5-oxoETE triggers nociception in constipation-predominant irritable bowel syndrome through MAS-related G protein–coupled receptor D

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Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is characterized by chronic abdominal pain concurrent with altered bowel habit. Polysaturated fatty acid (PUFA) metabolites are increased in abundance in IBS and are implicated in the alteration of sensation to mechanical stimuli, which is known as visceral hypersensitivity. We sought to quantify PUFA metabolites in patients with IBS and evaluate their role in pain. Quantification of PUFA metabolites by mass spectrometry in colonic biopsies showed an increased abundance of 5-oxoicosatetraenoic acid (5-oxoETE) only in biopsies taken from patients with IBS with predominant constipation (IBS-C). Local administration of 5-oxoETE to mice induced somatic and visceral hypersensitivity to mechanical stimuli without causing tissue inflammation. We found that 5-oxoETE directly acted on both human and mouse sensory neurons as shown by lumbar splanchnic nerve recordings and Ca2+ imaging of dorsal root ganglion (DRG) neurons. We showed that 5-oxoETE selectively stimulated nonpeptidergic, isolecitin B4 (IB4)–positive DRG neurons through a phospholipase C (PLC)– and pertussis toxin–dependent mechanism, suggesting that the effect was mediated by a G protein–coupled receptor (GPCR). The MAS-related GPCR D (Mrgprd) was found in mouse colonic DRG afferents and was identified as being implicated in the noxious effects of 5-oxoETE. Together, these data suggest that 5-oxoETE, a potential biomarker of IBS-C, induces somatic and visceral hyperalgesia without inflammation in an Mrgprd-dependent manner. Thus, 5-oxoETE may play a pivotal role in the abdominal pain associated with IBS-C.

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder in which recurrent abdominal pain is associated with a change in bowel habit, typically constipation (IBS-C), diarrhea (IBS-D), or a mixed (constipation and diarrhea) bowel habit (IBS-M) (1). IBS is a common disorder in Western populations, which affects around 11% of the global population (2), with a higher prevalence in women than in men (1). Although the etiology of IBS remains unclear, low-grade inflammation has been widely described in this disorder, with several fundamental studies implicating proinflammatory molecules in the pathophysiology of IBS symptoms (3). We previously showed that the amounts of several polysaturated fatty acid (PUFA) metabolites, also defined as bioactive lipids, are statistically significantly altered in biopsy samples from patients with IBS compared to those in samples from healthy controls (HCs) (4). This is in agreement with previous studies focused on the prostanoïd subtype of PUFA metabolites (5-7).

The functional relationship between PUFAs and pain has been the subject of many studies (8). Both basic and clinical studies have revealed that a dietary intake of n-3 series PUFAs results in a reduction in pain associated with rheumatoid arthritis (9, 10), dysmenorrhea (11), inflammatory bowel disease (12), and neuropathy (13), whereas n-6 series PUFAs are high in abundance in patients with chronic pain, including patients with IBS (4, 14, 15). The n-3 PUFA metabolites, such as resolvins (Rvs), are analgesic in multiple pain models, an effect attributed to inhibition of certain transient receptor potential (TRP) channels (16). For example, RvE1 specifically inhibits TRPV1 signaling (17), whereas resolvin D1 (RvD1) attenuates the function of TRPA1 and TRPV4 (18), and RvD2 inhibits TRPV1 and TRPA1 activity (19). These effects have been observed with other types of n-3 PUFAs, such as maresin 1 (Mar1), which also has inhibitory effects on TRPV1 channel function (20) and reduces pain. The quantification of Rvs in the knee synovia of patients suffering from inflammatory arthritis suggests that synthesis of specialized proresolving mediators (SPMs) at the site of inflammation may be a mechanism of endogenous pain relief in humans. In contrast, n-6 PUFA metabolites are pronociceptive by stimulating nerve fibers through the activation of immune cells (21, 22). Nonetheless, several n-6 PUFA metabolites, such as thromboxane A2 (TXA2), prostaglandin E2 (PGE2),

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leukotriene B\(_4\) (LtB\(_4\)), and PGD\(_2\), can directly stimulate sensory nerve fibers (23–26). However, some n-6 PUFA metabolites, such as lipoxins, can inhibit pain (27). Consistent with the role of TRP channels in the transduction of noxious stimuli, we previously showed a correlation between PUFA metabolites and TRP channel activation, particularly for the TRPV4 agonist 5,6-epoxyeicosatrienoic acid (5,6-EET) and pain intensity in IBS-D patients (4). Furthermore, PUFA metabolites from colonic biopsies of patients with IBS-C induced Ca\(^{2+}\) influx in sensory neurons independently of TRPV4, suggesting that the PUFA metabolites produced in IBS-C and IBS-D are distinct (4). Thus, the aim of this study was to identify algogenic PUFA metabolites specifically produced in patients with IBS-C and decipher the mechanism by which they may activate sensory nerves. Herein, we showed that 5-oxoeicosatetraenoic acid (5-oxoETE), an n-6 PUFA subtype selectively increased in abundance in colonic tissues from patients with IBS-C, induced hypersensitivity in a manner dependent on the MAS-related G protein–coupled receptor D (Mrgprd).

**RESULTS**

5-oxoETE is increased in abundance in colonic biopsies from patients with IBS-C

PUFA metabolites were quantified in colonic biopsies from patients with IBS and HCs using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Hierarchical clustering of PUFA metabolite amounts quantified in biopsies (picograms per milligram of protein) was used to reveal the main differences between HCs and patients with IBS-M, IBS-C, and IBS-D (Fig. 1A). PUFA metabolites formed five different clusters. The first cluster contained products of arachidonic acid (AA) metabolism [PGE\(_2\), thromboxane B\(_2\) (TxB\(_2\)), 5,6-EET, and 14,15-EET], eicosapentaenoic acid metabolism [18-hydroxyeicosapentaenoic acid (18-HEPE), LtB\(_5\), and PGE\(_3\)], and protectin Dx (PDx). Metabolites belonging to this first cluster were most abundant in biopsies from patients with IBS-D (Fig. 1A). Note that TxB\(_2\), PGE\(_2\), and 5,6-EET were only increased in biopsies of patients with IBS-D (Fig. S1). In contrast, TxB\(_2\) was decreased in abundance in biopsies from patients with IBS-C (Fig. S1). The second cluster discriminated only 5-oxoETE, which was substantially increased in biopsies from patients with IBS-C (Fig. 1, A and B). The concentrations of 7-Mar1 and 15dPGJ\(_2\), which delineated a third cluster, showed an increasing trend but did not reach statistical significance in biopsies from any group of patients with IBS (Fig. S2). The fourth cluster, grouping most of the lipoygenase-derived metabolites, was decreased in biopsies from all subtypes of patients with IBS compared to HCs (Fig. 1A). 15-Hydroxyeicosatetraenoic acid (15-HETE), 5-HETE, 12-HETE, 14-hydroxy-docosahexaenoic acid (14-HDoHE), and 17-HDoHE were statistically significantly decreased in abundance in all patients with IBS (Fig. S2). In addition, 12-HETE was statistically significantly decreased in abundance in biopsies from patients with IBS-C (Fig. S1). The metabolites included in the fifth cluster were reduced in amount only in biopsies from patients with IBS-C and IBS-D (Fig. 1A). RvD1 and RvD2 were not detectable in any colonic biopsies. Thus, among all of the PUFA metabolites quantified in colonic biopsies from patients with IBS, 5-oxoETE was the only one to be statistically significantly increased in patients with IBS-C compared to the other IBS subtypes (Fig. 1B) and thus warranted further investigation.

Local administration of 5-oxoETE induces somatic and visceral hyperalgesia without inflammation

Because PUFA metabolites can stimulate the immune system, directly stimulate nerves, or both, we first assessed the effect of 5-oxoETE on pain and inflammation processes in vivo. In a first set of experiments, 5-oxoETE was subcutaneously injected into the paws of mice, and the paw-withdrawal threshold to mechanical stimuli was estimated using calibrated von Frey filaments. The time course of mechanical hypersensitivity of the mice that received 5-oxoETE was compared with that of mice injected with vehicle (HBSS). Basal mechanical sensitivity, measured in the paw before injection, was identical in both groups of mice (Fig. 2A). Injection of 5-oxoETE into the hind paws resulted in a decrease in the paw-withdrawal threshold (Fig. 2A) and was observed from 15 min up to 2 hours after 5-oxoETE injection, with maximal reduction at 30 min. The
filtration was observed even 6 hours after the injection of 100 μM of inflammation. Similarly, neither tissue disruption nor cellular in-

Moreover, histological analysis of paw tissue did not reveal any sign

of inflammation and histological analysis were investigated to verify whether injection of 5-oxoETE induced an inflammatory process. Injection of 5-oxoETE or HBSS.

Data are means ± SEM of two experiments with five mice per group. Error bars indicate SEM. (B) The von Frey test was performed 30 min after injection of the indicated concentrations of 5-oxoETE. Data are means ± SEM of two experiments with six mice per group. (C) Mouse paw tissue samples were stained with hematoxylin and eosin (H&E) 6 hours after the administration of HBSS or 100 μM 5-oxoETE as indicated. Images are representative of two experiments with five mice per group. (D) Visceromotor response (VMR) to increasing pressures of colorectal distension (CRD) before and 30 min after intracolonic administration of 10 μM 5-oxoETE (black bars) or vehicle (40% ethanol; white bars). Data are means ± SEM of two experiments with 10 mice per group and are relative to the baseline recorded before treatment. (E) Colon tissue samples stained with H&E from mice treated with 40% ethanol or 10 μM 5-oxoETE as indicated. Images are representative of two experiments with five mice per group. Statistical analysis was performed using Kruskal-Wallis ANOVA and subsequent Dunn’s post hoc test. ***P < 0.01 and **P < 0.001 compared to control mice.

mechanical pain threshold was decreased in a dose-dependent manner 30 min after 5-oxoETE administration with a median effective concentration of 0.6 μM (Fig. 2B). In addition, paw edema formation and histological analysis were investigated to verify whether injection of 5-oxoETE induced an inflammatory process. Injection of 5-oxoETE into the hind paw did not induce paw edema (Fig. S3). Moreover, histological analysis of paw tissue did not reveal any sign of inflammation. Similarly, neither tissue disruption nor cellular infiltration was observed even 6 hours after the injection of 100 μM 5-oxoETE (Fig. 2C). Thus, at the somatic level, 5-oxoETE increased paw sensitivity to mechanical stimulation without inducing a quantifiable inflammatory reaction.

Intracolonic administration of 5-oxoETE resulted in an increased intensity of abdominal contractions in response to colorectal distension (CRD) (Fig. 2D). Moreover, the increased intensity of abdominal contractions was observed in response to both innocuous (15 mmHg; allostynia) and noxious (30 to 60 mmHg; hyperalgesia) stimuli 30 min after 5-oxoETE treatment (Fig. 2D). Intracolonic treatment with vehicle (40% ethanol) did not alter abdominal contraction response (Fig. 2D). As was observed after the subcutaneous injection of hind paws, intracolonic administration of 5-oxoETE did not induce inflammation of the colon. Colonic inflammation was assessed by macroscopic scoring and myeloperoxidase activity, which were not increased by 5-oxoETE administration when compared to vehicle (fig. S3). Moreover, histological analysis did not reveal intestinal wall thickening, leukocyte infiltration into the lamina propria, the presence of ulceration, or goblet cell depletion (Fig. 2E). Thus, in vivo local administration of 5-oxoETE induced visceral hyperalgesia in the absence of inflammation, thus suggesting a direct effect on nociceptors.

5-oxoETE stimulates visceral and somatic nociceptors: Translation to human dorsal root ganglia

To confirm a direct effect of 5-oxoETE on sensory nerve terminals innervating the colon, we examined its effects upon action potential firing in mouse colonic nociceptors. Specifically, we made teased-fiber electrophysiological recordings from the lumbar splanchnic nerve innervating the distal colon, which is primarily composed of colonic nociceptors with endings on serosal blood vessels penetrating the colon and in the mesentery. On defined populations of nociceptors with receptive fields isolated in flat sheet preparations, we applied 100 μM 5-oxoETE to a small chamber placed directly over the receptive field. 5-oxoETE stimulated action potential firing greater than vehicle in 38% of colonic nociceptor endings assessed (Fig. 3). In a second set of experiments, we determined the effect of 5-oxoETE on Ca2+ mobilization in primary cultures of neurons from mouse dorsal root ganglia (DRGs). In preliminary experiments performed with a working solution containing Ca2+ and Mg2+, we observed a transient increase in the concentration of intracellular Ca2+ ([Ca2+]i) (fig. S4). To determine whether this transient increase was the consequence of intracellular Ca2+ release or influx of external Ca2+, experiments were performed without Ca2+ and Mg2+ in the extracellular solution. Even without Ca2+ in the extracellular compartment, 5-oxoETE evoked a transient increase in [Ca2+]i, that was maximal after 10 to 20 s and declined thereafter (Fig. 4A). The mobilization of intracellular Ca2+ by 5-oxoETE was concentration dependent (Fig. 4B). Similarly, 5-oxoETE induced an increase in [Ca2+]i, and the percentage of responding neurons in a concentration-dependent manner in human primary sensory neurons (Fig. 4C). Thus, our data suggest that 5-oxoETE directly activates colonic DRG neurons from mice, as well as human sensory neurons, inducing an increase in nociceptor firing (Fig. 3) and in [Ca2+]i (Fig. 4).

Because 5-oxoETE induced somatic pain without inflammation in vivo, we hypothesized that 5-oxoETE predominantly activates isolectin B4–positive (IB4+) sensory neurons, which do not release neuropeptides involved in neurogenic inflammation. To assess our hypothesis, we labeled mouse sensory neurons with IB4 and treated them with 10 μM 5-oxoETE in the absence of extracellular Ca2+. We found that 5-oxoETE induced an increase in [Ca2+]i in >50% of the
5-oxoETE activates sensory neurons and induces visceral hypersensitivity through Mrgprd

Because we found that 5-oxoETE specifically activated IB4+ sensory neurons through Gαι/o-mediated signaling pathways, we focused our attention on the Mrgprd, which couples to Gαι/o and Gαq proteins. The expression and function of Mrgprd in polymodal nociceptors innervating the skin are well established (28); however, for visceral tissues, this remains less clear. To comprehensively assess this, we retrogradely labeled sensory afferents from the colon using microinjection of Fast Blue (FB) in wild-type (WT) mice and MrgprdEGFP mice. Single-cell quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed on FB-expressing cells from the DRGs of WT mice. We were able to detect Mrgprd mRNA at some level in 40% (18 of 45) of the FB-labeled sensory neurons projecting to the colon through the splanchnic nerve originating from the thoracolumbar (TL; T10-L1) DRG (Fig. 5A). We detected Trpv1 mRNA in 82% (37 of 45) of cells, with 41% (15 of 37) of the Trpv1-positive neurons also expressing Mrgprd mRNA (Fig. 5A). Immunohistochemistry was performed on the thoracic position 13 (T13) DRG from the MrgprdEGFP-expressing mice to determine the incidence of green fluorescent protein (GFP) expression and the peptidergic marker calcitonin gene-related peptide (CGRP) in FB-labeled cells, which revealed two distinct non-overlapping populations (Fig. 5B). Consistent with previous studies, those sensory neurons labeled from the colon with the retrograde tracer FB were predominantly CGRP+ (~70%). In contrast, GFP immunoreactivity was observed in a restricted subset of colonic sensory neurons, accounting for only 7% of FB-labeled cells (Fig. 5B and Table 1). Of the 274 FB+ cells assessed, only 1 cell coexpressed both Mrgprd and CGRP. Those neurons projecting to the viscera represented ~10% of the total population of T10-L1 DRG neurons. Thus, only a very small population (between less than 1 and 4%) of T10-L1 DRG neurons are likely to be both colon projecting and Mrgprd positive.

In experiments using an antibody against Mrgprd, we observed infrequent, yet reproducible, colocalization of Mrgprd with PGP9.5 in the colon sections of 6 of 10 WT mice that were assessed (Fig. S5). Note that Mrgprd immunoreactivity was not observed in the colons of Mrgprd-deficient mice (Fig. S6). The expression of Mrgprd in human sensory neurons was also assessed. We found that Mrgprd immunoreactivity was present in 22% of human T11 DRG neurons (Fig. 5C), which also coexpressed the pan-neuronal marker PGP9.5. In a culture of human sensory neurons, 20% of PGP9.5+ neurons showed Mrgprd immunoreactivity (Fig. 5D).

To demonstrate the role of Mrgprd in 5-oxoETE–induced neuronal firing, we knocked it down by transducing primary cultures of

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Fig. 3. 5-oxoETE induces lumbar splanchnic nerve firing. (A) Example of a teased-fiber recording showing the lumbar splanchnic (that is, colon innervating) nerve response to ring application (7 min) of 5-oxoETE in mouse serosal afferents. Arrows indicate application and removal of 5-oxoETE. Data are representative of eight experiments in which 5-oxoETE elicited nerve discharge above baseline from a total of 21 teased fibers isolated from seven mice. (B) Mean change in firing per second in serosal receptive fields in response to 5-oxoETE compared with the response to vehicle (Krebs buffer). Data are means ± SEM of eight teased-fiber recordings (N = 7 mice) for 5-oxoETE and five teased-fiber recordings (N = 5 mice) for vehicle. Statistical analysis was performed using a Mann-Whitney test. **P < 0.01 compared to vehicle. (C) Proportion of responses in lumbar splanchnic afferents to application of 5-oxoETE (n, number of teased-fiber recordings; N, number of mice).
mouse sensory DRG neurons with a recombinant lentivirus expressing a short-hairpin RNA (shRNA) directed against Mrgprd and the gene reporter red fluorescent protein (RFP). As a control, neurons were transduced with a lentivirus expressing a scrambled shRNA. As expected, the percentage of neurons that responded to 5-oxoETE was statistically significantly reduced in sensory neurons expressing the Mrgprd-specific shRNA compared to those neurons expressing the scrambled shRNA (Fig. 6A). Accordingly, application of 5-oxoETE on sensory neurons from Mrgprd-deficient mice had no greater effect than the application of HBSS alone (Fig. 6B). In contrast, treatment of sensory neurons from Mrgprd-deficient mice with a mixture of GPCR agonists (bradykinin, serotonin, and histamine, each at 10 μM), which was used as a positive control, induced an increase in [Ca²⁺], (Fig. 6B). Reciprocally, 5-oxoETE induced a concentration-dependent increase in [Ca²⁺], only in transfected, Mrgprd-expressing Chinese hamster ovary (CHO) cells (Fig. 6C). Last, we assessed the sensitivity of Mrgprd-deficient mice to CRD 30 min after intracolonic administration of 10 μM 5-oxoETE. 5-oxoETE did not induce hypersensitivity in response to CRD in Mrgprd-deficient mice (Fig. 6D).

**DISCUSSION**

In this study, we showed that (i) concentrations of the PUFA metabolite 5-oxoETE were statistically significantly increased in biopsies from patients with IBS-C compared to biopsies from patients with other IBS subtypes or from HCs; (ii) 5-oxoETE induced somatic, as well as visceral, hyperalgesia, without promoting inflammation; (iii) 5-oxoETE activated both mouse and human sensory neurons; and (iv) in mice, 5-oxoETE signaled in a manner dependent on Mrgprd. Together, these data suggest a role for 5-oxoETE and Mrgprd-expressing, IB4+ sensory neurons in visceral hypersensitivity in patients with IBS-C.

Eicosanoids and docosanoids are the most important lipids implicated in inflammatory processes. They derive from the oxidation of 20 and 22 carbon PUFAs, respectively (29). Several PUFA metabolites are increased in abundance in the intestinal mucosa from patients with inflammatory bowel diseases (IBDs) (including TXA₂, PGE₂, LTB₄, and PGD₂), which induce visceral afferent fiber activation (23-26). Here, we showed that PGE₂, 5,6-EET, and TxB₂ were statistically significantly increased in abundance in the intestinal mucosa of patients with IBS-D, whereas no alteration in PUFA metabolism was observed in patients with IBS-M. Furthermore, when...
Fig. 5. Expression of Mrgprd in sensory neurons. (A) Expression of Mrgprd (red) and Trpv1 (blue) mRNA transcripts as detected by single-cell qRT-PCR analysis (middle) of retrogradely labeled mouse colonic sensory neurons (left). Pie chart representation (right) of the expression (dark color) or not (light color) of Mrgprd and Trpv1 mRNA in FB+ neurons. Each segment represents a single colonic sensory neuron. (B) Representative images of GFP (green), CGRP immunoreactivity (red), and FB labeling (blue) in a T13 DRG from an MrgprdEGFP mouse in which FB was injected into the colon. Scale bar, 50 μm. Inset images are magnifications of the boxed areas in the largest images. (C and D) Expression of Mrgprd by immunostaining in a whole human T11 DRG (C) and in a primary culture of human sensory neurons using confocal microscopy (D). Pie chart representation of the immunoreactivity (dark color) or not (light color) of Mrgprd in Pgp9.5+ neurons. Each segment represents a single sensory neuron. Scale bars, 10 μm. Images are representative of two experiments with 10 slides per experiment (C) and of five experiments with two wells per experiment (D).
lipid extracts from HCs and all patients with IBS were compared, we observed a statistically significant decrease in the amounts of 14-HDoHE and 17-HDoHE, which are SPM precursors (30). Because SPMs have an analgesic effect (31), the pain associated with IBS could also be the consequence of a decrease in SPM abundance, leading to sensory neuron activation. A complete characterization of the different SPMs produced by the metabolism of eicosapentaenoic acid, docosahexaenoic acid, or docosapentaenoic acid will be of interest for the characterization of bioactive lipids potentially linked with pain in patients with IBS.

We showed that the concentration of 5-oxoETE increased in only the colonic biopsies of patients with IBS-C, highlighting its potential relevance as a new marker of this disease. 5-oxoETE, which derives from AA metabolism, is produced by various inflammatory cells. In addition, it can also be synthesized from 5-HETE by stromal cells, possibly by transcellular biosynthesis (32). 5-oxoETE is formed by the oxidation of 5-HETE by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) (33), a microsomal enzyme that is highly selective for 5S-HETE and requires nicotinamide adenine dinucleotide phosphate (NADP+) as a cofactor (34). 5-HEDH is found in neutrophils as well as in various other inflammatory and stromal cells, including monocytes (35), dendritic cells (36), and intestinal epithelial cells (37). 5-oxoETE is a potent chemoattractant for human and rat eosinophils, and it indirectly promotes the survival of these cells (38). However, we observed no cellular infiltration either in the paw or in the intestinal mucosa of mice administered with 5-oxoETE. This discrepancy may be due to the rapid metabolism of 5-oxoETE in vivo (32) or the absence of other molecules, such as interleukin-5, which act in synergy to attract inflammatory cells during inflammatory processes or allergies (39). In our experiments, the injection of 5-oxoETE alone, without cofactors, could thus explain the absence of infiltration of tissues by polymorphonuclear cells. The formation of 5-oxoETE requires NADP+ (40). Accordingly, oxidative stress associated with IBS-C (41) may improve the conversion rate of NADPH (reduced form of NADP+) into NADP+.
agonist of Mrgprd, increases \([\text{Ca}^{2+}]_i\) (45). By contrast, no PUFA metabolite with TRP activity, specifically associated with the IBS-D subgroup (4). Because we found that only 5-oxoETE was increased in biopsies of patients with IBS-C, we hypothesized that this PUFA metabolite might be responsible for the activation of sensory neurons and hypersensitivity associated with IBS-C. As previously reported in humans, 5-oxoETE may interact with the oxoeicosanoid receptor 1 (OXER1). However, there is no homologous OXE receptor in rodents (40). Because the observed 5-oxoETE–induced increase in \([\text{Ca}^{2+}]_i\) in mouse sensory neurons was inhibited by both a PLC inhibitor and PTX, we hypothesized that 5-oxoETE led to the activation of G\(\alpha_Q/G\alpha_k\)-coupled GPCRs. Given that 5-oxoETE acts selectively on IB4+ sensory neurons, the targeted receptor should be specifically expressed on this neuronal subclass. Accordingly, we observed the role of Mrgprd, a GPCR specifically expressed on IB4+ sensory neurons, which may be coupled to G\(\alpha_Q\) proteins and to PTX-sensitive G\(\alpha_{i/o}\) proteins (42) and was previously reported as a key player in mechanical hypersensitivity (43–45).

Stimulation of Mrgprd+ neurons with \(\beta\)-alanine, the prototypical agonist of Mrgprd, increases \([\text{Ca}^{2+}]_i\) (46), as was observed here after 5-oxoETE treatment. Moreover, in a FLIPR (fluorescent imaging plate reader) assay developed for the simultaneous identification of Mrgprd agonists and antagonists, a PLC inhibitor completely blocked the FLIPR response to \(\beta\)-alanine, whereas PTX treatment resulted in 50% reduction in \([\text{Ca}^{2+}]_i\) (47). Again, similar results were obtained here in experiments with PTX or a PLC inhibitor to inhibit 5-oxoETE–induced activation of primary mouse sensory neurons. Using tissue from adult Mrgprd\(_{\text{EGFP}}\) mice stained with antibodies to GFP, a previous study showed that Mrgprd is expressed in nonpeptidergic neurons that innervate the epidermis; however, Mrgprd+ fibers were not observed in any other visceral organs, including both the small and large intestine (28). By contrast, numerous studies using different retrograde tracers have identified a minor population (20 to 26%) of IB4+ sensory neurons that innervate the colon (48–50). A study also identified Mrgprd mRNA in colonic sensory neurons by single-cell RNA sequencing (51). To confirm the presence of both Mrgprd mRNA and Mrgprd protein in colonic sensory neurons in the present study, we applied a similar retrograde neurotracing approach using single-cell qRT-PCR analysis and anti-GFP immunostaining in Mrgprd\(_{\text{EGFP}}\) mice. We observed Mrgprd mRNA and Mrgprd protein expression in sensory DRG neurons projecting to the colon at a similar frequency to that observed in previous studies (51), thereby not only confirming the presence of an Mrgprd+ colonic neuronal subtype but also reinforcing Mrgprd as a potential target of 5-oxoETE. The activity of 5-oxoETE toward Mrgprd was attested by its ability to induce an increase in \([\text{Ca}^{2+}]_i\), in IB4+ sensory neurons but not in mouse neurons in which Mrgprd was knocked down by shRNA or in neurons from Mrgprd-deficient mice. Conversely, whereas \(\text{Ca}^{2+}\) transients were stimulated by 5-oxoETE in CHO cells transfected with a plasmid expressing Mrgprd, CHO cells transfected with a control plasmid were not responsive to 5-oxoETE.

Activation of Mrgprd inhibits a fraction of the total M-current, carried primarily by the voltage-gated Kv7 (KCNQ2/3 K+ channel, contributing to an increase in the excitability of DRG neurons (52). Thus, Mrgprd activation by 5-oxoETE might promote the excitability of primary nociceptive afferents by KCNQ inhibition. Several groups have demonstrated that retigabine, a KCNQ opener, is effective in reducing neuropathic (53) and inflammatory pain (54). At the visceral level, retigabine reduces capsaicin-induced visceral pain and can inhibit noxious chemosensitivity in human tissue, suggesting that KCNQ channels play an inhibitory role in the transmission of visceral nociception (55, 56). Given that human sensory DRG neurons express Mrgprd and are activated by 5-oxoETE, we can speculate that 5-oxoETE modulates KCNQ channels through Mrgprd activation, leading to neuronal activation that contributes to the pain symptoms associated with IBS-C. Nevertheless, because OXER1 is expressed in human tissue, we cannot exclude the possibility that this receptor is activated by 5-oxoETE in human tissue. Together, our current findings build on our previous studies to suggest a pivotal role for PUFA metabolites in the visceral pain associated with IBS (4). Specifically, our study identifies 5-oxoETE with pronociceptive activity, as a hallmark of the IBS-C subtype.

### MATERIALS AND METHODS

#### Chemicals

6-keto-prostaglandin F1\(_a\) (6kPGF\(_1\alpha\)), TxB\(_2\), PGE\(_2\), prostaglandin A\(_1\) (PG\(_A\)_1), 8-iso PG\(_A\)_2, prostaglandin E\(_3\) (PG\(_E\)_3), 15-deoxy-\(\Delta^{12,14}\) prostaglandin J\(_2\) (15d-PGJ\(_2\)), lipoxin A\(_4\) (LxA\(_4\)), lipoxin B\(_4\) (LxB\(_4\)), LxA\(_4\) deuterated (LxA\(_4\)-d5), RvD1, RvD2, 7-maresin 1 (7-Mar1),

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<th>CGRP*</th>
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<tr>
<td>Total (mean ± SD)</td>
<td>7.2 ± 4.6%</td>
<td>68.8 ± 6.7%</td>
<td>0.3 ± 0.5%</td>
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</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>IBS</th>
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</thead>
<tbody>
<tr>
<td>Number</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
<td>49 (20–76)</td>
</tr>
<tr>
<td>Sex ratio (F/M)</td>
<td>8/6</td>
</tr>
<tr>
<td>Bowel movements</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
</tr>
<tr>
<td>Constipation</td>
<td>0</td>
</tr>
<tr>
<td>Mix</td>
<td>0</td>
</tr>
</tbody>
</table>
Lx3, Lx4, Lx6 deuterated (Lx3-d4, 10S17S-protectin (Pdx), 18-
HEPE, dihydroxy-eicosatetraenoic acid (5,6-DiHETE), 15-
HETE, 12-HETE, 8-HETE, 5-HETE, 5-HETE-d8, 17-HDoHE, 14-HDoHE,
14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, and 5-oxoETE were pur-
chased from Cayman Chemical.

Patients
Patients (Table 2) were recruited from outpatient clinics of the
Department of Medical and Surgical Sciences of the University of
Bologna (Italy) and were included according to the Rome III crite-
rion for IBS. HCs were asymptomatic subjects undergoing colonos-
cy for colorectal cancer screening. In this group, we excluded subjects based on the presence of the following symptoms in the pre-
vious 12 months: abdominal discomfort or pain, bloating, and bowl
habit changes. Exclusion criteria for both IBS and HC included major
abdominal surgery, any organic syndrome, celiac disease (excluded by
detection of anti-transglutaminase and anti-endomysial antibodies),
asthma, food allergy, or other allergic disorders. None of these pa-
tients or HCs were taking nonsteroidal anti-inflammatory drugs or
any other anti-inflammatory drugs (including steroids, antihistamines,
and mast cell stabilizers). Patients and HCs gave written informed
consent. The study protocol was approved by the local Ethics Com-
mittee (64/2004/O/Sper and EM14/2006/O) and conducted in accor-
dance with the Declaration of Helsinki. Patients underwent colonoscopy, and in all cases, six mucosal biopsies were obtained from
the proximal descending colon. One biopsy was sent to the pathology
department for exclusion of microscopic colitis or other
microscopic tissue abnormalities, and four were used in other studies.
One biopsy was snap frozen in liquid nitrogen for lipid extraction
and PUFA quantification for the purpose of our study.

Lipid extraction
Biopsies were crushed with a FastPrep-24 Instrument (MP Bio-
medicals) in 500 μl of HBSS (Invitrogen) and 5 μl of internal stan-
dard mixture (LxA4-d5, LT3B-d4, and 5-HETE-d8 at 400 ng/ml in
MeOH). After two crush cycles (6.5 m/s, 30 s), 10 μl was withdrawn
for protein quantification and 300 μl of cold methanol was added.
Samples were centrifuged at 1000 g for 15 min at 4°C. Supernatants
were collected, adjusted to 2 ml in H2O, and submitted to solid-
phase extraction using HRX-50 mg 96-well (MACHEREY-NAGEL).
Briefly, after plate conditioning, the sample was loaded at a flow rate
of 0.1 ml/min. After complete loading, the plate was washed with
H2O/MeOH (90:10, 2 ml), and lipid mediators were eluted with
MeOH (2 ml). The solvent was evaporated under nitrogen, and sam-
ples were dissolved with MeOH and stored at −80°C for LC-MS/MS
measurements.

LC-MS/MS measurements
6kPGFlα, Txβ2, PGE2, PGA1, 8-isoPGE2, PGE3, 15d-PGJ2, LxA4,
LxB4, RvD1, RvD2, 7-Mar1, Lx7a, Lxβ6, PDX, 18-HEPE, 5,6-DiHETE,
15-HETE, 12-HETE, 8-HETE, 5-HETE, 17-HDoHE, 14-HDoHE,
14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, and 5-oxoETE were quan-
tified in human biopsies (57). To simultaneously separate 28 lipids
of interest and three deuterated internal standards, LC-MS/MS
analysis was performed on an ultrahigh-performance liquid chro-
matography system (UHPLC; Agilent LC1290 Infinity) coupled to an
Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped
with electrospray ionization operating in negative mode. Reverse-
phase UHPLC was performed using a ZorbAX SB-C18 column
(Agilent Technologies) with a gradient elution. The mobile phases
consisted of water, acetonitrile (ACN), and formic acid (FA) [75:25:0.1
(v/v/v)] (solution A) and ACN and FA [100:0.1 (v/v)] (solution B).
The linear gradient was as follows: 0% solution B at 0 min, 85%
solution B at 8.5 min, 100% solution B at 9.5 min, 100% solution B
at 10.5 min, and 0% solution B at 12 min. The flow rate was 0.35 ml/min.
The autosampler was set at 5°C, and the injection volume was 5 μl.
Data were acquired in multiple reaction monitoring (MRM) mode
with optimized conditions. Peak detection, integration, and quanti-
tative analysis were performed with MassHunter Quantitative anal-
ysis software (Agilent Technologies). For each standard, calibration
curves were built using 10 solutions at concentrations ranging from
0.95 to 500 ng/ml. A linear regression with a weight factor of 1/X
was applied for each compound. The limit of detection (LOD) and
the limit of quantification (LOQ) were determined for the 28 compounds
using signal-to-noise (S/N) ratios. The LOD corresponded to the
lowest concentration leading to an S/N value >3, and LOQ corre-
sponded to the lowest concentration leading to an S/N value >10.
All values less than the LOQ were not considered. Blank samples
were evaluated, and their injection showed no interference (no peak
detected), during the analysis. Hierarchical clustering was performed,
and heat maps were obtained with R (www.r-project.org). PUFA
metabolite amounts were transformed to z scores and clustered based
on 1 – Pearson correlation coefficient as distance and the Ward al-
gorithm as agglomeration criterion.

Animals
C57BL/6 male mice (3 weeks old) were purchased from JANVIER
LABS. Mrgrp drctg mice were a gift from D. J. Anderson (Caltech,
Pasadena). These mice were previously generated as described by
Rau et al. (44) and were in an almost pure C57/B16 background
when we received them at the Institut de Biologie du Développe-
demelle mouse facility. There, the mice were kept as heterozygotes
and were backcrossed to C57/Bl6J for another eight
reasons. Heterozygous mice used in this study were ob-
tained by intercrossing Mrgrp drctg heterozygous mice. Animals
were maintained in ventilated cages (four mice per cage) in a specific pathogen–
free room at 20° to 24°C and relative humidity (40 to 70%) with a
12-hour light/12-hour dark cycle and given free access to food and
water. The Animal Care and Ethics Committee of US006/CREFE
(CEEA-122) approved the whole study protocol (permit no.
05/01/64/09/12). Mrgrp dEGFP mice [B6129SP2-Mrgrp dtm41(COP4)Sj/
Mmnc; Mutant Mouse Resource and Research Centers, North Carolina,
USA] were raised and maintained at the University of Glasgow and
were characterized previously (58). Experiments conducted at the
University of Glasgow were approved by the University’s Ethical
Review Process Applications Panel and were performed in accor-
dance with the European Community directive 86/609/EC and the
United Kingdom Animals (Scientific Procedures) Act 1986.

Measurement of somatic nociception
Paw-withdrawal thresholds were measured using calibrated von Frey
filaments with forces ranging from 0.04 to 2 g (Stoelting), which were
applied onto the plantar surface of the mice. An ascending series of
von Frey filaments was applied with each monofilament being tested
five times for about 1 s. Threshold to mechanical stimuli was calcu-
lated as the force value of the von Frey filament triggering three paw
withdrawals over five applications (59). Responses to mechanical
stimuli were recorded before and 15 min, 30 min, 1 hour, 2 hours,
and 6 hours after an intraplantar injection of 0.1, 1, 10, or 100 μM 5-oxoETE or vehicle (HBSS). In a second set of experiments, paw edema was measured using a digital caliper (resolution, 0.01 mm; Mitutoyo, Aurora, IL, USA) at 1, 2, 3, and 4 hours after intraplantar injection of 100 μM 5-oxoETE. At the end of the experiment, paws were collected for histological analysis by H&E staining.

**CRD and electromyography recordings**

Mice were administered with either 100 μl of 5-oxoETE (10 μM) or vehicle (40% ethanol) intracolonically. We performed a session of CRD and recorded VMRs from implanted electrodes before and 30 min after treatment as previously described (60). Data are presented as the difference between the VMR induced by the distension performed before and after intracolonic treatments. After distension, mouse colons were harvested to perform histological (H&E) analysis and myeloperoxidase activity assay.

**Lumbar splanchnic nerve recording**

The distal colon with associated lumbar splanchnic nerves was removed from male C57BL/6 mice (12 weeks old). The colon was then opened along the antimesenteric border and pinned flat, mucosal side up. The tissue was perfused (7 ml/min; 32° to 34°C) with carbonated Krebs buffer (124 mM NaCl, 4.8 mM KCl, 1.3 mM NaH2PO4, 2.4 mMCaCl2, 1.2 mM MgSO4, 7H2O, 11.1 mM glucose, and 25 mM NaHCO3) and supplemented with 10 μM nifedipine and 10 μM atropine to block smooth muscle contraction, and 3 μM indomethacin to inhibit endogenous prostanoid production. Single unit activity was discriminated using waveform analysis software (Spike 2 Cambridge Electronic Design) from fibers teased from the lumbar splanchnic nerve (rostral to the inferior mesenteric ganglia), recorded using borosilicate glass suction electrodes. Signals were amplified, band pass filtered (gain 5 K; 100 to 1300 Hz; Neurology, Digitalis Ltd., UK), digitally filtered for 50 Hz noise (Humbug, Quest Scientific, UK), digitally filtered at 20 kHz (micro1401; Cambridge Electronic Design, UK), and displayed on a computer using Spike 2 software. Individual receptive fields of afferent nerve fibers were identified by systematically probing the tissue with a 1-g von Frey filament. Receptive fields that responded to probing and not to stretch were identified as serosal units (61). Once a serosal unit was identified, a metal ring was placed over the receptive field, and the baseline activity was observed for 3 min. The Krebs solution within the ring was then removed and replaced by 100 μM 5-oxoETE warmed to bath temperature. After a 7-min challenge period, the 5-oxoETE and ring were removed.

**Immunofluorescence in mouse colon**

The descending colons of 10 WT and 10 Mrgprd-deficient mice were cryoprotected in optimum cutting temperature compound, sectioned at a thickness of 10 μm (one every 0.1 cm, 20 per mouse) on a cryostat (Leica CM1950, Nanterre, France), and mounted on Superfrost slides (Thermo Fisher Scientific, Villebonne-sur-Yvette, France). Slides were washed in phosphate-buffered saline (PBS), 0.5% Triton X-100, and 1% bovine serum albumin (BSA) solution (Sigma-Aldrich) and incubated overnight at 4°C with anti-Mrgprd (1:500; Alomone Labs, catalog no. AMR-061; CliniSciences, Nanterre, France) and anti-PGPR9.5 (1:500; Abcam, catalog no. AB86808; Coger SAS, Paris, France) as primary antibodies. After washing, slides were incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Thermo Fisher Scientific), washed, and mounted with ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Images were acquired using a Zeiss LSM-710 confocal microscope (Carl Zeiss Microlmaging, Jena, Germany) with 20× objective in the inverted configuration.

**Single-cell qRT-PCR analysis of retrogradely labeled mouse sensory neurons**

DRG neurons projecting to the colon were selectively labeled and individually harvested by pulled glass pipette. After RNA extraction, single-cell qRT-PCR analysis for the presence of Mrgprd mRNA transcripts was performed as previously described (62). Briefly, adult mice were subjected to laparotomy under anesthesia, and six to eight injections of FB (~0.2 μl, 2% in saline; Polysciences GmbH) were made into the wall of the distal colon. Five days after surgery, TL (T10-L1) DRGs were collected and enzymatically dissociated (62). Individual cells were isolated by pulled glass pipette and collected into a premplification mastermix containing 0.1 μl of SUPERAse-in (Ambion, TX, USA), 0.2 μl of Superscript III Reverse Transcriptase/Platinum Taq mix (Invitrogen), 5 μl of CellDirect 2× reaction buffer (Invitrogen), 2.5 μl of 0.2× primer/probe mix, and 1.2 μl of Tris-EDTA (TE) buffer (AppliChem GmbH) before thermal cycling (50°C for 30 min, 95°C for 2 min, and then 21 cycles of 95°C for 15 s and 60°C for 4 min). TaqMan qPCR assays for Mrgprd (TaqMan Assay ID: Mm01701850_s1) and Trpv1 (TaqMan Assay ID: Mm01246300_m1) were performed on diluted cDNA products (1:5 in TE buffer) using the following cycling protocol: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldedheyde-3-phosphate dehydrogenase (Gapdh) mRNA acted as an internal positive control, and a sample of the bath solution was used as a no-template negative control. Single-cell RT-PCR products contained Gapdh mRNA, whereas bath control samples did not. The quantitative assessment of gene expression was determined by quantification of cycle values less than the threshold of 35 that were considered as positive. In total, 15 single cells per spinal region (TL) per mouse (n = 3 mice) were isolated; therefore, the expression of mRNA transcripts was determined in 45 colonic sensory neurons.

**Immunohistochemistry of FB-labeled colonic sensory neurons from MrgprdEGFP mice**

From TL regions, DRG T13 were stained from four MrgprdEGFP mice retrogradely labeled with FB to the colon, as described earlier. A single T13 DRG was sectioned sequentially across 10 slides at a thickness of 12 μm. Therefore, on a given slide, the T13 DRG was sampled at 120-μm intervals for the full thickness of the DRG. In total, 16 sections from four animals were analyzed, yielding 274 FB-labeled cells. Slides were stained with chicken anti-GFP (1:1000; Abcam, catalog no. Ab13790) and rabbit anti-CGRP (1:10,000; Sigma-Aldrich, catalog no. C8198) antisera. The secondary antibodies used were goat anti-chicken 488 (1:1000) and donkey anti-rabbit 594 (1:1000). Each probe (that is, MrgprdEGFP and CGRP) per section had a background reading subtracted out and then normalized between the maximum and minimum intensity cells. A threshold of mean + 3× SD for the minimum intensity cells (from all 16 sections) was used to differentiate positive from negative cells. Positive cells were then manually confirmed.

**Ca2+ imaging of mouse sensory neurons**

DRGs of WT and Mrgprd-deficient mice were rinsed in cold HBSS (Invitrogen) and enzymatically dissociated as described previously (63). Neurons were plated in 96-well plates (fluorescence Greiner
Bio-One, Dominique Dutsch, Brumath, France) and cultured for 24 hours. In a first set of experiments, neurons were treated with 5-oxoETE (1, 5, 25, 50, and 100 μM) or vehicle (HBSS). In a second set of experiments, neurons were incubated for 1 hour with IB4 (10 μg/ml) from *Griffonia simplicifolia* conjugated to Alexa Fluor 594 (Thermo Fisher) to differentiate IB4 from IB4 sensory neurons. Ca²⁺ flux was monitored by recording the changing emission intensity of Fluor-4 (Molecular Probes) after treatment with 5-oxoETE (10 μM) or vehicle. In a third set of experiments, neurons were preincubated with PTX (250 ng/ml) overnight or with the U73122 PLC inhibitor (10 μM) 30 min before treatment with 5-oxoETE (50 μM) or vehicle.

Expression of shRNA directed against Mrgprd in sensory neurons

Lentiviral particles were produced as previously described (64). Briefly, HEK293T/17 cells (American Type Culture Collection) were cultured according to the supplier’s recommendations. Cells (1.7 × 10⁵) were seeded into a 175-cm² culture flask containing 30 ml of Dulbecco’s modified Eagle medium (DMEM; Gibco, USA) and then incubated at 37°C and 5% CO₂. On the next day, cells were transfected with a mixture of structural (psPAX2 and pMD2.G; Addgene, Cambridge, MA, USA) and transfer vectors (shRNA Mrgprd-RFP-CB or the control shRNA-RFP-CB; OriGene Technologies), with Genejuice (Millipore, USA) transfection reagent. Cells were incubated overnight at 37°C and 5% CO₂ before the medium was replaced with 18 ml of OptiMEM (Gibco, USA). Cell culture medium was harvested 48 hours later and cleared by centrifugation and filtration with a 0.45-μm syringe filter. Neurons were plated in 96-well plates coated with poly-l-ornithine/laminin and cultivated in Neurobasal medium supplemented with B27 and 1-glutamine before being transfected with 50 μl of lentiviral supernatant. Three days later, a transduction efficiency of 35% was achieved and a calcium flux assay was performed in response to 5-oxoETE (10 μM), as described earlier.

Ca²⁺ flux in CHO cells expressing Mrgprd

CHO cells were transfected with a plasmid expressing mouse Mrgprd (OriGene Technologies, Rockville, USA) with Genejuice Transfection Reagent (1 μg of plasmid for 3 μl of Genejuice). The cells were incubated in Ham’s F12 Nutrient Mixture with 5% of fetal bovine serum (FBS). G418 (Sigma-Aldrich) was used as the selection antibiotic. Cells (50 × 10⁵ cells per well) in a 96-well plate were incubated with Fluo-8 loading solution (Fluo-8-AM; Invitrogen) according to the manufacturer’s instructions. The fluorescence was then measured at 530 nm on a microplate reader (NOVOstar; BMG Labtech) for 1 min. Five seconds after the beginning of the calcium measures, 5-oxoETE (1, 10, 25, 50, 100, and 200 μM) or β-alanine (1 mM; Sigma-Aldrich) was added. Data were collected and analyzed with the NOVOstar software.

Ca²⁺ imaging of human sensory neurons

Experiments were conducted according to opinion number 14-164 of the institutional review board (IRB00003888) of INSERM. Three human T11 DRGs were supplied through the National Human Tissue Resource Center from the NDRI. The DRGs were received unfixed in DMEM at 4°C. The DRGs were received unfixed in DMEM at 4°C. DRGs were dissected, minced in HBSS, and incubated in papain (27 μg/ml; Sigma-Aldrich, Saint-Quentin-Fallavier, France) for 20 min at 37°C. After washing with L-15 wash buffer [Leibovitz’s L-15 Medium (Invitrogen) and 10% FBS (Invitrogen)] and HBSS, the DRGs were incubated in HBSS containing collagenase type IV (1 mg/ml; Worthington, Lakewood, NJ, USA) and dispase II (4 mg/ml; Sigma-Aldrich). L-15 wash buffer was added to neutralize enzymatic activities, and the suspension was centrifuged at 1000g for 5 min. The cycle of digestion was repeated three times for 15 min. Neurons in the pellet were suspended in Neurobasal medium (Invitrogen) containing 2% B27, 2 mM glutamine, 1% penicillin/streptomycin, and 10 μM each of cytosine arabinoside, 5-fluoro-2′-deoxyuridine, and uridine (all from Sigma-Aldrich). The medium was changed every 3 days without cytosine arabinoside. Cells were plated in CC2 LabTek II (Nunc, Dominique Dutsch, Brumath, France) for the calcium signaling assay as described earlier in response to 5-oxoETE (0.1, 1, and 10 μM) and for immunochemistry.

Immunofluorescence in human DRG

Experiments were performed according to opinion number 12-074 of the institutional review board (IRB00003888) of INSERM. Two human T11 DRGs were supplied through the national human tissue resource center from the NDRI. The DRGs were received unfixed and cryoprotected. The DRGs were cut into 20-μm sections on a cryostat (Leica CM1950, Nanterre, France) and mounted on Superfrost slides (Thermo Fisher Scientific, Villebonne-sur-Yvette, France). Cultured sensory neurons and slides were washed in PBS, 0.5% Triton X-100, and 1% BSA solution (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and incubated overnight at 4°C with anti-Mrgprd (1:100; LifeSpan Biosciences, LS-A4123; CliniSciences, Nanterre, France) and anti-βIV-PG (1:500, AB86808, Abcam). After washing, the slides and cultured DRG were incubated with the appropriate secondary antibody conjugated with Fluor 488 or Alexa Fluor 555, washed, and mounted with ProLong Gold reagent containing DAPI (Molecular Probes). Images were acquired using a Zeiss LSM-710 confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) with a 20× objective in the inverted configuration.

Study approval

The study protocol for biopsy collection was approved by the local Ethics Committee (64/2004/O/Sper and EM14/2006/O) and conducted in accordance with the Declaration of Helsinki. Patients and HCs gave written informed consent. Fixed and fresh human DRGs were supplied through the National Human Tissue Resource Center from the NDRI (reference: DCEN1 001). Experiments on human DRGs were performed according to opinion number 14-164 of the institutional review board (IRB00003888) of INSERM. Animal experiments were conducted according to the Council of the European Union directive 2010/63/EU. The Animal Care and Ethics Committee of US006/CREFE (CEEA-122) approved the whole study protocol (permit no. MP/01/64/09/12). Experiments conducted at the University of Glasgow were approved by the University’s Ethical Review Process Applications Panel and were performed in accordance with European Community directive 86/609/EC and the United Kingdom Animals (Scientific Procedures) Act 1986.

Statistical analysis

Data are presented as means ± SEM. Analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Comparisons between groups were performed by Mann-Whitney test. Multiple comparisons within groups were performed by Kruskal-Wallis test, followed by Dunn’s post test. Statistical significance was accepted at P < 0.05.
SUPPLEMENTARY MATERIALS

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Fig. S1. Concentrations of PUFAs in metabolites from biopsies with IBS. Fig. S2. Concentrations of PUFAs in metabolites in biopsies of all patients with IBS. Fig. S3. 5-oxoETE does not induce somatic or visceral inflammation in vivo. Fig. S4. 5-oxoETE induces Ca2+ flux in mouse sensory neurons. Fig. S5. Mrgrpd immunoreactivity is observed in mouse colon. Fig. S6. Mrgrpd immunoreactivity is not observed in the colon of Mrgrpd-deficient mice.

REFERENCES AND NOTES


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M-currents and contributes to enhanced neuronal excitability. J. Neurosci.


Coupled receptor D- MAS-related G protein–coupled receptor D

C. Bulmer and Nicolas Cenac
Alexandre Denadai-Souza, Nathalie Vergnolle, Ewan St John Smith, David I. Hughes, Giovanni Barbara, Gilles Dietrich, David Raffaella Barbaro, Lilian Basso, Pauline Le Faouder, Corinne Rolland, Pascale Malapert, Aziz Moqrich, Helene Eutamene, Alexandre Denadai-Souza, Nathalie Vergnolle, Ewan St John Smith, David I. Hughes, Giovanni Barbara, Gilles Dietrich, David

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Inducing pain in IBS

A symptom of irritable bowel syndrome (IBS) that accompanies altered bowel function is abdominal pain. Noting that various polyunsaturated fatty acids (PUFAs) are implicated in modulating inflammation in patients with IBS, Bautzova et al. found that the abundance of the PUFA 5-oxoETE was selectively increased in colonic biopsies from patients with a subtype of IBS characterized by constipation (IBS-C). 5-oxoETE increased pain sensitivity in mice without eliciting inflammation and stimulated both mouse and human dorsal root ganglia neurons expressing the GPCR Mrgprd. Knockdown of Mrgprd in mice reduced the percentage of neurons that responded to 5-oxoETE and decreased pain sensitivity, suggesting that this PUFA may mediate abdominal pain in patients with IBS-C.