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FAAH inhibitor URB597 shows anti-hyperalgesic action and increases brain and intestinal tissues fatty acid amides in a model of CRF_1 agonist mediated visceral hypersensitivity in male rats

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Abstract

Background & Aims: The endocannabinoid (eCB) system includes ligands (anandamide and 2-arachidonoyl glycerol, 2-AG), receptors and catabolizing enzymes (fatty acid amide hydrolase, FAAH and monoacylglycerol lipase) expressed in both the brain and gut. We investigated whether the FAAH inhibitor, URB597, influenced visceral pain to colorectal distension (CRD) in an acute stress-related model of visceral hypersensitivity induced by the selective corticotropin releasing factor receptor subtype 1 (CRF_1) agonist, cortagine.

Methods: Male Sprague-Dawley rats were injected subcutaneously (SC) with URB597 (3 mg/kg) or vehicle and 2h later, intraperitoneally with cortagine (10 μg/kg) or vehicle. The visceromotor responses (VMR) were assessed to a 1st CRD (baseline) before injections, and to a 2nd CRD 15 min after the last treatment. Brain, jejunum and proximal colon were collected

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Authors contributions

ML, CH, AM performed the *in vivo* research; SA, PG performed the eCBs assays; ML, AM, SA, PG, JPP analyzed the data; ML, MM, JPP, MGC, YT designed the research study; ML and YT wrote the paper, ML, AM, CH, MM, SA, PG, JPP, MGC, YT revised the paper and approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Conflict of interest statement

No competing interests to declare. At time of work, JPP, MGC, PG and SA were employed at Ironwood Pharmaceuticals Inc., Boston, MA, USA. MGC is currently board member at Ironwood Pharmaceuticals Inc., Boston, MA, USA.

from treated and naïve rats for levels quantification of 3 fatty acid amides (FAAs) [anandamide (arachidonyl-ethanolamide, AEA), oleoyl-ethanolamide (OEA) and palmitoyl-ethanolamide (PEA)], and 2-AG. In separate animals, defecation/diarrhea were monitored after URB597 and cortagine.

Key Results: URB597 inhibited cortagine-induced increased VMR at 40 mmHg (89.0 ± 14.8% versus 132.5 ± 15.6% for vehicle SC, $p<0.05$) and 60 mmHg (107.5 ± 16.1% vs. 176.9 ± 24.4% for vehicle SC, $p<0.001$) while not influencing basal VMR. In URB597 plus cortagine group, FAAs levels increased in the brain and intestinal tissue while 2-AG did not change. URB597 did not modify cortagine-induced defecation/diarrhea *vs* vehicle.

Conclusions & Inferences: URB597 shows efficacy to elevate brain and intestinal FAAs and to counteract the colonic hypersensitivity induced by peripheral activation of CRF_1 signaling supporting a potential strategy of FAAH inhibitors to alleviate stress-related visceral hypersensitivity.

Keywords

anandamide; cortagine; defecation; irritable bowel syndrome; URB597; rat; visceral pain

1 | INTRODUCTION

The endocannabinoid (eCB) system includes the endogenous lipid ligands, arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol, their target receptors (CB_1 and CB_2) and the enzymes involved in their biosynthesis and degradation.[1,2] The eCB system is distributed in both the central and peripheral nervous systems and also expressed in the gastrointestinal tract, namely in the enteric nervous system, smooth muscle, epithelial, glial and immune cells.[3–6] Cannabinoid ligands have been implicated in a broad range of gut function (secretion, motility, ion transport, intestinal barrier integrity and gastroprotection) as well as in the modulation of pathophysiological processes such as intestinal inflammation and pain signaling (see reviews).[4,5,7–10]

Endocannabinoids are rapidly degraded mainly by two enzymes, the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase.[11–13] FAAH is the major enzyme responsible for the catabolism of anandamide (AEA) and several other acylethanolamides (fatty acid amides, “FAAs”), including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) which do not activate cannabinoid receptors, while monoacylglycerol lipase degrades 2-arachidonoylglycerol (2-AG).[11,12,14] Pharmacological inhibition of FAAH to increase endogenous eCB ligands and other non-eCB FAAs (OEA, PEA) has become an attractive therapeutic option in circumstances where endocannabinoid or other FAAs modulation may be beneficial.[15,16] This is mainly due to the lack of psychotropic side effects and motor impairments of endocannabinoids in contrast to exogenous cannabinoid agonists administration.[17–21] In particular, it is well documented that the administration of FAAH inhibitors exert anti-nociceptive actions in a wide range of preclinical somatic pain models induced by inflammatory, thermal, or mechanical noxious stimuli and in some neuropathic pain models.[22–24] Clinical studies showed that the orally active and highly selective FAAH inhibitor, PF-04457845[25,26] given orally to healthy

subjects is safe and well-tolerated in Phase 1 clinical trials, and induces a robust and sustained elevation of FAAs (AEA, OEA, PEA) levels in plasma collected from peripheral blood.[27] PF-04457845 was also reported to exert beneficial effects on fear extinction and stress-related behaviors in a controlled clinical trial.[28]

Irritable bowel syndrome (IBS) is a stress-sensitive prevalent bowel disorder categorized into three major clinical subtypes IBS-constipation (IBS-C), IBS-diarrhea (IBS-D) and IBS-mixed (IBS-M),[29] each with complex underlying mechanisms, including disturbances along the brain-gut axis.[30–33] Abdominal pain is the most important determinant of IBS severity, impairment of life quality and healthcare utilization in patients suffering from IBS.[34] Although advances in treatments have been made,[35] to date there is still an unmet clinical therapeutic need in IBS.[36] Visceral hypersensitivity to rectosigmoid distension is observed in nearly half of IBS patients and is thought to underlie abdominal pain.[37,38]

Experimental studies with FAAH inhibitors showed beneficial effects in visceral pain models induced by the intraperitoneal or intracolonic administration of chemical noxious irritants (acetic acid, phenyl-*p*-benzoquinone, lactic acid) while monoacylglycerol lipase inhibitors had no effect.[39–44] In the context of IBS, the influence of FAAH inhibitors has not yet been examined under the condition of acute stress-related hypersensitivity by monitoring the visceromotor response (VMR) to colorectal distension (CRD), a robust model of visceral nociception.[45] Previous studies indicate that the activation of the corticotropin releasing factor receptor subtype 1 (CRF_1) signaling pathway in the brain and/or the gut plays a key role in acute stress-related gastrointestinal alterations and visceral pain.[46,47] Of relevance to IBS, we initially reported and others that the CRF_1 peptide agonist, cortagine, or CRF injected intraperitoneally in rodents reproduces IBS-D-like symptoms including visceral hypersensitivity to ascending phasic CRD and defecation/diarrhea in rats.[48–50]

In the present study, we test the hypothesis that increasing endogenous cannabinoids will modulate visceral pain. This was achieved by investigating the influence of the well-established selective FAAH inhibitor, URB597,[51,52] administered peripherally on visceral pain using the cortagine-induced hypersensitivity to CRD model in rats.[53,54] The associated brain and intestinal changes of anandamide and 2-arachidonoylglycerol and non-cannabinoid fatty acid amides including palmitoylethanolamide and oleoylethanolamide levels were determined. In separate experiments we also monitored whether URB597 modified defecation and diarrhea under basal and cortagine treated rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments were performed in adult male Sprague-Dawley rats (Harlan Laboratory, San Diego, CA, USA) weighing 200–250 g. The use of male rats only was in keeping with sex differences in brain activation and visceral responses to CRD and stress,[55,56] thereby avoiding sex-related variability.[55–57] Animals were kept under controlled conditions of illumination (12:12h light-dark cycle starting at 6 a.m.), temperature (21–23°C) and humidity (30–35%) and allowed free access to water and food (Purina rat chow, USA). Animals were acclimated to the animal facility for 1 week after their arrival. Experiments

were initiated between 7 a.m. and 9 a.m. and ended no later than 2 p.m. to avoid circadian influence on parameters under study. Experiments followed NIH guidelines according to protocol # 06016–08 approved by the Institutional Animal Care and Use Committee (IACUC) of the VA Greater Los Angeles Healthcare System under the auspice of the Office of Laboratory Animal Welfare - Assurance of Compliance (A3002–01).

2.2 | Compounds and treatments

Cortagine ([Glu^{21},Ala^{40}][sauvagine$_{1–12}$]x[rat CRF$_{14–30}$]x[sauvagine$_{30–40}$]) (Peptide Biology Laboratories, Salk Institute, La Jolla, CA) stored in powder form at −80°C, was weighed and dissolved in sterile water immediately before use as previously described.[58] URB597 [(3'-(aminocarbonyl)([1,1'-biphenyl]-3-yl)-cyclohexylcarbamate)] (Cayman Chemical, Ann Arbor Michigan) was dissolved in dimethyl sulfoxide (DMSO)/cremophor El/saline (10/10/80 v/v). The volumes of intraperitoneal (IP) and subcutaneous (SC) injections were 0.2 and 0.3 ml, respectively.

2.3 | Visceral pain studies

Colorectal distension procedure.—The VMR to CRD was monitored as previously described using a non-invasive method we developed based on the measurement of intraluminal colonic pressure (ICP).[53,54,59] Rats were trained to the experimental conditions (Bollman cage 4h/day and handling for SC and IP injections) for 3 days before the experiment. The day after the end of training, animals were briefly anesthetized with isoflurane (3% in O_2) and the modified miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX) equipped with a custom-made polyethylene plastic balloon (2 cm width × 5 cm length) tied below the pressure sensor was inserted into the colorectum up to 1 cm past the anal verge. The catheter was secured to the tail with tape, and rats were placed in Bollman cages and left to rest for 30 min before the CRD procedure. Then, the balloon was unfolded by two distensions at 60 mmHg, immediately followed by the 1st set of CRD consisting of phasic distensions at incremental pressure of 10, 20, 40 and 60 mmHg (20-s duration; 4-min inter-stimulus interval, twice each) and a 2nd similar set of CRD after treatment. The distensions were always in the same ascending order and not randomized. Such a CRD paradigm is standard and has been previously used to assess visceral pain-related responses in rats.[48,54,60]

Experimental protocol.—See experimental design (Fig. 1A). In conscious rats, the VMR to the 1st set of CRD was obtained and taken as baseline response. Immediately after the end of the distension, rats received SC injection of URB597 (3 mg/kg) or vehicle. After a 2-h rest, animals were injected IP with cortagine (10 μg/kg) or vehicle and 15 min later, the VMR to the 2nd set of CRD was recorded. The visceral pain experiments were repeated in 3 different cohorts of rats (n=6, n=6, n=5, total n=17) treated with vehicle SC + cortagine IP and 2 cohorts (n=11, n=7, total n=18) for URB597 SC + cortagine IP which led to similar results, so data were pooled together. We selected the maximal effective dose of cortagine based on previous dose response studies to increase VMR to CRD[48] and that of URB597 based on its maximal antinociceptive effect in somatic tests of allodynia and hyperalgesia.[61] The time interval between URB597 administration and testing is taking into account the report of slow and reliable accumulation of AEA in the nervous system with a

maximal effect at 2 hours post-injection[51] therefore we selected 2 h post injection to test the antinociceptive effect on cortagine-induced visceral hyperalgesia.

Signal acquisition.—The modified miniaturized pressure transducer was connected to a preamplifier (model 600; Millar Instruments, Houston, TX) and the balloon to an electronic barostat (Distender Series II, G&J Electronics Inc, Toronto, Canada). The barostat controlled for balloon pressure variation and minimized any interference of colonic motor activity changes during balloon inflation. The signal was acquired using CED Micro1401/SPIKE2 program (Cambridge Electronic Design, Cambridge, UK). The phasic component of the ICP signal was extracted from the original signal recorded by applying the DC Remove Process in Spike 2 (CED, Ltd., Cambridge) component with a time constant of 1 s to exclude the slower, tonic changes in ICP resulting from colonic smooth muscle activity, and by applying the root mean square (RMS) amplitude process with a time constant of 1 s to the resulting trace. ICP activity was recorded for 20 s before, during and after termination of CRD. The VMR was defined as the increase area under the curve (AUC) of ICP during CRD over the mean of pre- and post-distension periods (20 s each) and quantified using the "modulus" process in Spike 2. To examine the pressure-response relationship and adjust for inter-individual variations of the signal, ICP amplitudes were normalized for each rat to the highest pressure (60 mmHg) in the 1st set of CRD and taken as 100%. The VMR to the 2nd CRD with or without treatment in each animal is shown as % from their normalized control values (% control) at different pressures of distension as validated in our previous studies.[60]

2.4 | Assessment of colonic motor function

See experimental design (Fig. 1B). In four separate groups of naïve conscious rats (n=10, n=10, n=11, n=11), fecal pellet output (FPO) and incidence of diarrhea (defined as the percentage of rats displaying at least one watery stool during the observation period) were recorded. Defecation was monitored for the first 2 h post SC injection of URB597 (3 mg/kg) or vehicle then every 15 min for 1h after the IP injection of cortagine (10 μg/kg) or vehicle. In all experiments, animals were handled daily for 5 days before the first administration of cortagine or vehicle. The IP dose of cortagine was based on previous dose-response studies showing defecation and shortening of colonic transit time in rats[48] and that of URB957 was similar to the dose used for VMR experiment.

2.5 | Assessment of FAAs (AEA, OEA, PEA) levels and 2-AG levels

Brain and intestinal tissues: collection and sample preparation.—Naïve rats and experimental groups were deeply anesthetized with isoflurane 30 min after the last set of CRDs and decapitated. The brain was flash-frozen on dry ice. The proximal colon and jejunum (5 cm each) were collected, each opened by a longitudinal incision, and rinsed in ice-cold saline before being flash-frozen on dry ice. Tissues were stored at −80°C until eCBs assays using a modified published method.[62,63] Tissue samples were weighed and extracted in 7 ml ethyl acetate: hexanes (9:1 vol/vol) containing N-palmitoyl propanolamide, as internal standard. Samples were homogenized by an electric-powered mechanical tissue disrupter (Omni International, Kennesaw, GA) in 3 ml water. Tubes were vortexed and centrifuged [10°C, 1875xg, 20 min (intestine), 30 min (brain)]. The organic layer was transferred to glass tubes and was evaporated under azote gas until

dry. Extracts were reconstituted in chloroform:methanol (1:3 vol/vol), transferred to 1.5 ml capacity polypropylene tubes, and centrifuged (room temp, 16,000xg, 3 min). Supernatants were transferred to 96 well plates, diluted 1:1 (vol/vol) in ice-cold methanol containing anandamide labeled with 4 deuterium atoms, d4-AEA (Cayman Chemical, Inc., Ann Arbor, MI) and were mixed. Diluted samples were placed in a chilled (6°C) autosampler and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS).

Liquid chromatography-tandem mass spectrometry assay.—The concentrations of endogenous anandamide and 2-arachidonoylglycerol and non-cannabinoid fatty acid amides including palmitoylethanolamide and oleoylethanolamide, levels in samples were determined by LC-MS compared to standard curves generated with respective synthetic standards (Cayman Chemical Inc.) diluted in methanol. Analytes were detected and quantified by tandem mass spectrometry in positive ion mode on a Waters Acquity/TQD system in positive ion (ES+) mode. The samples were injected (20 μl) on a Clipeus C8 reverse phase HPLC column (2.1 mm × 30 mm dimensions; 5 μm particle size; Higgins Analytical, Mountain View, CA) and were chromatographed using a gradient system with 0.1% formic acid in water and 0.1% formic acid in acetonitrile/ isopropanol/ water (85:10:5, vol:vol:vol). Chromatograms were integrated and quantified by peak area against a standard curve using Quanlynx software V4.0 SP4 (Micromass Ltd).

2.6 | Data analysis and statistics

Data were analyzed using the GraphPad Prism 7.00 software. The results are expressed as means ± SEM and p values <0.05 were considered statistically significant. Comparisons of the VMR to CRD in the URB597 + cortagine vs vehicle + cortagine groups and within each group of animals vs baseline were analyzed using two-way ANOVA and Bonferroni post-test. The defecation time-course was analyzed using a two-way ANOVA and Sidak post-hoc test comparisons and the mean cumulative FPO using a Mann-Whitney unpaired t test. For eCBs levels, statistical comparisons between treatment groups were made using an unpaired, two-tailed t–test. Fold change was determined against mean of biomarker concentration from tissues in naïve rats and in some cases, also in vehicle-treated rat tissues. Values were analyzed by Grubb's test to determine whether any value is a significant ($p < 0.05$) outlier from the group. For visceral pain, one outlier was removed from the cortagine + URB597 group due to abnormal response to distension characterized by a total absence of response to the CRD in baseline. Investigators were not blinded to the treatment groups.

3 | RESULTS

3.1 | URB597 SC inhibited IP cortagine-induced visceral hypersensitivity to colorectal distension in conscious rats.

Compared with the baseline 1st set of CRD, cortagine (10 μg/kg, IP, n=17) significantly increased the VMR to the 2nd set of CRD at 40 mmHg (132.5 ± 15.6 *vs.* 80.8 ± 7.2, p<0.01) and 60 mmHg (176.9 ± 24.4 vs 100.0 ± 0.0 p<0.001) while there was a non-significant trend to be decreased after the injection of IP vehicle group (VMR at 60 mmHg CRD in % control: 68.1 ± 12.4 *vs.* 100.0 ± 0.0, p>0.05, n=5) (Fig. 2A).

Pretreatment with URB597 SC, compared with vehicle SC, abolished the exacerbation of VMR induced by cortagine at 60 mmHg (107.5 ± 16.1 vs 176.9 ± 24.4, $p<0.01$) and showed a positive but not statistically significant trend at 40 mmHg (89.0 ± 14.8 vs 132.5 ± 15.6, n=18)(Fig. 2B). In addition, URB597 decreased the percentage of rats developing hypersensitivity in response to IP cortagine compared with vehicle pretreated (36% instead of 92% at 60 mmHg). By itself, URB597 had no significant effect on the VMR to CRD compared to baseline (79.8 ± 18.1 vs 82.1 ± 19.0 at 40 mmHg and 82.7 ± 8.6 vs 100 ±0.0 at 60 mmHg, n=7).

3.2 | URB597 SC increases the levels of fatty acid amides but not 2-AG in the brain, jejunum and proximal colon in rats treated with IP vehicle or IP cortagine.

In the groups undergoing the assessment of VMR detailed in 3.1, the endocannabinoid levels were increased by URB597 SC as monitored at the end of the experiment in several tissues.

Brain: Brain levels of anandamide increased significantly by 5.6-fold in the SC URB597 plus IP cortagine treated rats at 3h25 min after the pretreatment while they were not modified in SC vehicle plus cortagine group compared to naive rats (Fig. 3A). A 9.1 and 8.7-fold increases were observed in oleoylethanolamide and palmitoylethanolamide respectively compared to the naïve group (Fig. 3B and 3C, respectively). In contrast, the brain levels of 2-arachidonoylglycerol did not differ between groups (Fig. 3D).

Proximal colon: SC URB597 significantly increased the levels of anandamide by 2.2-fold in the proximal colon of IP cortagine-injected animals compared to SC vehicle + IP cortagine group and cortagine has no influence on anandamide levels compared to naive (Fig. 3E). SC URB597 increased levels of oleoylethanolamide and palmitoylethanolamide levels by 2.5 and 1.9-fold compared to the naïve group (Fig. 3F and 3G, respectively), while the levels of 2-arachidonoylglycerol did not differ between groups (Fig. 3H).

Jejunum: SC URB597 pretreatment in cortagine-injected rats increased by 1.6 levels of anandamide in the jejunum when compared with SC vehicle plus cortagine (Fig. 3I) and enhanced the levels of oleoylethanolamide (Fig. 3J) and palmitoylethanolamide (Fig. 3L) by 1.9-fold compared to naive. Interestingly, SC vehicle-treated rats but not SC URB597-treated rats injected with IP cortagine exhibited a small increase in jejunum levels of 2-arachidonoylglycerol compared to naïve rats (Fig. 3L).

3.3 | URB597 does not influence peripheral cortagine-induced increased defecation and diarrhea in conscious rats.

In naïve rats, cortagine injected IP at 10 μg/kg increased pellet output/h compared to vehicle (4.8 ± 1.5 vs 1.0 ± 0.7 pellets/h, $p<0.05$, n=10 for each group)(Fig. 4A) and induced diarrhea in 50% of vehicle-treated rats. The FAAH inhibitor, URB597 (3 mg/kg, SC) did not modify the pellet output (7.2 ± 1.4, Fig. 4A) or the diarrhea response (54.5%) induced by IP cortagine and did not influence basal output (0.6 ± 0.4 vs 1.0 ± 0.7 pellets/h, $p>0.05$, n=11 for each group)(Fig. 4A). The time course of the defecation response to cortagine IP showed a maximal response at 30 min that was similar in both URB597- and vehicle-treated rats (Fig. 4B).

4. Discussion

We provide evidence that the SC injection of the FAAH inhibitor, URB597, abrogates the visceral hypersensitivity to phasic CRD induced by peripheral injection of the CRF_1 agonist, cortagine while not influencing the basal VMR to CRD and defecation/diarrhea in rats. Under these experimental conditions, URB597 increased levels of fatty acid amides, anandamide, oleoylethanolamide and palmitoylethanolamide in the brain, proximal colon and jejunum of rats, while not modifying 2-arachidonoylglycerol levels. To the best of our knowledge, these data are the first to show an anti-hyperalgesic effect of a FAAH inhibitor in an experimental model of CRF receptor activation-induced visceral hypersensitivity to CRD.

In agreement with our previous studies,[48] the IP injection of cortagine results in a rapid hyperalgesia as shown by the 162% and 160% increase in the VMR to phasic CRD at 40 and 60 mmHg respectively compared to the IP saline group. This increased visceral sensitivity was inhibited by the peripheral administration of URB597. Previous reports showed that FAAH inhibitors including URB597 suppress visceral nociception in models of visceral pain elicited mainly by the IP injection of noxious chemical irritants such as acetic acid, lactic acid or phenyl-p-benzoquinone in mice.[22,40–42,44,64] The present findings extend the beneficial effect of FAAH inhibition to colonic hypersensitivity to CRD triggered by the activation of peripheral CRF_1 signaling known to be involved in colonic response to acute stressors including water avoidance or restraint.[46,65–69] This was shown by use of peripherally restricted peptide CRF antagonists[65–67] and the demonstration that partial restraint increased CRF expression and levels in the rat colon and that the inhibition of CRF expression selectively in the colon prevented partial restraint stress-induced increase in fecal output, ion secretion, and transepithelial tissue conductance.[46]

Under our experimental conditions, URB597 increased the levels of eCB ligand, anandamide, as well as those of oleoylethanolamide and palmitoylethanolamide in the brain, jejunum and colon while 2-arachidonoylglycerol levels were not modified in these tissues, confirming the selectivity of URB597 for inhibiting FAAH.[52] Changes in eCB levels in the rat gut by peripheral administration of FAAH inhibitors have been little investigated, however it has been reported previously to occur in the ileum and colon of mice after IP injection of the FAAH inhibitor, PF-3845.[22] The increases of anandamide in both the brain and intestine induced by URB597 could entail central and/or peripheral components contributing to the suppression of visceral hypersensitivity. In support of peripheral mechanism, first, the IP injection of cortagine- or CRF- induced visceral hyperalgesia to CRD has been established to be mediated peripherally by targeting CRF_1 receptors on colonic myenteric cholinergic neurons,[70] enterochromaffin cells[71] and mucosal mast cells[72] and increasing colonic permeability.[48,49] Second, there is evidence that the eCBs is distributed in the gut across key peripheral loci modulating pain transmission including primary enteric sensory neurons and sensory terminals of primary afferents.[4,73–76] Third, the peripherally restricted FAAH inhibitor, URB937, inhibited visceral pain induced by IP acetic acid.[64] Lastly, in preclinical models of inflammatory and neuropathic pain, the tissue specific CB_1 deletion of peripheral nociceptors accounts for the large proportion of cannabinoid analgesia.[77] Although this accumulated evidence points to a peripheral

mechanism, it cannot be ruled out that the elevated anandamide level in the brain induced by URB597 plays a role by modulating the neuronal circuitries activated by CRD.[57,78] The CB receptors involved are yet to be identified, however, previous studies point to a primary role of CB_1 receptor in the antinoceptive effect of the FAAH inhibitors URB597 and URB937 in the visceral pain models of IP injection of phenyl-p-benzoquinone or acetic acid.[40,64] CB_2 receptors have been involved mainly in modulating post-inflammatory visceral hypersensitivity to CRD in colitis models.[79–82] in keeping with their expression primarily on various immune cell populations.[83] By contrast, the model of cortagine-induced visceral hypersensitivity did not show signs of colonic inflammation histologically[48] and we found that cortagine alone did not modify brain and intestinal levels of anandamide levels contrary to what is observed in intestinal inflamed tissues.[4,84]

We further found that URB597 suppressed cortagine-induced visceral hypersensitivity to CRD without modifying the basal VMR. This suggest that the elevation of FAAs in the brain and intestine targets preferentially the hypersensitivity induced by IP cortagine unlike basal visceral pain response to mechanical distension of the colon. This is consistent with other report showing that the exogenous administration of peripherally acting CB_1-selective agonist, SAB-378 blocked the hypersensitivity induced by 12 repeated CRD at 80 mmHg while not influencing the response to the first basal CRDs.[85] The other FAAH inhibitor, PF3845 injected IP at the dose of 10–20 mg/kg similarly had no effect on the colonic mechanical distension.[44]

The finding that URB597 at a dose inhibiting cortagine-induced hyperalgesia did not influence the defecation and diarrhea is somewhat surprising considering the established inhibitory effect of endocannabinoids on gastrointestinal motility.[86] Other studies in mice showed that the FAAH inhibitor, AM3506 inhibited lipopolysaccharides induced fecal output.[87] These contrasting results may reflect differences in modalities of colonic propulsive activation or could be related to insufficient elevation of fatty acid amides at the synaptic level in the colon induced by URB597 to counteract the direct and robust activation of cholinergic myenteric and submucosal VIP neurons involved in the increased pellet output in response to cortagine.[70,88] The differential sensitivity to alleviate visceral pain and colonic motor response by the FAAH inhibitor, PF-3845was previously reported. The dose required to normalize the increase defecation triggered by exposure to a novel environment needed to be 30-fold higher than the one efficient to produce the antinociceptive effect in the writhing test of IP acetic acid.[22]

In summary, the peripheral administration of FAAH inhibitor, URB597 at 3 mg/kg increased the endogenous intestinal and brain levels of anandamide and acylethanolamides. This was associated with the suppression of the hypersensitivity to CRD induced by the activation of peripheral CRF_1 signaling pathway which contributes to the colonic motor and visceral alterations induced by water avoidance or restraint stressors.[46,67–69] By contrast the increased defecation was not modified. In view of mounting evidence of endocannabinoids as an emerging therapy in IBS,[86,89,90] our data support the potential benefit of FAAH inhibitors recently tested for their safety profile[91,92] to alleviate visceral pain in stress-sensitive IBS patients.

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Key points

- Two decades ago, it was hypothesized that endocannabinoid (eCB) deficiency contributes to multiple overlapping pain disorders in clinical setting, including irritable bowel syndrome (IBS). Since then, both alterations of the endocannabinoid system in IBS patients and the potential beneficial use of cannabinoid-related products to improve IBS symptoms have been reported.
- Despite this evidence, the role of eCB system in visceral pain is not well known. In this study, we demonstrate that URB597, which inhibits the eCB degrading enzyme fatty acid amide hydrolase (FAAH), increases the levels of 3 highly-characterized endogenous fatty acid amide substrates of FAAH in rat colon and brain (anandamide, OEA and PEA) and suppresses the visceral hypersensitivity to colorectal distension induced by peripheral CRF_1 receptor activation.
- Our results support the potential benefit of FAAH inhibitors to alleviate stress-related visceral hypersensitivity in IBS patients.

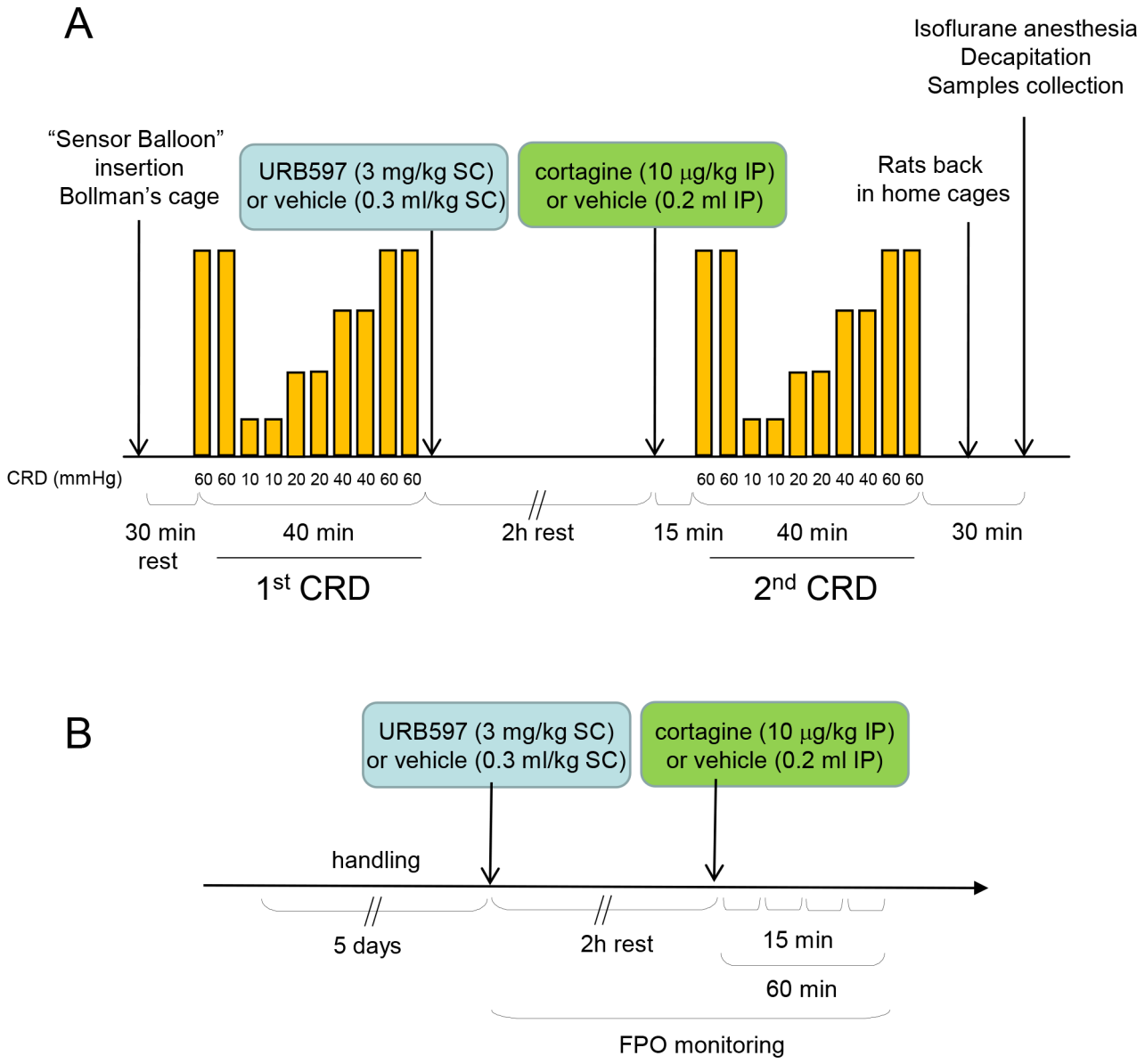

Figure 1:

Experimental design schema for visceral pain testing (**A**) and defecation response (**B**). **A)** In conscious rats, the VMR to the 1st set of CRD was obtained and taken as baseline response. Immediately after the end of the distension, rats received SC URB597 (3 mg/kg) or vehicle. After 2 h, animals were injected IP with cortagine (10 µg/kg) or vehicle and 15 min after the injection, the VMR to the 2nd set of CRD was recorded. Rats were returned to their cages and 30 min after the end of the 2nd CRD, were euthanized and their brains and colons collected for FAA assays. **B**) In two separate groups of naïve conscious rats, fecal pellet output (FPO) and the incidence of diarrhea (defined as the percentage of rats displaying at least one watery stool during the observation period) were recorded. Rats were handled for 5 days prior to the experiment to allow for habituation and reduce the stress levels. On the day of the experiment, defecation was monitored for the first 2h post SC injection of URB597 (3

mg/kg) or vehicle then every 15 min for 1 h after the IP injection of cortagine (10 μg/kg) or vehicle.

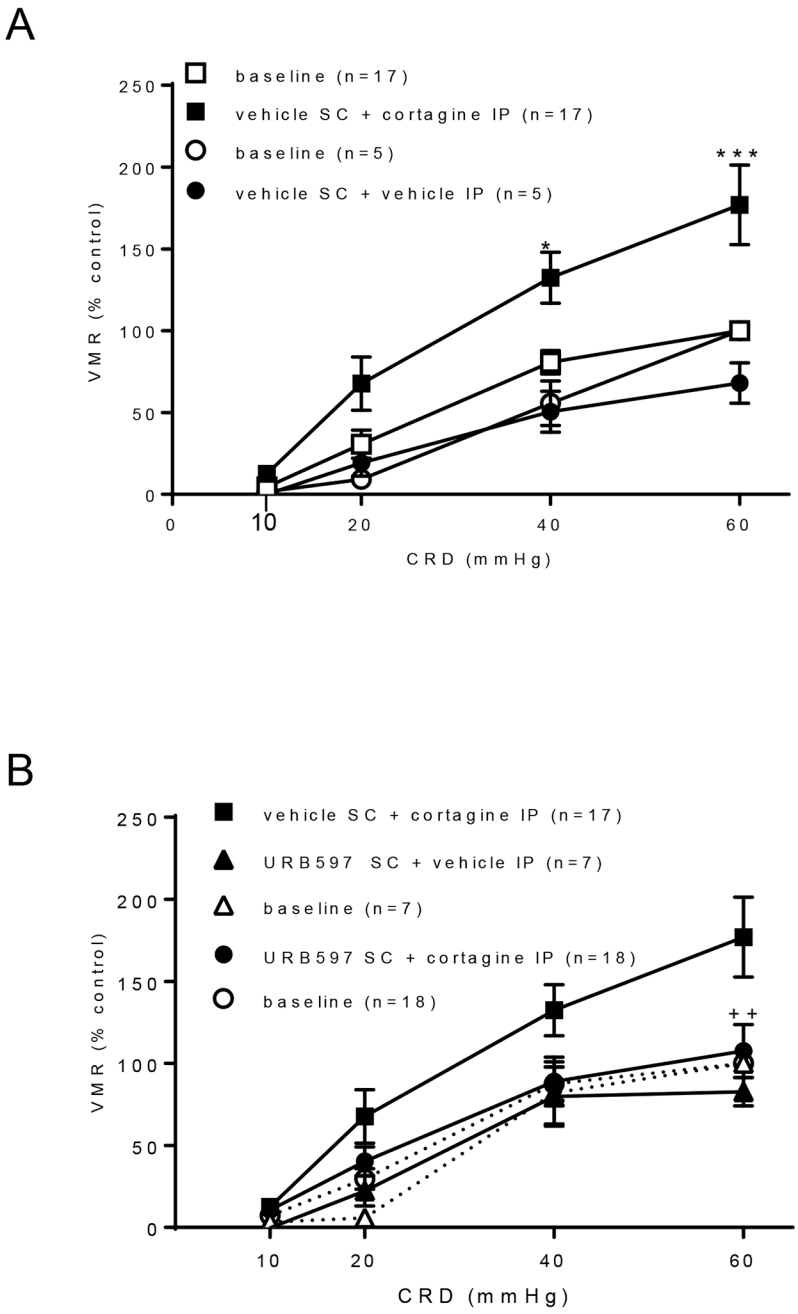

Figure 2:

Influence of URB597 pretreatment on cortagine-induced visceral hypersensitivity in male rats. **(A)** Visceromotor response (VMR) to colorectal distension (CRD) expressed in % control following the IP (0.2 ml) injection of cortagine (10 μg/kg) or vehicle (sterile water). Compared to IP vehicle-injected rats, IP cortagine significantly increases the VMR to CRD at 40 and 60 mmHg, while IP saline has no significant effect. Data are mean ± SEM, n as indicated in parentheses. *p<0.05, ***p<0.001 vs baseline, two-way ANOVA followed by Bonferroni post-test. **(B)** VMR to CRD expressed in % control following injections of vehicle SC + cortagine IP (10 μg/kg) (n=17), URB597 SC (3 mg/kg) + vehicle IP (n=7) or

URB597 SC (3 mg/kg) + cortagine IP (10 μg/kg) (n=18), or respective baselines. Following injection of cortagine IP, compared to vehicle SC, rats pretreated with URB597 SC exhibit a significant decrease of their VMR to CRD at 40 and 60 mmHg which is no longer different from baseline. URB597 SC per se has no significant effect on the VMR to CRD. Data are mean ± SEM, n as indicated in parentheses. ++$p<0.01$ vs vehicle SC + cortagine IP, two-way ANOVA followed by Bonferroni post-test.

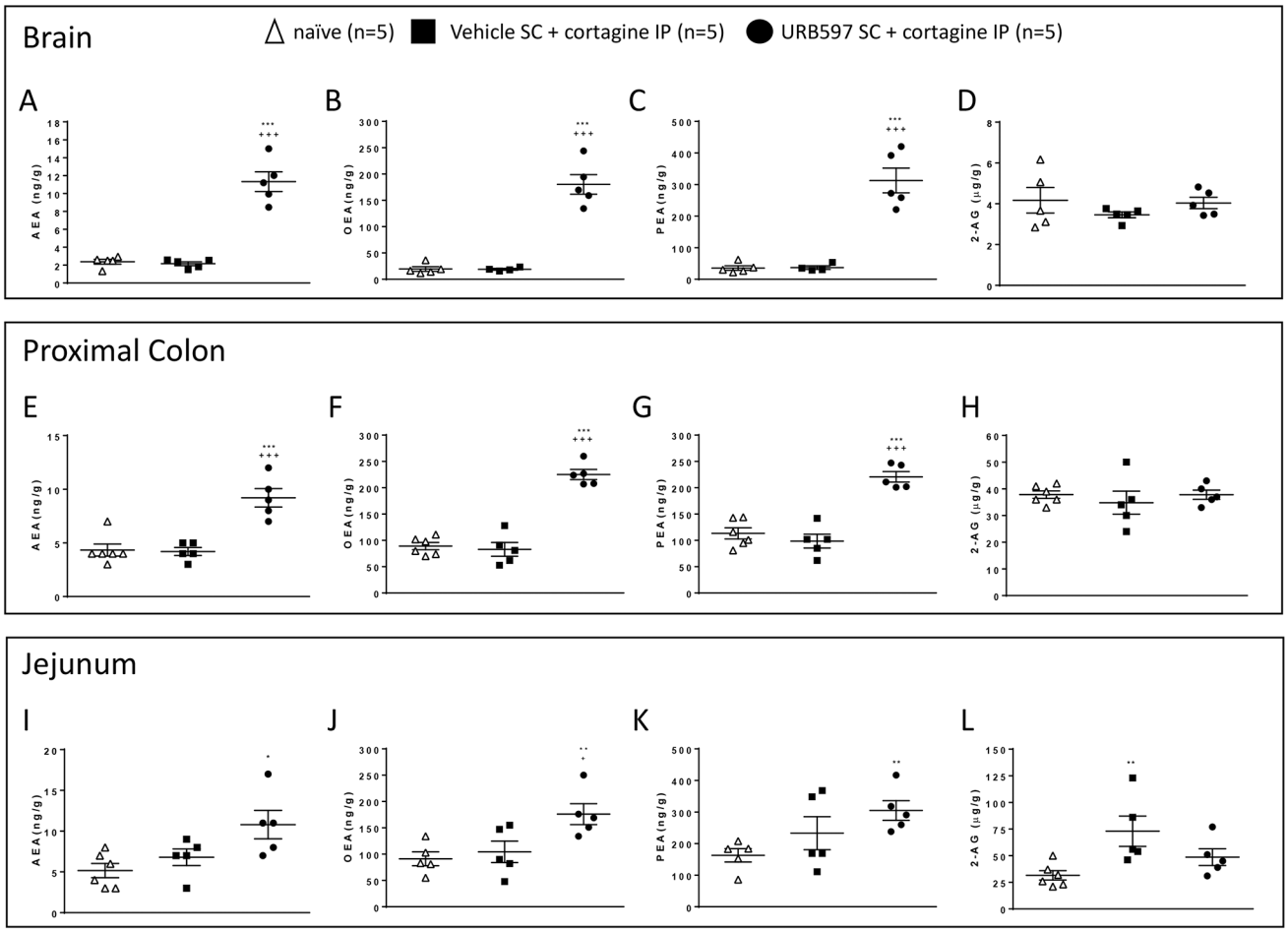

Figure 3:

Levels of anandamide (AEA) (**A, E, I**), oleoylethanolamide (OEA) (**B, F, J**) and palmitoylethanolamide (PEA) (**C, G, K**) and 2-arachidonoylglycerol (2-AG) (**D, H, L**) (left to right) in the brain, proximal colon and jejunum (top to bottom) of rats naïve or injected with cortagine and pretreated or not with URB597. Groups from Fig.1 were euthanized 30 min after the end of the 2nd CRD, after pretreatment with vehicle or URB597. AEA, PEA, OEA levels are expressed in ng/g and 2-AG levels in µg/g. Data are mean ± SEM, n as indicated in parentheses. *p<0.05, **p<0.01, ***p<0.001 vs naive, +p<0.05, +++ p<0.01 vs vehicle SC + cortagine IP, unpaired two-tailed *t* test.

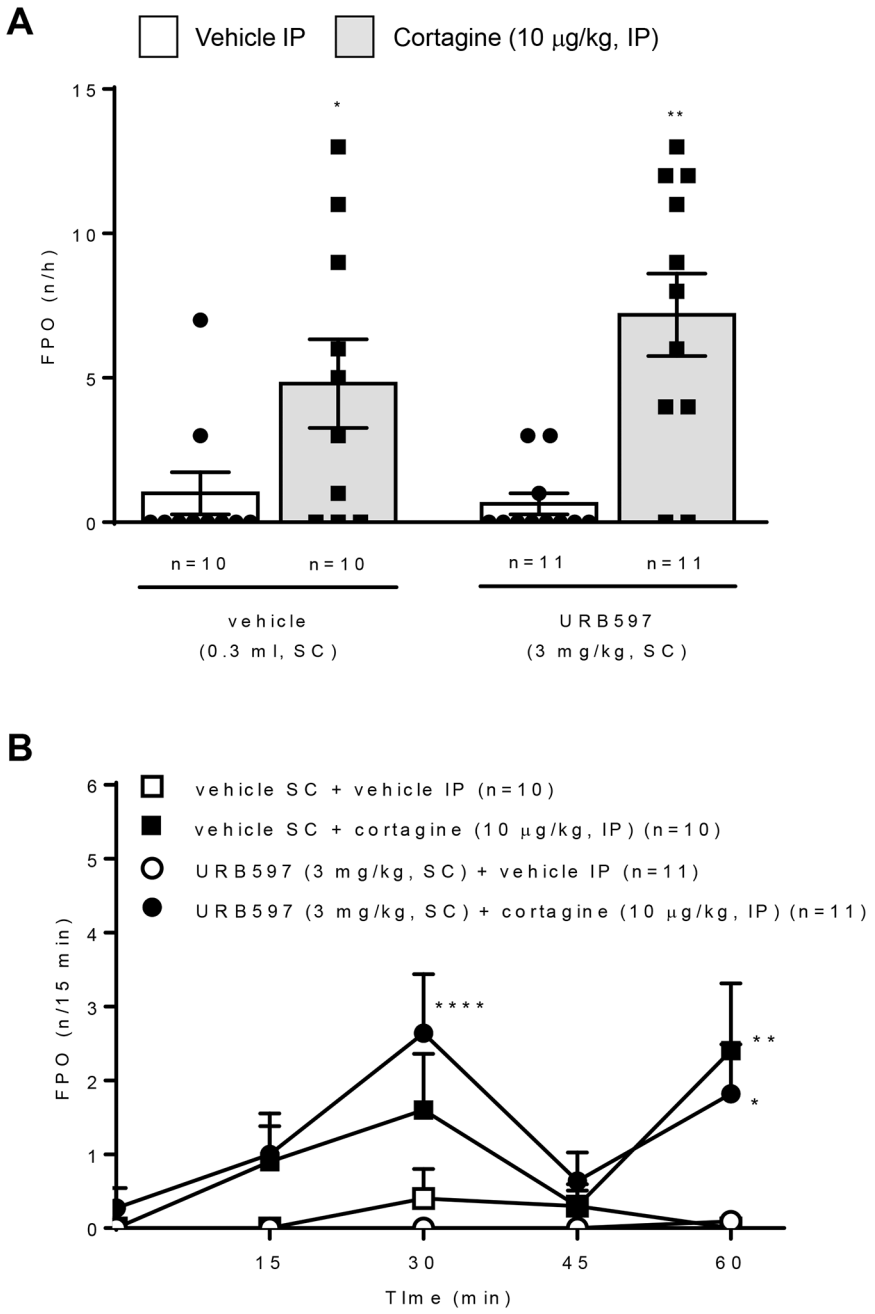

Figure 4:

Influence of URB597 pretreatment on cortagine-induced defecation in male rats. Mean fecal pellet output (FPO) over 1h following the IP injection of cortagine (10 μg/kg) or vehicle (sterile water) and SC (0.3 ml) pretreatment 2h before with URB597 (3 mg/kg) or vehicle (DMSO/cremophor El/saline). **(A)** Cortagine increases the defecation compared to vehicle treated rats, an effect that is not modified by pretreatment with URB597. Data are mean ± SEM, n as indicated at the bottom of the columns. *$p<0.05$, **$p<0.01$ vs respective control, Mann-Whitney unpaired t test. **(B)** The time-course response of defecation every 15 min for 1 h induced by cortagine is similar in rats pretreated with URB597 or vehicle. Data are

mean ± SEM, n as indicated in parentheses. *p<0.05, **p<0.01, ****p<0.0001 vs respective control, i.e. vehicle + vehicle or URB597 + vehicle, two-way ANOVA and Sidak post-hoc test comparisons.