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Dietary Marine n–3 PUFAs Do Not Affect Stress-Induced Visceral Hypersensitivity in a Rat Maternal Separation Model[1–3]

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Abstract

Background: Although never evaluated for efficacy, n–3 (ω-3) long-chain polyunsaturated fatty acids (LCPUFAs) are commercially offered as treatment for irritable bowel syndrome (IBS).

Objective: This study was designed to investigate, in a mast cell–dependent model for visceral hypersensitivity, whether this pathophysiologic mechanism can be reversed by dietary LCPUFA treatment via peroxisome proliferator-activated receptor γ (PPARG) activation.

Methods: Maternally separated rats were subjected to hypersensitivity-inducing acute stress at adult age. Reversal was attempted by protocols with tuna oil–supplemented diets [4% soy oil (SO) and 3% tuna oil (SO–T3) or 3% SO and 7% tuna oil (SO–T7)] and compared with control SO diets (7% or 10% SO) 4 wk after stress. The PPARG agonist rosiglitazone was evaluated in a 1 wk preventive protocol (30 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}). Erythrocytes were assessed to confirm LCPUFA uptake and tissue expression of lipoprotein lipase and glycerol kinase as indicators of PPARG activation. Colonic mast cell degranulation was evaluated by toluidine blue staining. In vitro, human mast cell line 1 (HMC-1) cells were pretreated with rosiglitazone, eicosapentaenoic acid, or docosahexaenoic acid, stimulated with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore or compound 48/80 and evaluated for tumor necrosis factor α (TNF-α) and β-hexosaminidase release.

Results: Stress led to visceral hypersensitivity in all groups. Hypersensitivity was not reversed by SO–T3 or control treatment [prestress vs. 24 h poststress vs. posttreatment area under the curve; 76 ± 4 vs. 128 ± 12 ($P < 0.05$) vs. 115 ± 14 and 82 ± 5 vs. 127 ± 16 ($P < 0.01$) vs. 113 ± 19, respectively]. Comparison of SO–T7 with its control showed similar results [74 ± 6 vs. 103 ± 13 ($P < 0.05$) vs. 115 ± 17 and 66 ± 3 vs. 103 ± 10 ($P < 0.05$) vs. 117 ± 11, respectively]. Erythrocytes showed significant LCPUFA uptake in the absence of colonic PPARG activation. Rosiglitazone induced increased PPARG target gene expression, but did not prevent hypersensitivity. Mast cell degranulation never differed between groups. Rosiglitazone and LCPUFAs significantly reduced PMA/calcium ionophore–induced TNF-α release but not degranulation of HMC-1 cells.

Conclusion: Dietary LCPUFAs did not reverse stress-induced visceral hypersensitivity in maternally separated rats. Although further research is needed, claims concerning LCPUFAs as a treatment option in IBS cannot be confirmed at this point and should be regarded with caution. *J Nutr* 2015;145:915–22.

Keywords: diet, irritable bowel syndrome, long-chain polyunsaturated fatty acids, mast cells, maternal separation model, omega-3, omega-6, visceral hypersensitivity

Introduction

Dietary supplementation with n–3 long-chain polyunsaturated fatty acids (LCPUFAs)[9] has been demonstrated to elicit potential health effects ranging from decreased blood pressure in hypertension and cardiovascular disease to a decrease in inflammation in rheumatoid arthritis, inflammatory bowel disease, and asthma (1). Whereas n–3 FAs are thought to act in an anti-inflammatory manner, n–6 FAs are generally considered to be proinflammatory.

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The latter is in particular attributed to the role of n–6 arachidonic acid (AA) and its proinflammatory metabolites (2). In humans consuming a typical Western diet, the membrane phospholipids of blood inflammatory cells contain substantial amounts of AA (usually 10–20% of FAs), whereas the amounts of n–3 EPA (usually 0.5–1% of FAs) and DHA (usually 2–4%) are much lower (3–6). This shift toward n–6 PUFAs is thought to play a role in a variety of diseases (7), and it has recently been shown in female patients suffering from irritable bowel syndrome (IBS) (8). Presently, the beneficial effects of LCPUFAs in IBS treatment are often discussed on patient internet forums; despite the absence of scientific evidence of efficacy, LCPUFA preparations are sold online as a treatment option for this disorder (Internet search: "omega, IBS").

IBS is a gastrointestinal disorder that affects ~11% of the global population (9). Patients experience abdominal pain or discomfort associated with changes in bowel habits without a structural explanation (10). Although the prevalence and socioeconomic impact of IBS are high, the pipeline for novel drugs, especially those targeting abdominal pain, is limited. Visceral hypersensitivity, measured by an enhanced response to colonic distension, is considered an important pathophysiologic mechanism to explain abdominal pain. Indications are that stress-induced mast cell degranulation is an important trigger for visceral hypersensitivity (11). Admittedly, most of these data were obtained in animal models and their validity for IBS is sometimes questioned (12). However, in patient biopsies, mast cells were shown to be in closer proximity to colonic nerve endings, and this was shown to correlate with severity and frequency of abdominal pain (13). Further, supernatants from patient-derived mucosal biopsies were shown to release mast cell mediators capable of activating intestinal nerves in ex vivo settings (14, 15). In a clinical trial, we showed that treatment with the mast cell stabilizer and histamine-1 receptor antagonist ketotifen successfully increased the threshold of discomfort (meaning lowered sensitivity to colonic distension) and decreased other symptoms in hypersensitive IBS patients (16). Based on these trial results, we next evaluated the peripherally restricted histamine-1 receptor antagonists ebastine and fexofenadine in the rat maternal separation model for IBS. Both antihistaminics reversed poststress visceral hypersensitivity in rats (17) More recently, van Wanrooij et al. (18) translated these results into a successful double-blind randomized control trial. In concert, these results suggest that the activation of colonic mast cells and subsequent activation of sensory afferents by mast cell mediators plays an important role in visceral hypersensitivity and IBS symptom generation.

Although current knowledge of the effects of n–3 LCPUFAs on mast cell activation is limited, dietary supplementation of n–3 LCPUFAs has proven beneficial in several other diseases in which mast cells are thought to be relevant, e.g., rheumatoid arthritis, asthma, and allergic disorders (1–3, 7, 19, 20). In addition, in in vitro experiments, treatment with different n–3 LCPUFAs, including marine n–3 EPA and DHA, reduced both the production and release of a variety of mast cell mediators (21–23). The immune modulatory actions of n–3 LCPUFAs are elicited via several mechanisms, including competition with AA for the 2-acyl position of membrane phospholipids, thereby influencing concentrations of metabolites and/or hormones that regulate the behavior of cells. In addition, n–3 LCPUFAs activate nuclear peroxisome proliferator-activated receptor γ (PPARG), which negatively regulates inflammatory cytokine production by interfering with the activation of transcription factors (1). Importantly, PPARG agonists also reduce the production and release of histamine by mast cells (24, 25). Moreover, in the rat maternal separation model, we showed reversal of poststress visceral hypersensitivity with 2 different histamine-1 receptor antagonists (17). These data indicate that dietary n–3 LCPUFA supplementation may prove beneficial, possibly via PPARG-mediated mast cell modulation, in IBS. Given the current internet attention and premature commercialization of the LCPUFA hypothesis in relation to IBS, preclinical studies addressing this hypothesis are eagerly awaited. Thus, we investigated whether dietary supplementation with marine n–3 LCPUFAs, in the form of tuna oil, could reverse poststress hypersensitivity to colonic distension in the maternal separation model.

Methods

Ethics statement. All procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethical Committee of the Academic Medical Center at the University of Amsterdam (reference protocol number 100998).

Rats. Long-Evans rats (Harlan, Horst) were bred and housed at the animal facility of the Academic Medical Center in Amsterdam under conditions of controlled light (0600–1800), temperature (20–22°C), and humidity (45%). Water and food were consumed by the rats ad libitum.

Maternal separation. In earlier papers (26–28), we demonstrated that when subjected to maternal separation, Long-Evans rats showed an enhanced response to colorectal distension upon acute stress [i.e., water avoidance (WA)] later in life. In contrast, when nonhandled rats were subjected to the same acute stressor at an adult age, they did not become hypersensitive to distension. In the present investigation, we only evaluated maternally separated rats. On postnatal day 2, female pups were removed from the litter and dams were separated from the remaining pups for 3 h daily for a 12 d period (postnatal days 2–14) as described earlier (28).

Measurement of the visceromotor response to colonic distension and data analysis. In IBS patients, investigations of visceral sensitivity are performed by colorectal distensions: hypersensitive patients perceive pain during luminal distensions at lower volumes or pressures than normal controls. During distensions, pain scores in patients are often evaluated by self-rating questionnaires (i.e., visual analogue score) that cannot be assessed in rats. However, colorectal distension in rats leads to reproducible contractions of abdominal musculature—the so-called visceromotor response—and the quantification of these contractions by electromyography is often used to assess visceral pain. In the present investigation, we assessed abdominal electyromyography signals during distension protocols (1, 1.5, and 2 mL) by radiotelemetry. For details on colonic distensions, telemetric-tracing methodology, and data analysis, please refer to earlier papers (17, 26–28).

Experimental protocol and diet composition. The soy oil (SO) source of the cow milk protein–free AIN-93G diet (29) (Research Diet Services) was partially replaced by tuna oil (Bioriginals), the FA composition of which is shown in **Table 1**. In group 1, 10 rats were fed a 4% SO and 3% tuna oil (SO–T3) diet. In group 2, which was paired with group 1, 8 control rats were fed a 7% SO diet. In group 3, 9 rats

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[3] Supplemental Table 1 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[9] Abbreviations used: AA, arachidonic acid; CRM-E, Combined Rat and Mouse-expanded; HMC-1, human mast cell line 1; IBS, irritable bowel syndrome; LCPUFA, long-chain polyunsaturated fatty acid; SO, soy oil; SO–T3, 4% soy oil and 3% tuna oil; SO–T7, 3% soy oil and 7% tuna oil; WA, water avoidance.

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TABLE 1 FA composition of tuna oil batch used to partially replace the soy oil source of the AIN-93G diet in SO–T3 and SO–T7 diets[1]

FA	Value, g/100 g FA
SFA	27.8
MUFA	20.4
PUFA	39.4
n–6 PUFA	5.6
n–3 PUFA	33.8
EPA	6.2
DHA	24.7

[1] SO–T3, 4% soy oil and 3% tuna oil; SO–T7, 3% soy oil and 7% tuna oil.

received a 3% SO and 7% tuna oil (SO–T7) diet. In group 4, which was paired with group 3, 8 control rats were fed a 10% SO diet. In the latter 2 groups, an increase in the total fat percentage was needed to meet the requirements for an adequate amount and balance of the essential FAs Ala and linoleic acid (18:2n–6) (29); an appropriate amount of cornstarch thus was replaced by extra fat. Diets were stored at −20°C and refreshed weekly. Rats from all 4 groups were fed the tuna-oil–enriched or control diet post-WA only. Before dietary LCPUFA administration, intervention rats were fed a standard Combined Rat and Mouse-expanded (CRM-E) diet (Special Diet Services, Technilab-BMI). The approximate composition of the CRM-E diet was 10% moisture, 3% crude oil, 18.6% crude protein, 4.1% crude fiber, 5.8% ash, and 57.6% nitrogen-free extract. Distensions and concurrent electromyography recordings were performed 30 min before WA stress, 24 h post-WA, and on days 2, 8, and 28 or 29 post-WA. During WA, rats were positioned on a pedestal surrounded by water for 1 h (28).

Because symptoms are already present in IBS patients, we usually prefer post-WA treatment, with the purpose of reducing visceral hypersensitivity. However, when testing the possibility of activating PPARG to modulate visceral hypersensitivity, we chose a prestress treatment with a pharmacologic PPARG agonist to reach maximal efficacy. Rosiglitazone (30 mg · kg^{-1} · d^{-1}; Sigma-Aldrich) was added to a ground CRM-E unpurified diet, which was then converted into pellets. For the control rats, pellets were made from nonsupplemented CRM-E unpurified feed. Rats were fed these diets for a 7 d period pre-WA. In this experiment, distensions and concurrent electromyography recordings were performed before dietary treatment, 30 min before WA stress, and 24 h post-WA.

Erythrocyte FA profile. Immediately after rats were killed with the use of CO_2 inhalation, blood was collected with the use of heart puncture in lithium-heparin–coated blood tubes. Plasma and cells were separated with the use of centrifugation at 1000 × *g* for 15 min at 4°C. Erythrocytes were stored at −80°C until analysis. Lipids were extracted as described by Bligh and Dyer (30). The relative membrane FA composition was analyzed with the use of GC as described previously (31).

Histochemistry. Mucosal mast cells were stained following a staining protocol described by Wingren and Enerbäck (32). Distal colon paraffin sections (4 μm thickness) were incubated for 6 d in toluidine blue in 0.5 mol/L hydrochloric acid (pH 0.5). To semiquantitatively assess mast cell activation, every fifth section, with a total of 5 sections per rat, was evaluated in a blinded manner. A total of 50 mast cells were counted per section, and each mast cell was categorized as either dark staining intensity, medium staining intensity, or light staining intensity, with lightly stained mast cells indicating degranulation and dark stained cells representing nondegranulated mast cells.

Reverse transcriptase qPCR. Total RNA was isolated from white adipose tissue or the distal colon of rats treated with rosiglitazone and vehicle, or the SO–T7 and its control SO group, respectively, with the use of TriPure (Roche Diagnostics) and following the manufacturer's protocol. After deoxyribonuclease treatment, cDNA was obtained with the use of the RevertAid First Strand cDNA Synthesis Kit (Fermentas). qPCR was performed with SYBR Green in a LightCycler480 system (Roche) with the use of a default 60°C program. Primer pairs used are detailed in **Supplemental Table 1**.

In vitro mast cell TNF-α release and colonic TNF-α, Il-1β, and Il-6 concentrations. PPARG activation is known to interfere with NF-κB signaling and TNF-α is one of the genes regulated by NF-κB activation (33, 34). Therefore, and because mast cells are a major source of intestinal TNF-α (35), in order to evaluate the effects of EPA (20:5n–3), DHA (22:6n–3), and rosiglitazone on mast cell cytokine release, we used TNF-α as the prototype NF-κB–dependent cytokine. We evaluated TNF-α in human mast cell line 1 (HMC-1) cell culture supernatants. HMC-1 cells [human mast cell line kindly provided by Dr. Butterfield (36)] were pretreated with rosiglitazone, DHA, or EPA, which were all dissolved in DMSO, for 30 min. Concentrations used ranged from 0 to100 μM for rosiglitazone and 0 to 50 μM for DHA and EPA where indicated. Controls were pretreated with equal volumes of DMSO alone (1% final concentration). Next, cells were stimulated with a combination of phorbol 12-myristate 13-acetate (25 ng · mL^{-1}) and calcium ionophore A23187 (0.5 μM) for 4 h. The different EPA and DHA experimental conditions were tested in triplicate, rosiglitazone conditions in sextuplicate. Supernatant was analyzed with the use of a human TNF-α ELISA (R&D Systems). In addition, rat distal colons were homogenized in Green Berger Lysis buffer with added protease inhibitor cocktail (Sigma-Aldrich) with the use of Tissue Lyzer (Qiagen). Cytokine concentrations were determined with the use of rat TNF-α, Il-1β, and Il-6 ELISAs (R&D Systems).

In vitro mast cell degranulation. As a readout for mast cell degranulation, we used the β-hexosaminidase assay that is often used as a marker enzyme for histamine-containing granules [described earlier (23)]. In short, HMC-1 cells were pretreated with different concentrations of rosiglitazone (0–100 μM), DHA (0–50 μM), or EPA (0–50 μM), and then stimulated with compound 48/80 (1 mg · mL^{-1}; Sigma-Aldrich). The different EPA and DHA experimental conditions were tested in sextuplicate, rosiglitazone conditions in nonuplicate. Upon centrifugation, supernatants were collected and remaining cell pellets were lysed with Triton X-100. Supernatants and cell pellets were then incubated with 4-methylumbelliferyl glucosaminidase, and release of fluorescent 4-methylumbelliferone was measured as the readout for β-hexosaminidase activity (release calculated as a percentage of total cellular content).

Statistical analysis. The analysis was performed with the use of SPSS for Windows version 16.0. The normality and homogeneity distribution of data were assessed with Bartlett¨s test. Data concerning the visceromotor response to colonic distension and in vitro β-hexosaminidase and TNF-α release by HMC-1 cells were subjected to log transformation and analyzed statistically with the use of 2-factor repeated measures ANOVA followed by Bonferroni's multicomparison post hoc test. Data regarding reverse transcriptase qPCR, toluidine blue mast cell staining, in vivo cytokine concentrations, and FAs were analyzed with the use of the Mann–Whitney *U* test for independent samples. In all cases, differences were considered to be significant when $P \leq 0.05$. Data in all figures are presented untransformed as means ± SEMs.

Results

Effects of dietary n–3 LCPUFAs on visceral hypersensitivity and mucosal mast cell activation. WA stress significantly increased the visceromotor response to colorectal distension in all experimental and control groups (**Figure 1**A and 1C). Similar to SO control diets, dietary treatment with the SO–T3 (Figure 1A) or the SO–T7 (Figure 1C) diet did not reverse post-WA hypersensitivity. Colonic tissue sections were used to establish mast cell activation status in a semiquantitative manner by evaluating toluidine blue staining intensity of mucosal mast cells. When comparing tuna oil diets with respective control diets, we observed no differences between the percentages of cells scored in the 3 staining intensity categories (Figure 1B and 1D).

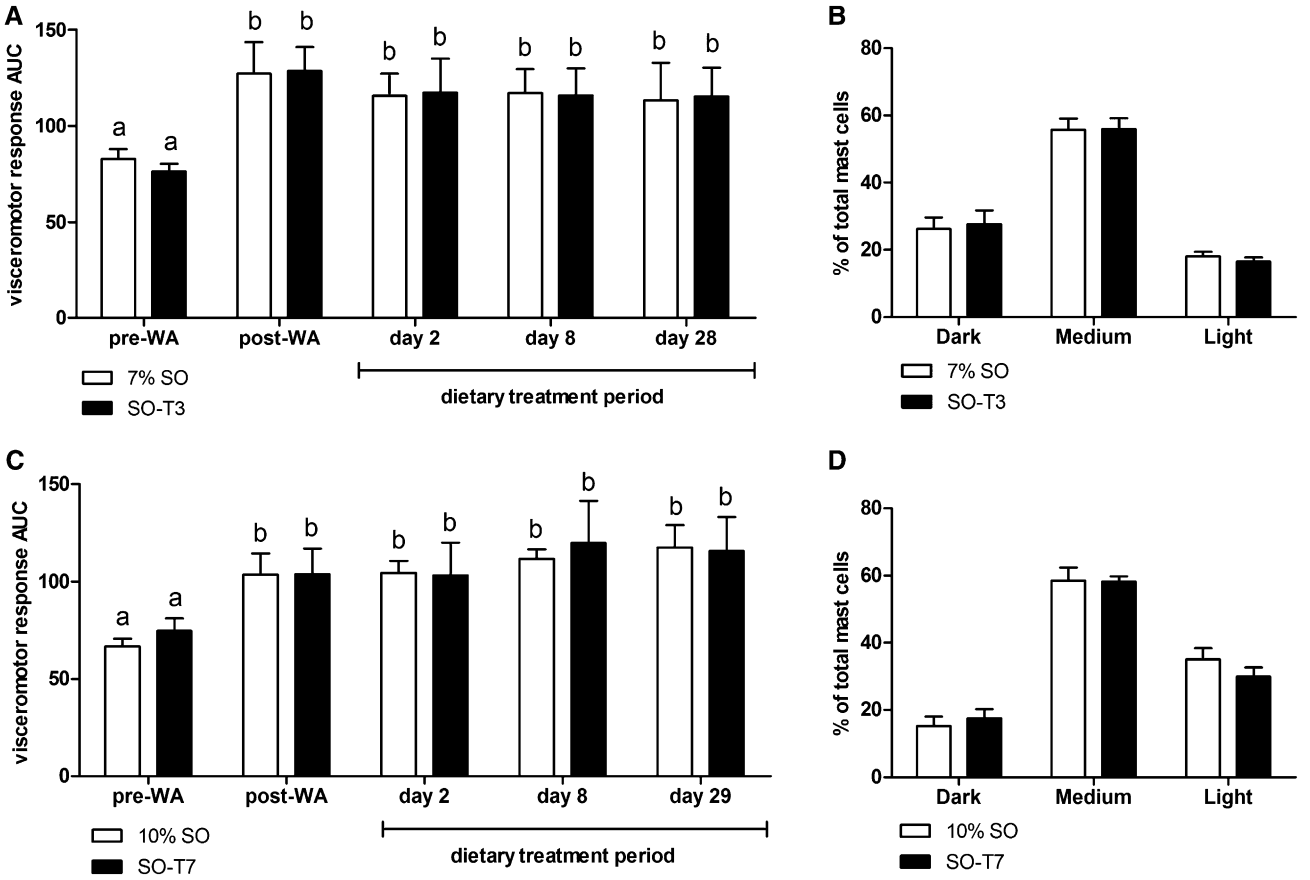

FIGURE 1 Visceromotor response and mast cell degranulation status of rats treated with SO–T3 or SO–T7 or their respective control SO diets. Visceromotor response data for SO–T3 and 7% SO control diets (A) and SO–T7 and 10% SO control diets (C) are presented as mean AUCs ± SEMs, n = 8–10 rats. Means in the same treatment group labeled without a common letter differ, $P < 0.05$. Different toluidine blue staining intensities of mucosal mast cells in the distal colon are presented as percentage of total stained cells. Comparisons were made between rats fed SO–T3 (B) and SO–T7 diets (D) and their respective control groups. SO, soy oil; SO–T3, 4% soy oil and 3% tuna oil; SO–T7, 3% soy oil and 7% tuna oil; WA, water avoidance.

LCPUFAs induced changes in erythrocyte FA profile. Compared with those of rats fed the control diet, in rats fed SO–T3 or SO–T7 diets, the relative content of EPA and DHA in the erythrocyte membrane was significantly increased (**Figure 2**A, 2B, 2D, and 2E). Importantly, in rats fed SO–T7, the relative content of AA (20:4n–6) in the erythrocyte membrane was significantly decreased (Figure 2F). Rats fed SO–T3 showed no significant changes in relative AA content compared to control 7% SO diet (Figure 2C).

Effects of rosiglitazone-mediated PPARG activation on visceral hypersensitivity and mucosal mast cell activation. Comparison of pretreatment and pre-WA measurements did not show significant rosiglitazone-induced changes in baseline sensitivity to distension. Subsequent WA stress significantly increased the visceromotor response in rats fed the rosiglitazone-supplemented diet, as well as in control rats (**Figure 3**A). In accordance with these sensitivity results, treatment with rosiglitazone did not affect mucosal mast cell activation (Figure 3B).

Dietary LCPUFAs and rosiglitazone administration induced mRNA expression of PPARG target genes. We investigated the expression of lipoprotein lipase and glycerol kinase, 2 target genes of PPARG that are involved in lipid metabolism. mRNA expression of both genes was significantly increased in the abdominal fat tissue of rats treated with rosiglitazone compared with control rats (Figure 3C and 3D), but not in the colonic tissue of rats fed the SO–T7 diet (Figure 3E and 3F).

In vivo release of TNF-α, Il-1β, and Il-6 in the colon. When comparing colon homogenates of rats fed the control 10% SO diet with those fed the SO–T7 diet, we observed no significant differences in TNF-α, Il-1β, or Il-6 concentrations (**Supplemental Figure 1**).

In vitro modulation of mast cell mediator release by EPA, DHA, and rosiglitazone. Although rosiglitazone did not affect degranulation as measured by release of β-hexosaminidase (**Figure 4**A), ≥25 μM rosiglitazone (Figure 4D) did significantly inhibit release of TNF-α. Rosiglitazone treatment did not affect basal release of β-hexosaminidase or TNF-α in unstimulated cells. Treatment with different concentrations of EPA and DHA did not affect release of β-hexosaminidase in unstimulated and 48/80-stimulated cells (Figure 4B and 4C). Release of TNF-α, however, was significantly reduced in DHA-treated phorbol 12-myristate 13-acetate/calcium ionophore–stimulated cells (Figure 4F), but not in EPA-treated cells (Figure 4E). Basal TNF-α release did not change in EPA- or DHA-treated cells.

Discussion

Internet-based IBS patient groups and forums often discuss the use of LCPUFAs as a treatment option, and LCPUFA preparations

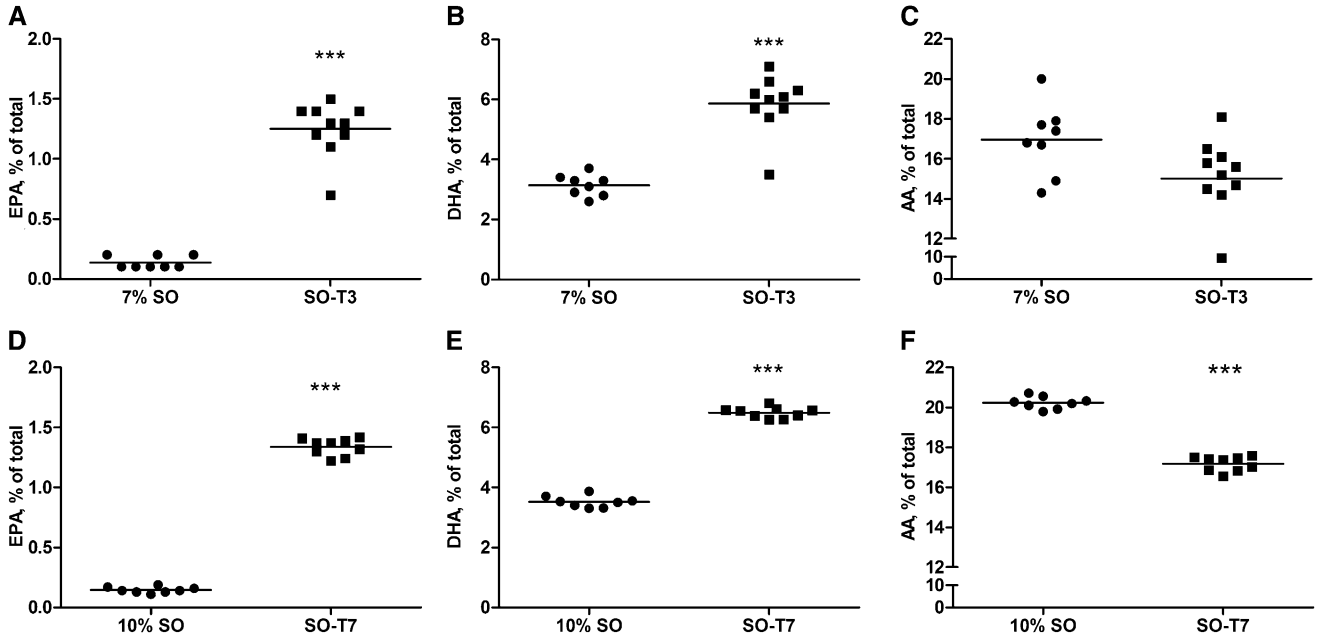

FIGURE 2 Dietary LCPUFA-mediated changes in rat erythrocyte membrane FA composition. Relative content of EPA (A, D), DHA (B, E), and AA (C, F) in the erythrocyte membranes of rats fed SO–T3 (A, B, C), SO–T7 (D, E, F), or control SO diets. Data are presented as mean percentages EPA, DHA, or AA of total FAs ± SEMs, $n = 8$–10 rats. ***Different from control diet, $P < 0.001$. AA, arachidonic acid; LCPUFA, long-chain polyunsaturated fatty acid; SO, soy oil; SO–T3, 4% soy oil and 3% tuna oil; SO–T7, 3% soy oil and 7% tuna oil.

are sold online for this purpose. However, although preclinical evidence exists for treatment efficacy in other disorders and diseases, such evidence is completely lacking for IBS. Because visceral hypersensitivity is a hallmark trait in the majority of patients and is thought to be a pathophysiologic mechanism, we evaluated the use of marine LCPUFAs to reverse poststress visceral hypersensitivity, possibly via mast cell modulation, in the rat maternal separation model. Our results suggest that LCPUFAs do not reverse existing stress-induced visceral hypersensitivity in this model.

Our previous animal experiments and a successful clinical trial with the mast cell stabilizer ketotifen indicated that mast cells may be an attractive target in IBS therapy (16, 17, 26), and others have shown that LCPUFAs are capable of modulating mast cell mediator release (21–23). In search of nutritional routes to modulate mast cell activation, and fueled by internet

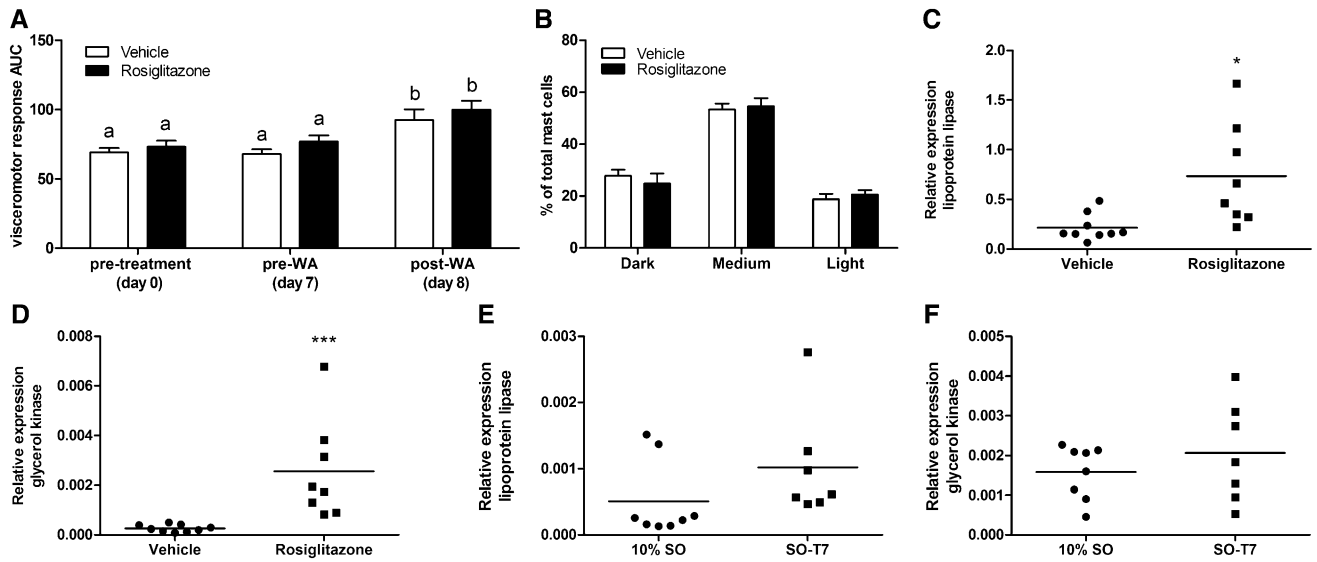

FIGURE 3 Visceromotor response and mast cell degranulation status of rosiglitazone-treated rats plus PPARG target gene expression in rosiglitazone-treated and SO–T7-treated rats. Visceromotor response data of rosiglitazone-treated rats are presented as mean AUCs ± SEMs, $n = 9$–10. Means in the same treatment group labeled without a common letter differ, $P < 0.05$ (A). Toluidine blue staining intensities of mucosal mast cells in the distal colon of rosiglitazone-treated rats are presented as percentages of total stained cells ± SEMs, $n = 9$ (B). Relative mRNA expression of the PPARG target genes lipoprotein lipase (C) and glycerol kinase (D) measured in rat adipose tissue of rosiglitazone-treated rats, and lipoprotein lipase (E) and glycerol kinase (F) in the colonic tissue of SO–T7-treated rats. Relative mRNA expression data are means ± SEMs, $n = 7$–9. Different from vehicle treatment, *$P < 0.05$ and ***$P < 0.001$. SO, soy oil; SO–T7, 3% soy oil and 7% tuna oil; WA, water avoidance.

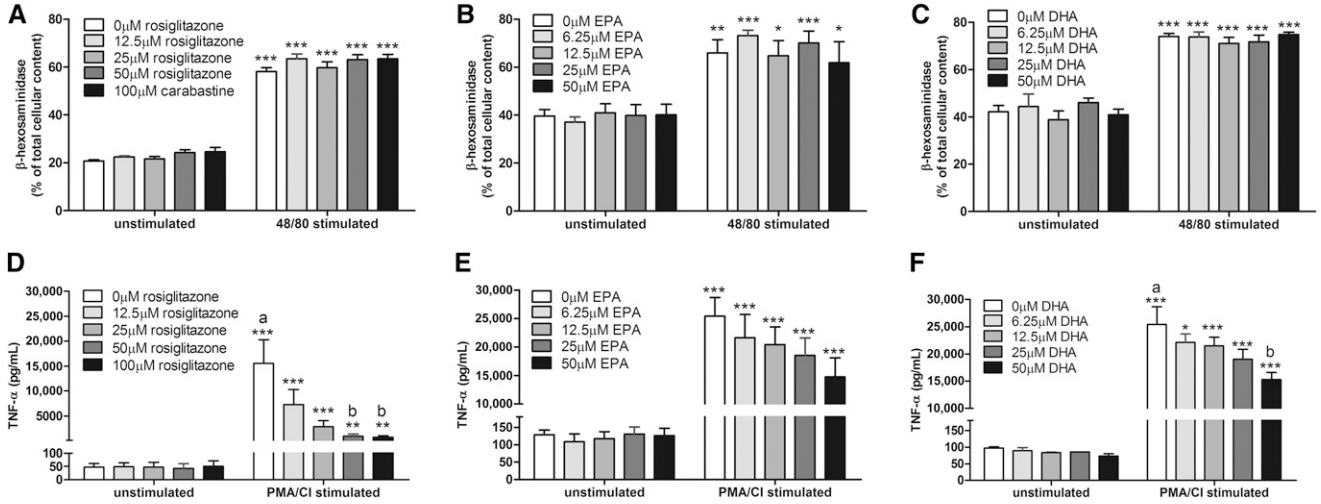

FIGURE 4 In vitro effects of rosiglitazone, EPA, and DHA on a human mast cell line. Compound 48/80-induced β-hexosaminidase release by HMC-1 cells preincubated with rosiglitazone (A), EPA (B), and DHA (C). PMA/CI-induced TNF-α release by HMC-1 cells preincubated with rosiglitazone (D), EPA (E), and DHA (F). Data are means ± SEMs, n = 3–9 per condition. Different from unstimulated controls, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Means within a group labeled without a common letter differ, $P < 0.01$. CI, calcium ionophore; HMC-1, human mast cell line 1; PMA, phorbol 12-myristate 13-acetate.

attention, we evaluated the use of marine n–3 LCPUFAs in our rat model. However, in contrast to earlier experiments in which we successfully used the mast cell stabilizer doxantrazole (37) and the histamine-1-receptor antagonist ebastine (17) to induce reversal of poststress visceral hypersensitivity in maternal separated rats, such reversal was not achieved with LCPUFA treatment. As mentioned earlier, one of the mechanisms by which n–3 LCPUFAs might elicit their beneficial effect is by competing with AA for the 2-acyl position within the phospholipid membrane, thus interfering with the production of proinflammatory AA metabolites. Hence, an imbalance toward n–6 FAs and their metabolites is thought to play a role in a variety of inflammatory states. Importantly, an n–6/n–3 FA imbalance was also shown in female IBS patients (8) and the maternal separation model (38). In patients, the observed imbalance correlated with increases in the AA metabolites PGE_2 and leukotriene B4. Because PGE_2 and leukotriene B4 are biologically active at very low concentrations, profound downstream effects can be induced by even small changes in AA concentrations (39). Thus, similar to other diseases (19, 20), neutralizing the n–6/n–3 FA balance might beneficially affect IBS symptoms. In our experiments, incorporation of EPA and DHA into the phospholipid membrane was assessed by monitoring the FA profile of erythrocyte membranes. Although not specifically evaluated here, it is known that other cell types, including those of the immune system, also incorporate these FAs into their cell membranes (3). Our data showed a profound increase in membrane DHA and EPA concentrations at the expense of AA, but without affecting poststress hypersensitivity to colorectal distension. These results are in line with the study by Clarke et al. (8), in which plasma AA concentrations did not correlate with symptom severity in IBS patients. Combined with the present data on LCPUFA supplementation, it may be concluded that increased AA concentrations are not a probable cause for IBS symptoms.

Another possible mechanism by which LCPUFAs reportedly modulate mast cells is via PPARG agonistic activity (1, 24, 25). Consequently, our negative in vivo results on visceral hypersensitivity reversal may suggest that the LCPUFA treatment protocol insufficiently activated PPARG. Therefore, we used the pharmacologic PPARG agonist rosiglitazone as a positive control. Indeed, evaluation of the PPARG target genes lipoprotein lipase and glycerol kinase indicated effective in vivo PPARG activation by rosiglitazone that was not observed in the high-dose LCPUFA experiment. Nevertheless, rosiglitazone also did not affect visceral hypersensitivity in our model. Celinski et al. (40) showed that the same rosiglitazone dosage (30 mg rosiglitazone $\cdot$ $kg^{-1} \cdot d^{-1}$) was adequate to suppress inflammation and inflammatory cytokine response in dextran sodium sulphate colitis. Thus, we suggest that the inability of rosiglitazone to prevent a stress-induced increase in sensitivity was unlikely to be because of insufficient dosing. In relation to the tuna oil dosage scheme used in the present study, it should be mentioned that we previously used 6% tuna oil and 4% SO to successfully prevent allergic sensitization to cow milk protein in mice (41). Although in the present experiments we used the same batch of tuna oil, an important difference with the current protocol was the preventive approach in the former experiments. Mice were fed the LCPUFA-rich diet starting 2 wk before and during the cow milk sensitization protocol. Because IBS patients are hypersensitive to begin with, our aim in the present investigations was a treatment strategy for reversal of symptoms instead of prevention. This may have influenced the outcome of our in vivo investigations. However, we did use a "prevention protocol" to test the efficacy of rosiglitazone and this strategy was also ineffective.

The inability of LCPUFAs and rosiglitazone to affect visceral hypersensitivity suggests that mast cell degranulation was not impaired by these treatment protocols and this was confirmed by toluidine blue staining of mucosal mast cells. This observation was also supported by our in vitro experiments, in which rosiglitazone, EPA, and DHA showed no effect on compound 48/80-induced degranulation of HMC-1 cells. Although these negative results confirm our earlier observations on IgE-mediated degranulation of LAD2 cells (23), they also add to the existing controversy. Some studies report suppressive effects of n–3 PUFAs on mast cell mediators and cytokine release (22, 23, 42), whereas others report augmenting effects (43, 44). Because our data on erythrocyte n–3 LCPUFA concentrations confirm proper uptake of dietary LCPUFAs, the lack of effect on mast cell–dependent visceral hypersensitivity suggests that their in vivo effect on degranulation is limited. In contrast to the

negative results on degranulation, we did observe in vitro inhibitory effects on TNF-α release, with moderate modulation by EPA and DHA, and strong inhibition by rosiglitazone. Because mast cells are a major source of TNF-α in the intestine (35), we expected to confirm these inhibitory effects in vivo. However, when comparing TNF-α content of colon homogenates, there was no significant difference between vehicle and rosiglitazone treatment groups, suggesting that rosiglitazone did not alter TNF-α production in vivo. However, because we used whole-colon homogenates, relatively small differences may have been rendered undetectable. Irrespective of this, at present there are no indications that TNF-α plays a role in this rat model.

On the internet, LCPUFAs are offered as a possible treatment option for IBS, but preclinical studies on efficacy are lacking. To our knowledge, this rat study is the first to evaluate the possible effect of LCPUFAs on an important pathophysiologic mechanism of this disorder. In summary, this study shows that neither the increased intake of n–3 LCPUFAs nor the pharmacologic activation of PPARG can modulate poststress visceral hypersensitivity in maternally separated rats. These results should not be regarded as definitive proof that LCPUFA supplementation in IBS will be ineffective, but more extensive investigations are required to strengthen that conclusion. Our results do, however, indicate that current claims should be regarded with caution.

Acknowledgments

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