using specific antibodies against phosphorylated form (P-NF-κB) and total forms of NF-κB, COX-2, and β-actin. Staining densities in the epithelial cell lysates were expressed as a function of NF-κB signals for P-p65 NF-κB and as a function of β-actin signals for COX-2. Experiment was repeated 6 times, with 6 replicates per group; *P < 0.05. Typical Western blot (D) and subsequent quantitative analysis (E) in smooth muscle cell lysates derived from control, TNF-α, and TNF-α + MAG-EPA by using specific antibodies against phosphorylated form (P-NF-κB) and total forms of NF-κB, COX-2, and β-actin. Staining densities in the smooth muscle cell lysates were expressed as a function of NF-κB signals for P-p65 NF-κB and as a function of β-actin signals for COX-2. Experiment was repeated 6 times, with 6 replicates per group; *P < 0.05.]

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easy-to-use, well-absorbed ω3 PUFA that exerts a resolving effect and consequently has the ability to reduce UC severity.

Enhanced role for ω3 PUFA-derived mediator in UC. The ω3 fatty acids, such as EPA, are highly polyunsaturated and readily undergo oxidation. Certain oxidized lipids, such as resolvins, protectin, and epoxyeicosanoids, which are enzymatically synthesized from EPA (cyclooxygenase, lipoygenase, CYP450 epoxygenase), promote the resolution of inflammation with greater potency than their parent precursors (54). Moreover, it has been proposed that selected anti-inflammatory lipid mediators may reflect organ function and differentiate individual patient status based on their lipidomic profiles (54). Moreover, we also established that MAG-ω3 compounds were metabolized by lipoxigenases and CYP450 to generate metabolites mediating anti-inflammatory effects in our experimental models (35, 36, 37). Hence, we propose that MAG-EPA not only improves the plasma and cell/tissue content of EPA but also increases the production of beneficial oxidized metabolites and thus exerts resolving actions in our model of UC. Our study suggests that the beneficial effects of MAG-EPA in chronic inflammatory UC disease may be due to the oxidative metabolism of EPA and its subsequent inhibition of NF-κB and downstream proinflammatory products in this rat model of UC. The inhibition of EPA oxidative metabolization by vitamin E corroborates the hypothesis of a converse interaction between ω3 PUFA and vitamin E intake on disease severity and inflammatory biomarkers in DSS model of UC. Experimental studies and large clinical trials quite convincingly suggest that antioxidants, including isoflavones, carotenes, and vitamins, should not be recommended for the prevention of lung cancer and that their use may promote tumor growth (45, 51, 57, 66). Moreover, an epidemiological study by Julia et al. (24) demonstrated an inverse relationship between ω3 PUFA intake and elevated levels of CRP in individuals taking vitamin E supplements. Sethi et al. (55, 56) demonstrated that oxidized EPA products but not native EPA inhibited leukocyte adhesion receptor expression and leukocyte-endothelial interactions through the activation of PPARα and subsequent inhibition of NF-κB. Resolvin E1 pretreatment significantly improved the histological damage and clinical score and inhibited the ex-
pression of proinflammatory cytokine including TNF-α, IL-1β, and IL-6, which are associated with DSS-induced colitis (7, 21). One noteworthy characteristic of oxidized lipids such as resolvins, however, is that they contain several double bonds and three hydroxyl groups such that this unstable structure is vulnerable to metabolic inactivation. The use of biochemical precursors or stable analogs such as MAG-EPA therefore represents an interesting alternative approach to overcome such metabolic and structural features of these metabolites. Further investigations would be required to determine which specific oxidized EPA metabolites would be responsible for the anti-inflammatory activity of MAG-EPA in our model of UC.

MAG-EPA prevents decreased intestinal motility in DSS-inflamed colon. Clinical studies suggest that active colitis is accompanied by a decrease in contractile activity in the inflamed area (26, 43, 64). The mechanisms underlying the colonic dysmotility are unclear but may involve changes in colonic smooth muscle contractility (18, 69), enteric neurotransmission (22), or afferent sensory input from the bowel wall (43). Our data demonstrated that, following MAG-EPA treatment, colonic smooth muscle displays increased contractile responses to MCh compared with the tension developed by colonic smooth muscle derived from DSS rats. In vitro studies using colonic muscle strips obtained from patients with UC and from animal models of colitis support the idea that colitis is accompanied by a decrease in the contractility of smooth muscle from the inflamed area (18, 25, 69). Several studies have demonstrated increased concentrations of NO in intestinal mucosa of patients with IBD (34, 42, 61). Increased concentrations of NO not only could result in damage of epithelial cells and apoptosis but also could lead to mucosal vasodilatation, an increased vasopermeability, and a decreased colonic
motility (6). As a result of downregulatory mechanisms, smooth muscle response to NO is inhibited by high endogenous NO production and could lead to a reduced relaxation. Our finding of reduced responses to SNP in tissues from DSS rats supports the finding that colitis alters relaxing properties of colonic smooth muscles. In contrast, our data revealed that MAG-EPA treatment prevented the reduced relaxing responses to SNP in tissues induced by DSS.

Study limitation. In the present work, despite the fact that we have clearly demonstrated that MAG-EPA given per os at a dose of 318 mg·kg⁻¹·day⁻¹ was able to exert anti-inflammatory effects and reduce severity of DSS-induced colitis in rats, we have not ruled out a dose-response curve to determine working concentrations for MAG-EPA in this model. Moreover, tension measurements were performed on intact colon rings, and the presence of the mucosa includes the possibility that the tension induced by pharmacological agents was due to mediators released from other layers of colon wall, rather than via a direct action on the smooth muscle. The effects of MAG-EPA have not been directly assessed in vitro on purified cells, such as epithelial, smooth muscle, and leukocytes, to determine the nuclear translocation of NF-κB by immunofluorescence. It would be of prime interest to assess the specific cellular mode of action to explain the effects of MAG-EPA in related IBD in vitro and in vivo models.

In summary, the present results show for the first time that MAG-EPA without vitamin E effectively ameliorates DSS-induced colitis in rats. Additionally, MAG-EPA is clearly proven effective in modulating intestinal inflammation, which was related to its inhibition of NF-κB pathway and downregulation of some proinflammatory mediators. Furthermore, when administered per os, MAG-EPA represents a stable compound that could serve as a precursor to generate a variety of PUFA-derived mediators such as oxidized EPA metabolites known to directly mediate anti-inflammatory and proresolving effects. Therefore, the present data support the notion that MAG-EPA formulations without vitamin E may constitute a novel strategy to reduce severity of DSS-induced colitis in rats.

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DISCLOSURES

Only S. Fortin declares a potential conflict of interest, since he is the owner of SCF Pharma including a worldwide exclusive license on patented uses of MAG-EPA and composition containing MAG-EPA.

AUTHOR CONTRIBUTIONS

C.M. and S.F. conception and design of research; C.M. and S.F. performed experiments; C.M. and S.F. analyzed data; C.M., P.U.B., and S.F. interpreted results of experiments; C.M. prepared figures; C.M. drafted manuscript; C.M., P.U.B., and S.F. approved final version of manuscript; P.U.B. and S.F. edited and revised manuscript.

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