Synergy between Enzyme Inhibitors of Fatty Acid Amide Hydrolase and Cyclooxygenase in Visceral Nociception

Pattipati S. Naidu, Lamont Booker, Benjamin F. Cravatt, and Aron H. Lichtman

Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia (P.S.N., L.B., A.H.L.); and Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, Scripps Research Institute, La Jolla, California (B.F.C.)

Received July 12, 2008; accepted December 31, 2008

ABSTRACT

The present study investigated whether inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for anandamide catabolism, produces antinociception in the acetic acid-induced abdominal stretching model of visceral nociception. Genetic deletion or pharmacological inhibition of FAAH reduced acetic acid-induced abdominal stretching. Transgenic mice that express FAAH exclusively in the nervous system displayed the antinociceptive phenotype, indicating the involvement of peripheral fatty acid amides. The cannabinoid receptor 1 (CB₁) receptor antagonist, rimonabant, but not the cannabinoid receptor 2 (CB₂) receptor antagonist, SR144528, blocked the antinociceptive phenotype of FAAH(–/–) mice and the analgesic effects of URB597 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) or OL-135 (1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenyl heptane), respective irreversible and reversible FAAH inhibitors, administered to C57BL/6 mice. The opioid receptor antagonist, naltrexone, did not block the analgesic effects of

either FAAH inhibitor. URB597, ED₅₀ [95% confidence interval (CI) = 2.1 (1.5–2.9) mg/kg], and the nonselective cyclooxygenase inhibitor, diclofenac sodium [ED₅₀ (95% CI) = 9.8 (8.2–11.7) mg/kg], dose-dependently inhibited acetic acid-induced abdominal stretching. Combinations of URB597 and diclofenac yielded synergistic analgesic interactions according to isobolographic analysis. It is important that FAAH(–/–) mice and URB597-treated mice displayed significant reductions in the severity of gastric irritation caused by diclofenac. URB597 lost its gastroprotective effects in CB₁(–/–) mice, whereas it maintained its efficacy in CB₂(–/–) mice, indicating a CB₁ mechanism of action. Taken together, the results of the present study suggest that FAAH represents a promising target for the treatment of visceral pain, and a combination of FAAH inhibitors and NSAIDs may have great utility to treat visceral pain, with reduced gastric toxicity.

Visceral pain is a major cause of consulting in gastroenterology and the principal symptom of functional bowel disorders. This symptom is often associated with gut hypersensitivity to distension. The endogenous cannabinoid system possesses attractive targets for drugs that could potentially treat visceral and other types of pain. These targets include cannabinoid (i.e., CB₁ and CB₂) receptors and fatty acid

amide hydrolase (FAAH), the enzyme responsible for degradation of the endogenous cannabinoid, anandamide, and other fatty acid amides (Walker and Hohmann, 2005). Direct-acting cannabinoid receptor agonists, such as Δ^9 -tetrahydrocannabinol (THC), the primary psychoactive constituent of *Cannabis sativa*, and the irreversible FAAH inhibitor URB597 inhibited visceral nociception, as assessed in the phenyl-*p*-quinone model (Haller et al., 2006). The antinociceptive effects of both of these compounds were blocked by the CB₁ receptor antagonist, rimonabant, indicating a CB₁ receptor mechanism of action.

Although direct-acting cannabinoid receptor agonists possess analgesic properties similar to those of opioids, but without respiratory depressant effects, their psychomimetic side effects have dampened enthusiasm for their development as

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants P01-DA017259, R01-DA15197, R01-DA03672, T32-DA007027].

We dedicate this article to our beloved mentor and world renowned pharmacologist, the late Professor Billy R. Martin, whose keen insight, wisdom, and advice were invaluable.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.108.143487.

ABBREVIATIONS: CB₁, cannabinoid receptor 1; CB₂, cannabinoid receptor 2; FAAH, fatty acid amide hydrolase; THC, Δ^9 -tetrahydrocannabinol; URB597, 3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate; OL-135, 1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenyl heptane; CNS, central nervous system; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; FAAH-NS, nervous system FAAH-restricted; ANOVA, analysis of variance; 2-AG, 2-arachidonoyl glycerol; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*d,e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone.

therapeutic agents. In contrast, increasing endogenous cannabinoid levels by blocking FAAH represents an attractive alternate approach to elicit antinociception, but without eliciting cannabimimetic effects (Cravatt et al., 2001; Gobbi et al., 2005). Deletion of the FAAH gene leads to increased levels of anandamide, accompanied with CB₁ receptor-mediated hypoalgesic phenotypes in models of acute and inflammatory pain (Cravatt et al., 2001). Likewise, wild-type mice treated with FAAH inhibitors, such as URB597 (Kathuria et al., 2003) or the reversible FAAH inhibitor, OL-135 (Lichtman et al., 2004a), elicited hypoalgesic effects in acute models of pain that were accompanied with elevations of anandamide in the CNS. In addition, these FAAH inhibitors reduced hypersensitivity to thermal and mechanical hypersensitivity in neuropathic pain models (Chang et al., 2006; Russo et al., 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs), the mainstays in acute and chronic pain management, produce their beneficial actions by inhibiting cyclooxygenases (COXs): constitutive COX-1 and inducible COX-2 (Warner and Mitchell, 2004), which are responsible for the biosynthesis of the proinflammatory prostaglandins in the periphery (Vinegar et al., 1976) and CNS (Samad et al., 2001). However, the gastrointestinal adverse effects of nonselective COX inhibitors remain a major clinical concern (Wallace, 1996). On the other hand, FAAH deletion or inhibition leads to protective effects in animal models of colitis (Massa et al., 2004; Storr et al., 2008).

Of relevance, coadministration of locally applied anandamide and COX inhibitors produced synergistic antinociceptive effects in the rat formalin test (Guindon et al., 2006) and also reduced mechanical and thermal hyperalgesia in the rat partial sciatic nerve ligation model (Guindon and Beaulieu, 2006). In addition, coadministration of the NSAID, ketorolac, and the mixed CB₁/CB₂ receptor agonist, WIN55,212-2, elicited additive analgesic effects in the acetic acid abdominal stretching test, a preclinical model of visceral nociception (Ulugöl et al., 2006). However, the cannabimimetic properties of WIN55,212-2, which include hypomotility, catalepsy, hypothermia, and THC-like subjective effects in the drug discrimination paradigm (Compton et al., 1992), limit the development of this compound for therapeutic applications. Conversely, FAAH blockade does not elicit any of these THC-like side effects (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a; Gobbi et al., 2005).

Thus, simultaneous inhibition of FAAH and COX might offer an attractive therapeutic approach that maintains analgesic efficacy while minimizing untoward side effects associated with direct-acting cannabinoid receptor agonists and the NSAIDs. As a consequence, there were four goals of the present study. First, we examined whether FAAH(−/−) mice or wild-type mice treated with inhibitors of FAAH would display decreased nociceptive behavior in the acetic acid-induced abdominal stretching test. Second, we sought to determine the receptor mechanism of action underlying the antinociceptive phenotype of FAAH-compromised mice. Mice were evaluated with the respective CB₁ and CB₂ receptor antagonists, rimonabant and SR144528. In addition, because the antinociceptive effects of FAAH inhibitors have been suggested to include an opioid receptor mechanism of action (Chang et al., 2006), we also evaluated whether naltrexone would block the antinociceptive effects of URB597 and OL-

135. Third, we used an isobolographic approach to determine whether URB597, given in combination with diclofenac, a nonselective COX inhibitor, would elicit additive or synergistic antinociceptive effects. Fourth, we evaluated whether FAAH inhibition would offer gastroprotective effects against NSAID-induced gastric ulcers.

Materials and Methods

Subjects. The subjects consisted of male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), FAAH(−/−) mice backcrossed for at least 13 generations, CB₁(−/−) mice backcrossed for at least 13 generations, and CB₂(−/−) mice backcrossed for 5 generations onto a C57BL/6J background. In addition, male and female nervous system FAAH-restricted (FAAH-NS) (Cravatt et al., 2004) mice backcrossed onto a C57BL/6J background for at least 11 generations were used to determine whether the FAAH(−/−) antinociceptive phenotype was due to the deletion of this enzyme from neuronal tissue. All subjects weighed between 20 and 30 g and were housed four mice per cage in a temperature-controlled (20–22°C) facility, with food and water available ad libitum. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in concordance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Drugs. Diclofenac sodium and naltrexone HCl were purchased from Sigma-Aldrich (St. Louis, MO). URB597 was purchased from Cayman Chemical (Ann Arbor, MI). WIN55,212-2 and ranitidine were purchased from Tocris Bioscience (Ellisville, MO). Rimonabant (CB₁ receptor antagonist) and SR144528 (CB₂ receptor antagonist) were obtained from the National Institutes of Health National Institute on Drug Abuse (Rockville, MD). All the drugs were dissolved in a vehicle consisting of ethanol, alkamuls-620 (sanofi-aventis, Bridgewater, NJ), and saline in a ratio of 1:1:18, with the exception of naltrexone, which was dissolved in 0.9% saline. Acetic acid (0.6%) was diluted in saline and administered via the intraperitoneal route of administration. All other drugs were administered through the subcutaneous route of administration, with the exception of diclofenac in the gastric tolerability test. The injection volume for acetic acid and each drug was 10 µl/g body weight.

Acetic Acid-Induced Stretching. All animals were acclimated to laboratory environment for at least 2 h before testing. The acetic acid-induced stretching assay was carried out as described previously (Jain et al., 2002). In brief, each mouse was given an intraperitoneal injection of acetic acid. Beginning 3 min after the administration of acetic acid, the number of stretches [constriction of abdomen, turning of trunk (twist), and extension of the body and hind limbs] per mouse was counted for a 20-min period.

Diclofenac, WIN55,212-2, URB597, or OL-135 were given via the subcutaneous route of administration 60 min before acetic acid administration. In the antagonism studies, rimonabant (3 mg/kg) and SR144528 (3 mg/kg) were given 70 min before acetic acid, whereas naltrexone (1 mg/kg) was administered 30 min before acetic acid. Each of these doses and pretreatment times were based on previous reports from the literature and from previous studies from our laboratory.

Dose-response curves for URB597 and diclofenac were obtained using at least six animals at each dose. Mice were given subcutaneous injections of vehicle, diclofenac (3, 10, or 30 mg/kg), or URB597 (1, 3, or 10 mg/kg) and 60 min later were given an intraperitoneal injection of acetic acid. Dose-response curves were also obtained after coadministration of URB597 and diclofenac (ED_{50 MIX}) in fixed-ratio combinations based on fractions of their respective ED₅₀ values. The ratios of URB597 and diclofenac were 1:3, 1:1, and 3:1 (for specific doses of each drug, see Table 1). In the drug combination experiments, mice received a single injection containing both drugs.

TABLE 1

Fixed combinations of diclofenac and URB597 produce synergistic analgesic effects in the acetic acid abdominal stretch assay. Predicted additive ED₅₀ values (Z_{add}) and experimentally determined ED₅₀ values (Z_{mix}) for mixtures of diclofenac and URB597 in mice. Doses for each drug in combination at the three different ratios are presented in the first column.

Combination [Mixture (Doses in mg/kg)]	Mixture Ratio	ED ₅₀ mg kg ⁻¹ s.c. (95% Confidence Limit) Diclofenac/URB597 ^a	
		Z_{add} (Theoretical)	Z_{mix} (Experimental)
Diclofenac/URB597 0.6125/0.375 1.225/0.75 2.45/1.5 4.9/3.0	1:3	4.04 (3.37–4.80)	2.01 (1.68–5.49)*
Diclofenac/URB597 0.6125/0.125 1.225/0.25 2.45/0.5 4.9/1.0 9.8/2.0	1:1	5.91 (4.90–7.05)	2.16 (1.69–2.68)*
Diclofenac/URB597 1.875/0.125 3.75/0.25 7.5/0.5 15/1.0	3:1	7.81 (6.44–9.36)	4.14 (2.84–5.49)*

^a The Z_{add} and Z_{mix} values reflect the total amount of both drugs combined, in which diclofenac and URB597 were summed for each combination.

* $p < 0.05$ as compared with respective Z_{add} (theoretical value) using the Fisher test.

Ulcer Induction with Diclofenac and Scoring of Gastric Lesions. The mice were deprived of food for 18 to 24 h before the induction of ulcer but had free access to water. On the day of the ulcer induction, the mice were given a single dose of 30 or 100 mg/kg diclofenac via gavage and left in their cages for 6 h. The experimental procedure was conducted according to a previous report in which the damage induced by diclofenac peaks 6 h after oral administration (Sánchez et al., 2002). Ranitidine, a histamine H₂ receptor blocker and commonly used antiulcer agent, was used as a positive control. URB597 (10 mg/kg) or ranitidine (50 mg/kg) was administered subcutaneously 1 h before diclofenac.

At 6 h after gavage administration of diclofenac or vehicle, the mice were humanely euthanized by CO₂ asphyxiation. The abdomen was then opened through a midline incision, and the stomach was removed and opened along the greater curvature. After a gentle wash in phosphate-buffered saline, the stomach was spread for subsequent analysis and photography. Each of the following indices was observed, and scores were summed, as previously reported (Jain et al., 2002): red coloration (score = 0.5), spot ulcers (score = 1), and hemorrhagic streaks (score = 1.5). If three or four ulcers were found, a value of 2 was added to the score. If five or more ulcers occurred, a value of 3 was added to the score. Thus, each stomach could get multiple scores, and the ulceration index was the sum of its scores, with a maximal score of 6.

Statistical Analysis. Results are presented as means $\pm$ S.E.M. The data were analyzed using two-way ANOVA, one-way ANOVA, or the Student's *t* test, as indicated in the text. Dunnett's test was used for post hoc analysis in the dose-response experiments. In addition, planned comparisons were used to in the studies examining mechanism of action.

The ED₅₀ values with 95% confidence intervals were calculated using standard linear regression analysis of the dose-response curve for each drug alone or in combination. To determine the ED₅₀ values, the data were transformed to percentage maximal possible effect using the following equation: percentage maximal possible effect = $100 \times (\text{mean number of stretches in the control group} - \text{mean number of stretches in the test group}) / \text{mean number of stretches in the control group}$.

Isobolographic analysis was used to determine the nature of the drug interactions, as described previously (Tallarida, 2000). The dose

of diclofenac required to elicit a 50% effect was plotted on the abscissa, and the isoeffective dose of URB597 was plotted on the ordinate. The theoretical additive effect of the two drugs was represented by the straight line connecting the two points. If the experimentally determined data points and their confidence interval lie on this line, the drug effects are considered additive. If the points lie below this line, the interaction is considered to be superadditive (synergistic); however, if they lie above the line of additivity, the interaction is defined as subadditive (antagonistic). To determine whether the interaction between two drugs was synergistic, additive, or antagonistic, the theoretical additive ED₅₀ value of the two drugs combined (referred to as Z_{add}) was calculated from the dose-response curves of each drug administered individually, in which the combination is assumed to equal the sum of the individual effects of each drug. The experiment ED₅₀ value of the two drugs in combination (referred to as Z_{mix}) in which the two drugs were summed at each concentration was then determined by linear regression. The statistical difference between Z_{add} (the theoretical ED₅₀ value) and Z_{mix} (the experimental ED₅₀ value) were analyzed using Fisher's test. These calculations were performed using the program of Pharm Tools Pro (version 1.20; The McCary Group Inc., Elkins Park, PA), based on Tallarida (2000). *p* Values less than 0.05 were considered significant.

Results

Acetic Acid-Induced Abdominal Stretching. Intraperitoneal injection of 0.6% acetic acid in saline elicited a curving of the trunk and extension of limbs, which were scored as a stretch. In general, the first stretch occurred between 2 and 5 min after the acetic acid injection in the control mice. Pretreatment with WIN55,212-2 (2 mg/kg), a mixed CB₁/CB₂ cannabinoid receptor agonist, or diclofenac sodium (30 mg/kg), a nonselective COX inhibitor, significantly reduced the acetic acid-induced abdominal stretches in mice compared with vehicle controls, $F(3,16) = 156$, $p < 0.001$ (Fig. 1). However, WIN55,212-2-treated mice showed reductions in locomotor activity, one of many THC-like effects that has been described previously (Compton et al., 1992), thus making it difficult to delineate between antinociception and motor suppression. In contrast, none of the other drugs tested elicited any apparent alterations in behavioral activity.

Receptor Mechanisms Underlying the Antinociceptive Phenotype of FAAH-Compromised Mice. As shown

Fig. 1. Diclofenac sodium and WIN55,212-2 pretreatment reduces acetic acid-induced visceral nociception. Intraperitoneal administration of 0.6% acetic acid elicited a significant increase in abdominal stretches in mice. Pretreatment with diclofenac sodium (30 mg/kg s.c.) or WIN55,212-2 (2 mg/kg s.c.) attenuated the number of acetic acid-induced abdominal stretches compared with the vehicle-treated group. ***, $p < 0.001$ versus vehicle-treated group; data depicted as means $\pm$ S.E.M., $n = 6$ to 8 mice/group.

in Fig. 2A, FAAH(−/−) mice displayed a significant attenuation of acetic-induced nociception, $t(10) = 5.0$, $p < 0.001$. FAAH regulates endocannabinoid pathways in both the central nervous system and peripheral tissues, either of which could regulate nociception. Thus, we next evaluated acetic acid-induced abdominal stretching in transgenic mice that express FAAH exclusively in the nervous system (FAAH-NS

mice) (Cravatt et al., 2004). FAAH-NS mice retained the antinociceptive phenotype, $F(2,19) = 10.858$, $p < 0.001$ (Fig. 2B), implicating the involvement of nonneuronal endogenous cannabinoids in this phenotype.

Rimonabant and SR144528, respectively, were used to ascertain the involvement of CB₁ and CB₂ receptors in the antinociceptive phenotype exhibited by FAAH(−/−) mice. Rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), significantly blocked the antinociceptive phenotype of FAAH(−/−) mice, $F(2,33) = 6.1$, $p < 0.01$ (Fig. 2C). In contrast, these cannabinoid receptor antagonists administered alone to wild-type mice did not alter the number of abdominal stretches compared with vehicle-treated control mice. These results suggest that the antinociceptive phenotype of FAAH(−/−) mice in the acetic acid model of visceral nociception is mediated through a CB₁ cannabinoid receptor mechanism of action.

We next evaluated the effects of URB597, a well studied irreversible FAAH inhibitor (Kathuria et al., 2003), and OL-135, a reversible FAAH inhibitor (Boger et al., 2001), in the acetic acid stretching test. As shown in Fig. 3A, URB597 (10 mg/kg) significantly reduced acetic acid-induced abdominal stretching, $F(3,32) = 14.8$, $p < 0.001$. Pretreatment with rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), significantly blocked the antinociceptive effects of URB597. Likewise, administration of OL-135 (30 mg/kg) produced CB₁ receptor-mediated antinociceptive effects in the acetic acid model, $F(3,20) = 19.4$, $p < 0.001$ (Fig. 3B). As in the case of URB597, pretreatment with rimonabant (3 mg/kg), but not SR144528 (3 mg/kg), significantly blocked the antinociceptive effects of OL-135. The antinociceptive effects of URB597 or OL-135 [main effect of FAAH inhibitor, $F(2,30) = 68$, $p < 0.001$] was not blocked by naltrexone (1 mg/kg; see Fig. 3C). Finally, diclofenac sodium (30 mg/kg s.c.) significantly attenuated acetic acid-induced nociception, $F(3,16) = 12$, $p < 0.001$. The failure of rimonabant pretreatment to reverse the antinociceptive effects of this drug indicates that the underlying mechanism of action is independent of CB₁ receptors (see Fig. 3D). As previously shown in Fig. 2C), rimonabant alone did not affect acetic acid-induced stretching.

Dose-Response Analysis of URB597 and Diclofenac Sodium Alone and in Combination. Figure 4A depicts the dose-response curves for the antinociceptive effects of URB597 [$F(3,17) = 23$, $p < 0.001$] and diclofenac [$F(3,17) = 47$, $p < 0.001$] alone in mice. The ED₅₀ values and 95% confidence limit for URB597 and diclofenac were 2.1 (1.5–2.8) and 9.8 (8.2–11.7) mg/kg, respectively. Shown in Fig. 4B are the 1:3, 1:1, and 3:1 combinations of URB597 and diclofenac, with the dose of URB597 plotted on the abscissa. The dose-response curve of URB597 alone is plotted in this graph for comparison. The same data are also plotted in Fig. 4C, with the dose of diclofenac plotted on the abscissa. The dose-response curve of diclofenac alone is included in this graph for comparison. The plots of the combination ED₅₀ values for both fixed ratios (total dose) in relation to the ED₅₀ values of the drugs alone are shown in Fig. 4D. The isobologram suggests that a synergistic interaction occurs between URB597 and diclofenac because the experimental points lie significantly below the line of additivity. This graphic display of synergism is confirmed mathematically by statistical analysis of the predicted additive ED₅₀ values (Z_{add}) and experimentally derived ED₅₀ values (Z_{mix}) shown in Table 1. At

Fig. 2. The FAAH(−/−) antinociceptive phenotype in the acetic acid model of visceral nociception is mediated through a CB₁ receptor mechanism of action. A, FAAH(−/−) mice exhibit less abdominal stretching than FAAH(+/+) mice. $n = 8$ mice/group. B, FAAH-NS mice, which express FAAH only in neural tissue, show an equivalent reduction in acetic acid-induced abdominal stretches as global FAAH(−/−) mice. $n = 5$ –9 mice/group. C, pretreatment with rimonabant (SR1; 3 mg/kg s.c.), but not SR144528 (SR2; 3 mg/kg s.c.), prevented the FAAH antinociceptive phenotype in acetic acid-treated mice, $n = 12$ mice/group. **, $p < 0.01$ compared with respective control groups; data depicted as means $\pm$ S.E.M.

Fig. 3. Pharmacological inhibition of FAAH reduces acetic acid-induced visceral nociception through a CB_1 receptor mechanism of action. A, pretreatment with URB597 (URB; 10 mg/kg s.c.), an irreversible FAAH inhibitor significantly reduced the number of abdominal stretches in acetic acid-treated mice. Pretreatment with rimonabant (SR1; 3 mg/kg s.c.), but not SR144528 (SR2; 3 mg/kg s.c.), prevented the antinociceptive effects of URB597. $n = 9$ mice/group. B, OL-135 (OL; 30 mg/kg s.c.), a reversible FAAH inhibitor, significantly attenuated the number of abdominal stretches in acetic acid-treated mice. Pretreatment with SR1 (3 mg/kg s.c.), but not SR2 (3 mg/kg s.c.), prevented the antinociceptive effects of OL-135. $n = 6$ mice/group. C, antinociceptive effects of URB597 (10 mg/kg) or OL-135 (30 mg/kg) were not blocked by naltrexone (1 mg/kg). $n = 6$ mice/group. D, pretreatment with SR1 (3 mg/kg s.c.) failed to block the antinociceptive effects of diclofenac sodium (30 mg/kg s.c.). $n = 6$ mice/group. **, $p < 0.01$; ***, $p < 0.001$ compared with the appropriate control group; ##, $p < 0.01$ compared with URB-treated group; ###, $p < 0.001$ compared with the OL-treated group. Data depicted as means $\pm$ S.E.M.

each ratio tested, the Z_{mix} value is significantly less than the Z_{add} value.

FAAH(-/-) Mice and URB597-Treated Wild-Type Mice Show Gastroprotective Effects. Because one of the most common adverse effects associated with NSAID treatment is gastric ulcers, we evaluated the impact of FAAH deletion or inhibition on diclofenac-provoked gastric irritation. Diclofenac sodium treatment (100 mg/kg p.o.) caused a significant induction of gastric ulcers compared with saline-treated mice in food restricted mice (Fig. 6A). Pretreatment with the histamine H_2 receptor antagonist, ranitidine (50 mg/kg s.c.), as a positive control, significantly reduced diclofenac-induced gastric ulcers, $F(2,16) = 28$, $p < 0.001$. Shown in Fig. 5B are representative illustrations of stomachs from a mouse in each condition. Next, we compared the effects of diclofenac (100 mg/kg) between FAAH(+/+) and (-/-) mice. As can be seen in Fig. 5C, diclofenac provoked less gastric ulceration in FAAH(-/-) mice than in wild-type mice, $t(14) = 2.2$, $p < 0.05$.

In the final series of experiments, we evaluated the effects of URB597 on diclofenac-induced gastric ulcers. Subjects were treated with either vehicle or URB597 (10 mg/kg s.c.) 1 h before diclofenac (0, 30, or 100 mg/kg gavage). Two-way ANOVA revealed a significant interaction between URB597

and diclofenac, $F(2,40) = 5.9$, $p < 0.01$ (Fig. 6A). URB597 significantly reduced the severity of ulcers elicited by both concentrations of diclofenac. Stomachs from representative mice treated with vehicle or diclofenac (30 mg/kg s.c.) and vehicle or URB597 are shown in Fig. 6B. To examine whether the gastroprotective effects of URB597 are mediated through a cannabinoid receptor mechanism, we evaluated this drug in mice lacking CB_1 or CB_2 receptors. As illustrated in Fig. 6C, URB597 no longer offered gastric protection in $CB_1(-/-)$ mice ($p = 0.50$), although it continued to reduce ulcer index scores in $CB_1(+/+)$ mice ($p < 0.05$). Finally, URB597 significantly reduced diclofenac-induced ulcers to an equal magnitude in $CB_2(-/-)$ and $(+/+)$ mice, $F(1,20) = 18.9$, $p < 0.001$. In the absence of URB597, diclofenac elicited ulcers of a similar magnitude between $CB_1(-/-)$ and $(+/+)$ mice and between $CB_2(-/-)$ and $(+/+)$ mice (see Fig. 6, C and D).

Discussion

The first goal of the present study was to test the hypothesis that FAAH is a viable target to reduce visceral nociception. Complementary approaches of pharmacological blockade and genetic deletion of FAAH significantly reduced acetic acid-induced abdominal stretches. These findings are consis-

Fig. 4. Coadministration of URB597 and diclofenac sodium elicits synergic antinociceptive effects in the acid acetic model of visceral nociception. A, URB597 (1–10 mg/kg s.c.) or diclofenac sodium (3–30 mg/kg s.c.) dose-dependently reduced the number acetic acid-induced abdominal stretches compared with the control groups. **, $p < 0.01$; ***, $p < 0.001$ compared with the respective vehicle control groups. B, dose-effect curves for URB597 alone and in mixtures with diclofenac. C, dose-effect curves for diclofenac alone and in mixtures with URB597. D, isobologram showing the interactions between diclofenac sodium and URB597 in the mouse acetic acid-induced abdominal stretching test. The ED₅₀ values for diclofenac and URB597 are depicted on the x- and y-axes, respectively. The isobole of additivity is shown as a solid line drawn between the ED₅₀ values of diclofenac and URB597, which connects the x- and y-axes. The experimental ED₅₀ values with 95% confidence interval of mixtures of URB597 and diclofenac at the fixed-ratio combinations of 3:1, 1:1, and 1:3 were significantly below the theoretical isoboles of additivity (for statistics, see Table 1), indicating superadditive (synergistic) interactions. $n = 6$ mice/group.

tent with those of Haller et al. (2006), who showed that URB597 reduced abdominal stretching in the phenyl-*p*-quinone model of visceral nociception. Previous research has also found that FAAH(–/–) mice and mice treated with FAAH inhibitors possess elevated levels of anandamide in the CNS and periphery that reduce baseline pain thresholds to noxious thermal and chemical stimuli (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a). In addition, FAAH-compromised mice have been shown to display antihyperalgesic effects in the carrageenan paw edema model (Lichtman et al., 2004b). Thus, FAAH blockade elicits hypoalgesic effects in a wide range of preclinical pain models.

It is noteworthy that FAAH(–/–) mice possess increased brain levels of fatty acid amides, including *N*-palmitoyl ethanolamine (Cravatt et al., 2001), and the *N*-acyl taurines (Saghatelian et al., 2006), any of which could contribute to antinociceptive phenotype. In particular, *N*-palmitoyl ethanolamine has been well described to have anti-inflammatory actions (Mazzari et al., 1996; Conti et al., 2002; Lo Verme et al., 2005). Thus, it is plausible that elevated levels of this or other lipid signaling molecules, in addition to anandamide, may contribute to the antihyperalgesic phenotype observed in FAAH(–/–) mice or mice treated with FAAH inhibitors. However, anandamide is the only substrate of FAAH known

to bind and activate cannabinoid receptors. Thus, the second objective of the present study was to determine whether cannabinoid receptors mediate the antinociceptive phenotype of FAAH-compromised mice. It is notable that rimobant, but not SR144528, blocked the antinociceptive phenotype of FAAH(–/–) mice and OL-135- or URB597-treated mice. In contrast to the results of Chang et al. (2006), who reported that naloxone blocked the antihyperalgesic effects of OL-135 in rat spinal nerve ligation and thermal injury models, we found no evidence of opioid receptor involvement in the antinociceptive effects of URB597 and OL-135 in the acetic model of visceral nociception. Thus, these results indicate that CB₁ receptors play a critical role in the antinociceptive effects of FAAH blockade.

Although FAAH has been shown to metabolize a second endogenous cannabinoid, 2-arachidonoyl glycerol (2-AG), under certain in vitro conditions (Di Marzo et al., 1999), this enzyme does not seem to play a relevant role in regulating 2-AG (Blankman et al., 2007). Moreover, FAAH(–/–) mice do not display cannabinoid effects to exogenous administration of 2-AG, hydrolyze 2-AG in brain and liver homogenates at equivalent rates as wild-type mice, and possess similar levels of 2-AG as wild-type mice (Lichtman et al., 2002). Notwithstanding the fact that the present study did not quantify

Fig. 5. Diclofenac sodium (100 mg/kg p.o.) caused a significant induction of ulcers in mice fasted for 18 to 24 h. A, selective H_2 receptor antagonist, ranitidine (50 mg/kg; s.c.), significantly attenuated diclofenac sodium-induced gastric ulcers. ***, $p < 0.001$ compared with control and ranitidine groups; $n = 6$ mice/group. Data depicted as mean ulcer index $\pm$ S.E.M. B, representative stomachs from control (left; score = 0), diclofenac + vehicle (center; score = 6), and diclofenac + ranitidine (right; score = 1.5). C, FAAH(-/-) mice display a significant attenuation in diclofenac sodium-induced gastric ulcers compared with FAAH(+/+) mice. *, $p < 0.05$ compared with FAAH(+/+) mice; $n = 8$ mice/group. Data depicted as mean $\pm$ S.E.M. ulcer index.

specific substrates of FAAH, our overall results are consistent with the hypothesis that anandamide is responsible for the antinociceptive phenotype of FAAH-compromised mice. In addition, acetic acid elicited virtually the identical number of abdominal stretches in mice treated with rimonabant, SR144528, or vehicle, suggesting that endogenous cannabinoids do not tonically modulate basal nociceptive responses in this model. It is noteworthy that there were fluctuations in nociceptive behavior elicited by i.p. acetic acid administration across the different experiments. Several factors that may account for this variability include the variations in the distribution of acetic acid within the peritoneal cavity, individual differences among different lots of mice evaluated at different times, and the number of subcutaneous injections mice received before acetic acid.

The site of action of endocannabinoids in visceral nociception is not well understood. The reduction in pain behaviors seen in FAAH(-/-) mice persisted when FAAH was expressed exclusively under the control of a neuron-specific

promoter, suggesting that endocannabinoids were producing their effects through a peripheral site of action. These results are in accordance with other studies showing analgesic effects of cannabinoids after local administration (Guindon and Beaulieu, 2006; Guindon et al., 2006; Jhaveri et al., 2006).

The strategy of combining analgesics of different classes can facilitate patient compliance, simplify prescriptions, improve efficacy without increasing adverse effects, and decrease adverse effects without loss of efficacy (Raffa, 2001). Combination analgesic therapy is especially useful when the selected drugs have different mechanisms of action that provide additive or synergistic efficacy, reducing the required doses of the individual drugs compared with monotherapy and potentially limiting side effects. In accordance, the third goal of the present study was to determine whether combined administration of an FAAH inhibitor and a COX inhibitor would produce additive or synergistic antinociceptive actions in the acetic acid model of visceral nociception. Using an isobolographic analysis, our results revealed a synergistic antinociceptive interaction between the FAAH inhibitor, URB597, and the NSAID, diclofenac sodium. Thus, administration of a combination of FAAH and COX inhibitors can reduce the amount of drugs required to produce analgesic effects.

However, the isobolographic approach does not provide insight into the mechanism of action underlying this interaction. Although the mechanism of action underlying the synergistic interaction between diclofenac and URB597 is beyond the scope of the present study, it may be related to pharmacokinetic and/or pharmacodynamic factors. The NSAIDs elicit analgesia by inhibiting COX, the enzyme responsible for the biosynthesis of prostaglandins (Vane, 1971). In contrast, the antinociceptive effects caused by FAAH blockade were probably caused by elevated levels of anandamide acting at CB_1 receptors. The combination of URB597 and diclofenac would simultaneously block the production of prostaglandins in the viscera and activate CB_1 receptor-mediated antinociceptive pathways. In addition, several studies have found that a variety of NSAIDs, including indomethacin and ibuprofen, inhibit FAAH, particularly at low pH that occurs in inflamed tissues (Holt et al., 2001, 2007). However, NSAIDs only inhibit FAAH at considerably high concentrations with EC_{50} values greater than 50 μ M, although it should be noted that diclofenac has not been evaluated in FAAH activity assays. The failure of rimonabant to reverse the antinociceptive effect of diclofenac sodium rules out the involvement of CB_1 receptors for this NSAID. Conversely, other targets (e.g., CB_2 , transient receptor potential vanilloid type 1, or peroxisome proliferator-activated receptor α receptors) that are activated by substrates of FAAH may contribute to the interaction between these two drugs. Finally, the interactions are likely to involve peripheral and central pathways. Although the NSAIDs act mainly through peripheral inhibition of COX, a central action has also been described previously (Miranda et al., 2003). Likewise, cannabinoids have central and peripheral sites of action (Walker and Hohmann, 2005; Agarwal et al., 2007). In future studies, it will be of value to assess the degree to which FAAH inhibitors and COX inhibitors given alone and in combination reduce the inflammation caused by acetic acid in the viscera. In addition, it will be important to examine whether the syne-

Fig. 6. URB597 attenuates diclofenac sodium-induced ulcers through a CB₁ receptor mechanism of action. A, pretreatment with URB597 (10 mg/kg s.c.) significantly attenuated diclofenac sodium-induced (30 or 100 mg/kg gavage) gastric ulcers. B, representative stomachs from vehicle-vehicle (top left; score = 0), diclofenac (30 mg/kg)-vehicle (top right; score = 6), vehicle-URB597 (bottom left; score = 0), and diclofenac (30 mg/kg)-URB597 (bottom right; score = 3). C, CB₁(-/-) mice were resistant to the gastroprotective effects of URB597 in mice treated with diclofenac sodium (100 mg/kg gavage). D, URB597 ameliorated diclofenac sodium-induced (100 mg/kg gavage) gastric ulcers to a similar magnitude in CB₂(+/+) and (-/-) mice. *, $p < 0.05$ or **, $p < 0.01$ compared with corresponding control mice; $n = 6$ mice/group. Data depicted as mean $\pm$ S.E.M. ulcer index.

gistic interaction between diclofenac and URB597 extends to other pain models, and assess whether combinations of other FAAH inhibitors and other COX inhibitors produce similar effects.

A synergistic interaction for the antinociceptive effects of FAAH and COX inhibitors is highly appealing; however, this beneficial effect would be nullified, if combined administration of these drugs increases the magnitude of adverse effects. Gastric ulcers are the most frequently associated side effects with NSAID therapy and are generally related to dose. Therefore, the fourth objective of the present study was to determine whether pretreatment with URB597 would significantly reduce NSAID-induced gastric ulcers. FAAH(-/-) and URB597-treated wild-type mice displayed a significant amelioration in the magnitude of gastric irritation caused by diclofenac given via gavage in fasted mice. These findings are consistent with studies reporting that genetic deletion or pharmacological blockade of FAAH makes mice resistant to experimentally induced colitis (Massa et al., 2004; Storr et al., 2008). Our observations that CB₁(-/-) mice, but not CB₂(-/-) mice, were resistant

to the gastroprotective effects of URB597 suggests that both the antinociceptive and gastroprotective effects caused by FAAH blockade are mediated by CB₁ receptors. These findings, taken together, indicate that the combination of URB597 and diclofenac sodium not only shows a synergistic antinociceptive effect but also can significantly reduce a serious adverse effect of the NSAIDs.

In summary, we report that pharmacological inhibition or genetic deletion of FAAH produces CB₁ receptor-mediated antinociceptive effects in the acetic acid model of visceral nociception. Coadministration of URB597 and diclofenac sodium elicited a synergistic antinociceptive effect in this assay. Moreover, FAAH(-/-) mice and C57BL/6 mice treated with URB597 displayed a reduction in the caustic effects of diclofenac on the gastric mucosa. The observations that URB597 retained its efficacy in CB₂(-/-) and was ineffective in CB₁(-/-) mice indicates its gastroprotective effects were mediated through a CB₁ mechanism of action. In conclusion, the combination of FAAH inhibitors with NSAIDs may be particularly advantageous in maximizing antinociceptive effects, while minimizing gastric irritation.

Acknowledgments

We thank Dr. S. Stevens Negus for advice and guidance in analyzing the drug interaction experiments.

References

- Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, Rubino T, Michalski CW, Marsicano G, Monory K, Mackie K, Marian C, Batkai S, Parolaro D, Fischer MJ, Reeh P, Kunos G, Kress M, Lutz B, Woolf CJ, and Kuner R (2007) Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci* **10**:870–879.
- Blankman JL, Simon GM, and Cravatt BF (2007) A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem Biol* **14**:1347–1356.
- Boger DL, Miyauchi H, and Hedrick MP (2001) alpha-Keto heterocycle inhibitors of fatty acid amide hydrolase: carbonyl group modification and alpha-substitution. *Bioorg Med Chem Lett* **11**:1517–1520.
- Chang L, Luo L, Palmer JA, Sutton S, Wilson SJ, Barbier AJ, Breitenbucher JG, Chaplan SR, and Webb M (2006) Inhibition of fatty acid amide hydrolase produces analgesia by multiple mechanisms. *Br J Pharmacol* **148**:102–113.
- Compton DR, Gold LH, Ward SJ, Balster RL, and Martin BR (1992) Aminoalkylindole analogs: cannabimimetic activity of a class of compounds structurally distinct from Δ^9 -tetrahydrocannabinol. *J Pharmacol Exp Ther* **263**:1118–1126.
- Conti S, Costa B, Colleoni M, Parolaro D, and Giagnoni G (2002) Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. *Br J Pharmacol* **135**:181–187.
- Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, and Lichtman AH (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci U S A* **98**:9371–9376.
- Cravatt BF, Saghatelian A, Hawkins EG, Clement AB, Bracey MH, and Lichtman AH (2004) Functional disassociation of the central and peripheral fatty acid amide signaling systems. *Proc Natl Acad Sci U S A* **101**:10821–10826.
- Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA, and Kunos G (1999) Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *Eur J Biochem* **264**:258–267.
- Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, et al. (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci U S A* **102**:18620–18625.
- Guindon J and Beaulieu P (2006) Antihyperalgesic effects of local injections of anandamide, ibuprofen, rofecoxib and their combinations in a model of neuropathic pain. *Neuropharmacology* **50**:814–823.
- Guindon J, De Léan A, and Beaulieu P (2006) Local interactions between anandamide, an endocannabinoid, and ibuprofen, a nonsteroidal anti-inflammatory drug, in acute and inflammatory pain. *Pain* **121**:85–93.
- Haller VL, Cichewicz DL, and Welch SP (2006) Non-cannabinoid CB1, non-cannabinoid CB2 antinociceptive effects of several novel compounds in the PPQ stretch test in mice. *Eur J Pharmacol* **546**:60–68.
- Holt S, Nilsson J, Omeir R, Tiger G, and Fowler CJ (2001) Effects of pH on the inhibition of fatty acid amidohydrolase by ibuprofen. *Br J Pharmacol* **133**:513–520.
- Holt S, Paylor B, Boldrup L, Alajakku K, Vandevoorde S, Sundström A, Cocco MT, Onnis V, and Fowler CJ (2007) Inhibition of fatty acid amide hydrolase, a key endocannabinoid metabolizing enzyme, by analogues of ibuprofen and indomethacin. *Eur J Pharmacol* **565**:26–36.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC.
- Jain NK, Kulkarni SK, and Singh A (2002) Modulation of NSAID-induced antinociceptive and anti-inflammatory effects by alpha2-adrenoceptor agonists with gastroprotective effects. *Life Sci* **70**:2857–2869.
- Jhaveri MD, Richardson D, Kendall DA, Barrett DA, and Chapman V (2006) Analgesic effects of fatty acid amide hydrolase inhibition in a rat model of neuropathic pain. *J Neurosci* **26**:13318–13327.
- Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.
- Lichtman AH, Hawkins EG, Griffin G, and Cravatt BF (2002) Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. *J Pharmacol Exp Ther* **302**:73–79.
- Lichtman AH, Leung D, Shelton C, Saghatelian A, Hardouin C, Boger D and Cravatt BF (2004a) Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J Pharmacol Exp Ther* **311**:441–448.
- Lichtman AH, Shelton CC, Advani T, and Cravatt BF (2004b) Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. *Pain* **109**:319–327.
- Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, and Piomelli D (2005) The nuclear receptor PPAR-[alpha] mediates the antiinflammatory actions of palmitoylethanolamide. *Mol Pharmacol* **67**:15–19.
- Massa F, Marsicano G, Hermann H, Cannich A, Monory K, Cravatt BF, Ferri GL, Sibaev A, Storr M, and Lutz B (2004) The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* **113**:1202–1209.
- Mazzari S, Canella R, Petrelli L, Marcolongo G, and Leon A (1996) *N*-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur J Pharmacol* **300**:227–236.
- Miranda HF, Lemus I, and Pinardi G (2003) Effect of the inhibition of serotonin biosynthesis on the antinociception induced by nonsteroidal anti-inflammatory drugs. *Brain Res Bull* **61**:417–425.
- Raffa RB (2001) Pharmacology of oral combination analgesics: rational therapy for pain. *J Clin Pharm Ther* **26**:257–264.
- Russo R, Loverme J, La Rana G, Compton TR, Parrott J, Duranti A, Tontini A, Mor M, Tarzia G, Calignano A, et al. (2007) The fatty acid amide hydrolase inhibitor URB597 (cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester) reduces neuropathic pain after oral administration in mice. *J Pharmacol Exp Ther* **322**:236–242.
- Saghatelian A, McKinney MK, Bandell M, Patapoutian A, and Cravatt BF (2006) A FAAH-regulated class of *N*-acyl taurines that activates TRP ion channels. *Biochemistry* **45**:9007–9015.
- Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, and Woolf CJ (2001) Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* **410**:471–475.
- Sánchez S, Alarcón de la Lastra C, Ortiz P, Motilva V, and Martín MJ (2002) Gastrointestinal tolerability of metamizol, acetaminophen, and diclofenac in sub-chronic treatment in rats. *Dig Dis Sci* **47**:2791–2798.
- Storr MA, Keenan CM, Emmerdinger D, Zhang H, Yüce B, Sibaev A, Massa F, Buckley NE, Lutz B, Göke B, et al. (2008) Targeting endocannabinoid degradation protects against experimental colitis in mice: involvement of CB1 and CB2 receptors. *J Mol Med* **86**:925–936.
- Tallarida R (2000) *Drug Synergism and Dose-Effect Data Analysis*, Chapman & Hall/CRC Press, Boca Raton, FL.
- Ulugöl A, Ozyigit F, Yesilyurt O, and Dogrul A (2006) The additive antinociceptive interaction between WIN 55,212-2, a cannabinoid agonist, and ketorolac. *Anesth Analg* **102**:443–447.
- Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* **231**:232–235.
- Vinegar R, Truax JF, and Selph JL (1976) Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents. *Eur J Pharmacol* **37**:23–30.
- Walker JM and Hohmann AG (2005) Cannabinoid mechanisms of pain suppression. *Handb Exp Pharmacol* **509**–554.
- Wallace JL (1996) NSAID gastroenteropathy: past, present and future. *Can J Gastroenterol* **10**:451–459.
- Warner TD and Mitchell JA (2004) Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. *FASEB J* **18**:790–804.

Address correspondence to: Aron H. Lichtman, P.O. Box 980613, Richmond, VA 23298-0613. E-mail: alichtma@vcu.edu