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Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-κB in rats with TNBS-induced colitis

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Abstract

5-aminosalicylic acid (5-ASA) is widely used for the treatment of inflammatory bowel disease (IBD). Recent studies have evaluated the potential of nutritional intervention as adjunct therapy to 5-ASA in IBD. N-3 polyunsaturated fatty acids (PUFA) have shown potent anti-inflammatory properties in gut inflammation. Therefore, we aimed to evaluate the efficacy of the dual therapy (n-3 PUFA plus 5-ASA) in rats with 2, 4, 6-trinitrobenzen sulfonic acid (TNBS)-induced colitis. Colitis was induced by intrarectal injection of TNBS while control rats received the vehicle. Rats received by gavage a fish oil-rich formula (n-3 groups) or an isocaloric and isolipidic oil formula supplemented with 5-ASA for 14 days. A dose response of 5-ASA (5–75 mg. suppression mg kg^{-1} d^{-1}) was tested. Colitis was evaluated and several inflammatory markers were quantified in the colon. COX-2 expression ($P<.05$) and pro-inflammatory eicosanoids production of prostaglandin E_2 ($P<.001$) and leukotriene B_4 ($P<.001$) were significantly inhibited by n-3 PUFA or 5-ASA therapy. 5-ASA also reduces mRNA levels of tumor necrosis factor α ($P<.05$). n-3 PUFA or 5-ASA significantly inhibits nuclear factor κB (NF-κB) activation ($P<.01$ and $P<.05$, respectively). The dual therapy n-3 PUFA plus 5-ASA also inhibited inflammatory response by lowering NF-κB activation ($P<.01$) or inducing peroxisome proliferator-activated receptor-γ (PPARγ) expression ($P<.05$). These results indicate that 5-ASA plus n-3 PUFAs are more effective than a higher dose of 5-ASA alone to reduce NF-κB activation and to induce PPARγ. By contrast, the dual therapy did not improve the effects of individual treatments on eicosanoids or cytokine production. Use of n-3 PUFA in addition to 5-ASA may reduce dose of standard therapy.

Keywords: n-3 PUFA; 5-ASA; Colitis; Inflammation

1. Introduction

5-aminosalicylic acid (5-ASA) is a conventional treatment used in the management of inflammatory bowel diseases (IBD) [1]. 5-ASA administration in vivo can reduce inflammatory response through several mechanisms including (i) inhibition of nuclear factor κB (NF-κB) activity [2], (ii) inhibition of cyclooxygenase-2 (COX-2) and eicosanoids production [prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4)] [3,4] and (iii) a decrease of the oxidative stress [5,6].

Very recently, studies have evaluated the therapeutic potential of the addition of nutritional intervention to mainstream therapy (5-ASA). Tested nutrients were probiotics [7], curcumin [8] and fibres [9]. This novel therapeutics aimed (i) to improve the effects of the standard treatment (ii) to reduce the treatment dose and (iii) to limit their subsequent costs and side effects. In a small pilot clinical trial, addition of curcumin to standard therapy enables some patients to stop taking their medication [8]. In a double-blind, randomized, placebo-controlled clinical trial, use of probiotics VSL#3 as adjunctive to a standard treatment was safe and reduced inflammatory score in relapsing mild to moderate ulcerative colitis (UC) patients [10]. Probiotics seemed to reinduce remission in these patients but did not reach statistical significance [10]. By contrast, VSL#3 was not effective to reduce cost therapy in pediatric UC patients [7]. Curcumin is a major compound of food flavour turmeric and exhibited anti-inflammatory properties in experimental models of IBD [11–13]. In a small pilot study, curcumin therapy for 2 months improved clinical symptoms and 4 of 5 IBD patients reduced and even stopped their medication [8]. A recent case report has evaluated the effects of n-3 polyunsaturated fatty acids (PUFA) in addition to 5-ASA in a female UC patient [14].

We and others have demonstrated the anti-inflammatory properties on n-3 PUFA [15–17] in experimental models of IBD. A mechanism of action of 5-ASA and n-3 PUFA is the agonism of peroxisome proliferator-activated receptor-γ (PPARγ) [18]. PPARγ is highly expressed in the colon mucosa and regulates intestinal inflammation by interfering with inflammatory signaling pathways such as NF-κB [19,20]. Interestingly, PPARγ can be activated by nutrients such as n-3 PUFA, fibre, curcumin or probiotics [21]. We

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have previously shown in vitro that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were able to reduce production of interleukin (IL)-6 and IL-8 through PPARγ in enterocyte-like Caco-2 cells in response to a pro-inflammatory inducer IL-1β [22].

The present study aimed to evaluate whether the addition of n-3 PUFA can improve the effect of a conventional treatment (5-ASA) in rats with TNBS-induced colitis.

2. Materials and methods

2.1. Animals and study design

Animal care and experimentation complied with both French regulations and European Community regulations (Official Journal of the European Community L 358, 18/12/1986) and R.M.L. is authorized by the French government to use this rat model (Authorization no. 76-106). Sprague-Dawley male rats (Janvier, Le Genest St Isle, France) weighing 200–250 g were randomized into seven groups: control and control colitic (TNBS), TNBS+n-3, TNBS+5-ASA and three groups: TNBS+n-3+5-ASA at 5, 25 or 75 mg kg^{-1} d^{-1}. Each rat was individually kept in a metabolic cage to record daily food intake. Rats were weighed each day. Water and food were provided ad libitum. The rats were killed using an overdose of anesthesia (ketamine and xylasine).

2.2. Diets

Rats received 450 mg kg^{-1} day^{-1} per body weight of n-3 PUFA by gastric gavage because we have recently shown that this dose was efficient to inhibit inflammatory response and oxidative stress in rats with TNBS-induced colitis [15–17]. Rats received once a day, a fish oil formula (Omegaven, Fresenius-Kabi, TNBS+n-3 group and all TNBS+n-3+5-ASA groups), while the other colitic group (TNBS) and the control group received an isocaloric isolipidic formula (intralipides 10%, Fresenius-Kabi) for 14 d (from d−7 to d7). The 5-ASA was administered concomitantly to nutritional formula at: 75 mg kg^{-1} d^{-1} to TNBS+5-ASA group and 5, 25 or 75 mg kg^{-1} d^{-1} to TNBS+n-3 +5-ASA groups. These concentration ranges have been already tested to evaluate the therapeutic actions of 5-ASA in rat colitis [5]. Rats were food-deprived for 24 h prior to induction of colitis and were allowed free access to tap water throughout the study. The composition of both formulas is listed in Table 1.

2.3. Induction of colitis

Colitis was induced at day 0 by administration of 2, 4, 6-trinitrobenzen sulfonic acid (TNBS) (Sigma Aldrich-Company, Saint-Quentin Fallavier, France) as previously described [23]. Colitis was induced in 2 groups at day 0 by intrarectal injection of TNBS, whereas the control group received the vehicle (0.25 ml of 50% ethanol). Briefly, rats were anesthetized with an intraperitoneal injection of ketamine and chlorpromazine following 24 h food deprivation. TNBS dissolved in 50% (v/v) ethanol was instilled into the colon through a canula (25 mg in a volume of 0.25 ml) to induce chronic colitis. Following the instillation of the hapten, the rats were maintained in a head-down position for a few minutes to prevent leakage of the intracolon instillate. Control groups received the vehicle using the same volume.

Table 1
Formula composition

Ingredient (g/100 ml)	Intralipid 10%	Omegaven
Fish oil	–	10
Soybean oil	10	–
Glycerol	2.20	2.50
Egg purified phospholipids	1.20	1.20
Energy (kcal/100 ml)	110	112
Fatty acids composition of the fish oil (g/100 ml)		
14:0		0.1–0.6
16:0		0.25–1.0
16:1(n-9)		0.3–0.9
18:0		0.05–0.2
18:1(n-9)		0.6–1.3
18:2(n-6)		0.1–0.7
18:3(n-3)		≤0.2
18:4(n-3)		0.05–0.4
20:1(n-9)		0.05–0.3
20:4(n-6)		0.1–0.4
20:5(n-3)		1.25–2.82
22:1(n-9)		≤0.15
22:5(n-6)		0.15–0.45
22:6(n-3)		1.44–3.09
∑ (n-3) fatty acids		2.94–6.51
∑ (n-6) fatty acids		0.35–1.55
∑ (n-9) fatty acids		1.1–2.65

Data obtained from Fresenius Kabi.

2.4. Material

Phosphate-buffered saline (PBS) and Protease inhibitor cocktail were purchased from Sigma Aldrich-Company (Saint-Quentin Fallavier, France). The 4–12% Bis-Tris gels, Invitrolon PVDF membranes and Seeblue multi-colored standard were obtained from Invitrogen (Cergy Pontoise, France). The monoclonal antibody anti-PPARγ (sc-7273), the goat polyclonal antibody anti COX-2 (sc-1747), and the secondary antibodies IgG_1 horseradish conjugated were obtained from Santa Cruz biotechnology (Tebu, Le Perray-en-Yvelines, France).

2.4.1. Western blot

Frozen colon samples were homogenized in PBS with 0.1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail (Sigma). Homogenates were centrifuged (12,000×*g*, 15 min, 4°C), and the supernatants were collected. Protein concentration was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (30 μg) were separated on 4–12% NuPAGE gel (Invitrogen) and then transferred to a nitrocellulose membrane (Hybond, GE Healthcare, UK). After blocking, membranes were incubated with specific primary antibodies at the dilution of 1:500 (for COX-2, PPARγ). After three washes, filter was then incubated with the secondary horseradish peroxidase linked anti-goat IgG (for COX-2), and anti-mouse IgG for PPARγ antibody. To check equal loading, the blots were analysed for β-actin expression. Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (Amersham, Arlinghton Heights, IL, USA). Densitometric data were measured following normalisation to the control (housekeeping gene) by a Scientific Imaging Systems (KODAK 1D Image Analysis Software).

2.5. Colon tumor necrosis factor α (TNF-α) and IL-1β mRNA expression

Colon samples were frozen in liquid nitrogen and stored at −80°C before RNA preparation. Total RNA was isolated using the guanidium isothiocyanate method and reverse-transcribed into complementary deoxyribonucleic acids. The mRNA expression of IL-1β and, TNF-α and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured by quantitative RT-PCR.

2.6. Colon cytokines production

Concentrations of TNF-α and IL-1β in the colon were measured in duplicate by the LINCOplex assay (Linco Research, MO, USA). This assay relied on the use of polystyrene beads, each with a unique signature mix of fluorescent dyes that can be discriminated by a laser-based detection instrument (Luminex^{100}, Luminex, Austin, TX, USA). Each bead type was coated with a specific antibody to the cytokine of interest. The cytokine antibody pairs in this multiplex assay do not cross react with other analytes in the panel. The lowest limits of detection were 4.44 $ng \cdot L^{-1}$ for TNF-α and 2.32 $ng \cdot L^{-1}$ for IL-1β.

2.7. Colon PGE_2 and LTB_4 production

Samples of colon were weighed and homogenized in PBS on ice. The homogenates were then centrifuged and filtered before the assay. PGE_2 and LTB_4 concentrations were measured using a commercial rat ELISA kit according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

2.8. Colon NF-κB activation

Nuclear extracts from colon samples were prepared and NF-κB activity was measured according to the method reported in the TransAM NF-κB p65 Transcription Factor Assay Kit (Actif Motif, Rixensart, Belgium).

2.9. Histological studies

Fixed intestinal tissues were embedded in paraffin wax blocks and 5 μm sections were stained with hematoxylin-eosin. Sections were scored by the same pathologist and the samples were blinded. Epithelial necrosis, inflammatory cells infiltration and thickness of the mucosa were assessed using semi-quantitative scores which ranged from 0 to 3 for each variable (0, no inflammation; 1, very low level of inflammation; 2, moderate level of leukocyte infiltration; 3, high levels of leukocytes infiltration and vascular density, ulcerations) using the analysis software Leica QWin (Leica Microsystems, Bensheim, Germany).

2.10. Statistical analysis

Statistical comparisons were performed using GraphPadPrism 5. Data are expressed as mean±S.E.M. Body weight changes and food intake were analyzed with two-way analysis of variance (ANOVA) for repeated measures with Turkey's post hoc tests. All the other variables were analyzed by one-way ANOVA with Bonferroni