Regulation of Inflammatory Pain by Inhibition of Fatty Acid Amide Hydrolase^S

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ABSTRACT

Although cannabinoids are efficacious in laboratory animal models of inflammatory pain, their established cannabimimetic actions diminish enthusiasm for their therapeutic development. Conversely, fatty acid amide hydrolase (FAAH), the chief catabolic enzyme regulating the endogenous cannabinoid *N*-arachidonylethanolamine (anandamide), has emerged as an attractive target for treating pain and other conditions. Here, we tested WIN 55212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone], a cannabinoid receptor agonist, and genetic deletion or pharmacological inhibition of FAAH in the lipopolysaccharide (LPS) mouse model of inflammatory pain. WIN 55212-2 significantly reduced edema and hot-plate hyperalgesia caused by LPS infusion into the hind paws, although the mice also displayed analgesia and other central nervous system effects. FAAH(–/–) mice exhibited reduced paw edema and hyperalgesia in this model without apparent cannabimimetic effects. Transgenic mice expressing FAAH exclusively on neurons continued to display the antiedematous, but not the antihyperalgesic, phenotype. The CB₂ cannabinoid receptor (CB₂) antagonist SR144528 [*N*-[(1*S*)-endo-

1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide] blocked this non-neuronal, anti-inflammatory phenotype, and the CB₁ cannabinoid receptor (CB₁) antagonist rimonabant [SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] blocked the antihyperalgesic phenotype. The FAAH inhibitor URB597 [cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester] attenuated the development of LPS-induced paw edema and reversed LPS-induced hyperalgesia through the respective CB₂ and CB₁ mechanisms of action. However, the transient receptor potential vanilloid type 1 antagonist capsazepine did not affect either the antihyperalgesic or antiedematous effects of URB597. Finally, URB597 attenuated levels of the proinflammatory cytokines interleukin-1 β and tumor necrosis factor α in LPS-treated paws. These findings demonstrate that simultaneous elevations in non-neuronal and neuronal endocannabinoid signaling are possible through inhibition of a single enzymatic target, thereby offering a potentially powerful strategy for treating chronic inflammatory pain syndromes that operate at multiple levels of anatomical integration.

Increased pain sensitivity is one of the most common and debilitating symptoms of inflammatory disorders and is caused by various mediators, including neuropeptides, eicosanoids, and cytokines (Dray and Bevan, 1993). Cannabis

extracts and cannabinoid receptor agonists have long been known to elicit analgesic and anti-inflammatory actions (Sofia et al., 1973); however, the therapeutic utility of these drugs has been greatly limited by the occurrence of psychotropic side effects. The endogenous cannabinoid (endocannabinoid) system, consisting of naturally occurring ligands (e.g., anandamide) and 2-arachidonoyl glycerol (2-AG), enzymes regulating ligand biosynthesis and degradation, and two cloned cannabinoid receptors (i.e., CB₁ and CB₂) (Jhaveri et al., 2007; Ahn et al., 2008), provides multiple targets for the development of new pharmacological approaches for

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ABBREVIATIONS: FAA, fatty acid amide; FAAH, fatty acid amide hydrolase; 2-AG, 2-arachidonoylglycerol; CB₁, CB₁ cannabinoid receptor; CB₂, CB₂ cannabinoid receptor; URB597, cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester; WIN 55212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; rimonabant (SR141716), *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; TRP, transient receptor potential; TRPV1, TRP vanilloid type 1; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; PEA, *N*-palmitoylethanolamide; OEA, oleoylethanolamide; NS, nervous system; CNS, central nervous system; CPZ, capsazepine; THC, Δ 9-tetrahydrocannabinol.

treating inflammation and pain. In the present study, we tested whether one such endocannabinoid modulatory enzyme, fatty acid amide hydrolase (FAAH), can be targeted to treat inflammatory pain.

Several studies have demonstrated robust anti-inflammatory and antihyperalgesic phenotypes after genetic or pharmacological disruption of FAAH (Cravatt et al., 2001; Lichtman et al., 2004a,b; Massa et al., 2004; Holt et al., 2005), the principal degradative enzyme for anandamide and other bioactive FAAs, but does not play an appreciable role in 2-AG metabolism *in vivo* (Ahn et al., 2008). For instance, FAAH(−/−) mice display decreased ear swelling after repeated exposure to 2,4-dinitrofluorobenzene, which generates an antigen-specific cutaneous T cell-mediated allergic response (i.e., delayed-type hypersensitivity) (Karsak et al., 2007). The molecular basis for the anti-inflammatory and antihyperalgesic effects of FAAH disruption, including the relative contribution of peripheral and central cannabinoid systems and the role of specific receptors, remains unclear. However, with the availability of transgenic mice that express FAAH exclusively on neurons, we may now empirically address questions regarding the relative contribution of neuronal versus non-neuronal FAAH on outcome measures including pain and inflammation.

The primary objective of the present study was to examine whether FAAH regulates inflammatory and nociceptive responses in the lipopolysaccharide (LPS)-induced paw edema model. LPS is a bacterial endotoxin that causes the release of proinflammatory cytokines by immune cells, including macrophages and dendritic cells, thereby mimicking the innate immune response to bacterial infection (Rietschel et al., 1994), and can serve as a short-term model for investigating the actions of various classes of anti-inflammatory and analgesic drugs (Kanaan et al., 1997). Intraplantar injection of LPS induces central sensitization that reduces the threshold for nociception in the hot-plate and other pain tests (Kanaan et al., 1996). Peripheral mechanisms are well established to play a particularly important role in inflammatory pain sensitization, which include ion channels [e.g., sensory transient receptor potential (TRP) channels, acid-sensitive channels, and P2X receptors] and proinflammatory cytokines (Linley et al., 2010). LPS-induced hyperalgesia has a relatively short duration of action and is reversible (Kanaan et al., 1996). Here, we examined the mixed CB₁/CB₂ agonist WIN 55212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] and FAAH(−/−) mice and wild-type mice treated with the irreversible FAAH inhibitor URB597 [cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester] in the LPS model of inflammatory hyperalgesia.

To distinguish between the roles of neuronal and "non-neuronal" peripheral FAAH, we evaluated the development of LPS-induced edema in transgenic mice that express FAAH exclusively in the nervous system (FAAH-NS mice). These mice possess wild-type levels of anandamide and FAAs in brain and spinal cord, but significantly elevated levels of these lipid signaling molecules in non-neuronal peripheral tissues (Cravatt et al., 2004). In addition, we used complementary genetic [i.e., CB₁(−/−) and CB₂(−/−) mice] and pharmacological [i.e., rimonabant [SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide], a

CB₁ antagonist, and SR144528 [*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide], a CB₂ antagonist] approaches to determine whether cannabinoid receptors mediate the anti-inflammatory and antihyperalgesic phenotypes displayed by the FAAH-compromised mice. Because biochemical and pharmacological data have established that anandamide is also an agonist at TRPV1 receptors (Smart et al., 2000), we evaluated whether the TRPV1 receptor antagonist capsazepine (CPZ) would attenuate the protective effects of URB597 in the LPS model of inflammatory pain.

It is established that proinflammatory cytokines, including IL-1 β and TNF- α , play important roles in the pathogenesis of autoimmune and inflammatory disorders, such as arthritis. Substantial evidence from *in vitro* studies indicates that cannabinoid receptor agonists exert anti-inflammatory effects, in part by inhibiting proinflammatory cytokine release (Puffenberger et al., 2000; Chang et al., 2001; Roche et al., 2006). Given that LPS administration also increases production and release of these proinflammatory cytokines, in the final study we examined the effects of systemically administered URB597 on local levels of IL-1 β and TNF α in the paw, resulting from intraplantar LPS administration.

Materials and Methods

Subjects. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), male and female FAAH(−/−), CB₁(−/−), and CB₂(−/−) mice, and matched wild-type control mice on a C57BL/6 background served as subjects. In addition, FAAH-NS (nervous system FAAH restricted) mice were used to distinguish between FAAH in the nervous system and peripheral tissue. In these experiments, FAAH(+/-) mice served as controls because of the husbandry used to derive the mice (Cravatt et al., 2004). Although FAAH(+/-) mice possess half the amount of this enzyme as wild-type mice, they possess wild-type levels of anandamide and other FAAs, because more than approximately 90% suppression of FAAH is required to elevate FAAs *in vivo* (Fegley et al., 2005). All transgenic animals were back-crossed onto a C57BL/6J background for at least 13 generations. All subjects weighed between 20 and 30 g and were housed four animals per cage in a temperature-controlled (20–22°C) facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Food and water were available *ad libitum*. The animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Drugs. URB597 (Cayman Chemical, Ann Arbor, MI), capsazepine (Cayman Chemical), rimonabant (SR1; CB₁ antagonist; National Institute on Drug Abuse, Rockville, MD), SR144528 (SR2; CB₂ antagonist; National Institute on Drug Abuse), WIN 55212-2 (Tocris Bioscience, Ellisville, MO), and dexamethasone (Sigma-Aldrich, St. Louis, MO) each were dissolved in a mixture of 1:1:18 ethanol/alkamuls-620 (Rhône-Poulenc, Princeton, NJ)/saline. All drugs were administered intraperitoneally in a volume of 10 μ l/g body weight.

LPS-Induced Paw Edema. LPS from *Escherichia coli* 026:B6 was purchased from Sigma-Aldrich and dissolved in saline. Acute inflammation of mouse hind paws was induced according to published methods (Kanaan et al., 1996). For the edema studies, LPS (25 μ g in 50 μ l of saline) was injected subcutaneously into the plantar region of the left hind paw, and saline (50 μ l) was injected into the right hind paw. The thickness of the LPS-treated and control paws was measured both before and after LPS injection at the time points indicated in *Results*, by using digital calipers (Traceable Calipers, Friendswood, TX) and expressed to the nearest $\pm$ 0.01 mm. URB597

(10 mg/kg), WIN 55212-2 (2 mg/kg), or dexamethasone (2 mg/kg) was administered intraperitoneally 1 h before, 6 h after, and 23 h after LPS. In separate groups of animals, cannabinoid receptor mechanisms of action were evaluated by administering the CB₁ antagonist rimonabant (3 mg/kg), the CB₂ antagonist SR144528 (3 mg/kg), or the TRPV1 antagonist CPZ (5 mg/kg) 10 min before each injection 1 h before, 6 h after, and 23 h after LPS. The selection of each dose of these drugs was based on our previous experience showing activity in the relevant whole animal assays.

LPS-Induced Hyperalgesia. Hyperalgesia was induced by administering LPS (25 µg in 50 µl of saline) into both hind paws, and nociceptive behavior was assessed 24 h later in the hot-plate test. The plate (IITC Life Science Inc., Woodland Hills, CA) was maintained at 52°C because this temperature was demonstrated previously to elicit similar nociceptive latencies between FAAH(–/–) and FAAH(+/+) mice, whereas FAAH(–/–) mice displayed hypoalgesic responses at temperatures of 54°C and higher (Cravatt et al., 2001). The latency for each mouse to display one of the following five nociceptive behaviors was scored with a stop watch during a 30-s observation period by an observer who was blind to the drug treatment or genotype: 1) jump (i.e., all four paws are off the surface of the hot-plate), 2) licking of a hind paw, 3) shaking of a hind paw, 4) lifting of a hind paw and spreading of the phalanges, or 5) rapid repeated lifting of the hind paws.

Subjects were treated with URB597 23 h after LPS administration and assessed in the hot-plate test at 24 h. A cumulative dose-response curve was used to assess the antihyperalgesic/analgesic effects of WIN 55212-2 in which the mice were given cumulative doses of drug or an injection of vehicle every 30 min starting 20 h after LPS injection.

Proinflammatory Cytokine Assay. The levels of IL-1β and TNF-α from paw homogenate samples obtained from mice treated with LPS were measured by enzyme-linked immunosorbent assay. Hind limbs were severed at the level of calcaneus bone and weighed. Bone was separated from the limbs, the tissue was homogenized, and a 10% homogenate was prepared in phosphate-buffered saline, pH 7.4. The samples were spun by using an Eppendorf AG (Hamburg, Germany) tabletop centrifuge, then supernatants were collected and stored at –80°C until the cytokine assays. The concentrations of IL-1β and TNF-α were measured with mouse-specific enzyme-linked immunosorbent assay kits, per the manufacturer's protocol (BD Biosciences, San Jose, CA).

Statistical Analysis. Analysis of variance was used to analyze the data. The Scheffe test was used for post hoc analyses. In addition, Dunnett's test was used for post hoc analysis of the URB597 dose-response study. Differences were considered significant at $p < 0.05$.

Results

LPS-Induced Paw Edema and Hyperalgesia. Intraplantar administration of LPS produced paw swelling (Fig. 1A) and hyperalgesia (Fig. 1B) in the hot-plate test beginning within 6 h, peak effects occurred by 24 h, and all measures returned to baseline values within 96 h. Repeated administration of dexamethasone or WIN 55212-2, a mixed CB₁/CB₂ agonist, diminished the magnitude of paw edema (Fig. 2A), and acute administration at 23 h after LPS reversed the hyperalgesic responses (Fig. 2, B and C). However, WIN 55212-2 also provoked central cannabinoid effects, including hot-plate analgesia and an observed decrease in locomotor behavior in the home cage. WIN 55212-2 has been previously reported to elicit full cannabinoid effects, including hypomotility, catalepsy, hypothermia, and THC-like subjective effects in the drug discrimination paradigm (Compton et al., 1992).

Fig. 1. LPS treatment elicited significant paw edema and hyperalgesia in mice. A, intraplantar LPS (25 µg/50 µl per paw) significantly increased paw edema 3 h after injection, peak effects occurred at 24 h, and the edema was sustained for 72 h after LPS injection. B, a similar pattern of results was observed in the hot-plate test, but responses returned to baseline values by 48 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus mice given intraplantar injections of saline at the corresponding time points.

FAAH(–/–) Anti-Inflammatory and Antihyperalgesic Phenotypes in the LPS Model of Inflammatory Pain. Aside from hypoalgesic and anti-inflammatory effects, FAAH(–/–) mice do not display the array of cannabinoid activity typically elicited by WIN 55212-2 and THC (Cravatt et al., 2001). In the LPS model, FAAH(–/–) mice exhibited reduced paw edema (Fig. 3A) and hot-plate hyperalgesia (Fig. 3B) compared with FAAH(+/+) control mice, without apparent motor disturbances. To distinguish the role of FAAs in the nervous system and non-neuronal tissue, we compared LPS-induced edema and hyperalgesia between global FAAH(–/–) mice and FAAH-NS mice, which express the enzyme exclusively in neuronal tissue. FAAH(–/–) and FAAH-NS mice both showed significant reductions in paw edema compared with FAAH(+/–) mice, $F_{2,24} = 36.5$, $p < 0.001$ (Fig. 4A), implicating the involvement of non-neuronal FAAs. A significant genotype effect was also found in the hot-plate test, $F_{2,24} = 14.3$, $p < 0.001$ (Fig. 4B). FAAH(–/–) mice exhibited significant attenuation in LPS-induced hyperalgesia compared with FAAH(+/–) mice ($p < 0.001$) or FAAH-NS mice ($p < 0.05$). However, the hot-plate paw withdrawal latencies of

Fig. 2. A, pretreatment with dexamethasone (DEX; 2 mg/kg i.p.) or WIN 55212-2 (WIN; 2 mg/kg i.p.) administered 1 h before and 6 and 23 h after intraplantar LPS significantly prevented the development of paw edema. *, $p < 0.05$ versus mice treated with vehicle (VEH) at the corresponding time points. B, administration of DEX (2 mg/kg i.p.) significantly reversed LPS-induced hyperalgesia in the hot-plate test. Subjects were given a single injection of DEX or vehicle 23 h after LPS and assessed in the hot-plate test at 24 h. **, $p < 0.01$ versus mice given intraplantar saline injections; ††, $p < 0.01$ versus LPS-treated mice given intraperitoneal injections of saline. C, cumulative dose-response curve of WIN in mice given intraplantar injections of LPS. Low doses of WIN reversed the hyperalgesic effects of LPS treatment, but high doses showed significant analgesic effects in the hot-plate test. *, $p < 0.05$ versus preinjection latencies; †, $p < 0.05$; †††, $p < 0.001$ versus LPS-injected mice given vehicle. $n = 8$ to 10 mice per group. Data are depicted as means $\pm$ S.E.M.

Fig. 3. Comparison between FAAH(+/+) and FAAH(-/-) mice in the LPS model of inflammatory pain. LPS elicited less edema (A) and thermal hyperalgesia (B) in FAAH(-/-) mice than in FAAH(+/+) mice. *, $p < 0.05$; **, $p < 0.01$ versus corresponding FAAH(+/+) mice at each respective time point. $n = 8$ to 10 mice per group. Data are depicted as means $\pm$ S.E.M.

FAAH-NS mice did not differ from those of FAAH(+/-) mice ($p = 0.08$).

Rimonabant failed to attenuate the non-neuronally mediated FAAH(-/-) antiedematous phenotype (Fig. 5A), but completely blocked the antihyperalgesic effects (Fig. 5B), indicating a critical role for CB₁s in the FAAH knockout antihyperalgesic phenotype. Conversely, SR144528 completely blocked the FAAH(-/-) antiedematous phenotype (Fig. 5A) and concomitantly reduced the antihyperalgesic effects (Fig. 5B), thus implicating the involvement of CB₂s in both phenotypes. Rimonabant or SR144528 did not affect LPS-induced paw edema (Supplemental Fig. 1A) or hyperalgesic responses (Supplemental Fig. 1B) in wild-type mice.

The FAAH Inhibitor URB597 Produces Anti-Inflammatory and Antihyperalgesic Effects in the LPS Model of Inflammatory Pain. We next evaluated whether pharmacological inhibition of FAAH would also reduce LPS-induced paw edema and hyperalgesia. A single injection of URB597 (1, 3, or 10 mg/kg) given at 23 h after LPS administration did not reduce paw edema (Fig. 6A), but significantly decreased the hyperalgesic effects of LPS (Fig. 6B). However, only the highest dose (i.e., 10 mg/kg) of URB597 evaluated significantly attenuated LPS-induced hyperalgesia. Three injections of URB597 (10 mg/kg) given 1 h before, 6 h after, and 23 h after LPS administration significantly

Fig. 4. Dissociation between the antiedema and antihyperalgesic phenotypes in FAAH-deficient mice. A, FAAH(-/-) mice and FAAH-NS mice, which express FAAH exclusively in the nervous system, showed a significant reduction in LPS-induced paw edema compared with the FAAH(+/-) control mice, but did not differ between each other. B, LPS-induced hyperalgesia was reduced in FAAH(-/-) mice compared with FAAH(+/-) mice. However, hot-plate latencies did not differ between FAAH-NS and FAAH(+/-) mice. *, $p < 0.05$; ***, $p < 0.001$ versus control FAAH(+/-) mice. $n =$ nine mice per group. Data are depicted as means $\pm$ S.E.M.

ameliorated both effects of LPS in wild-type mice (Fig. 7) without eliciting any apparent overt motor deficits. The evaluation of URB597 in CB₁(-/-) and CB₂(-/-) mice revealed that distinct cannabinoid receptor mechanisms of action mediate the antihyperalgesic and antiedematous effects of this drug. URB597 retained its antiedematous effects in CB₁(-/-) mice (Fig. 7A), but CB₂(-/-) mice were resistant to this action (Fig. 7C). Conversely, the antihyperalgesic effects of URB597 were abated in CB₁(-/-) mice (Fig. 7B), but were maintained in CB₂(-/-) mice (Fig. 7D).

Because anandamide is also an agonist at TRPV1 receptors (Smart et al., 2000), we next evaluated whether the TRPV1 receptor antagonist capsazepine would attenuate the protective effects of URB597 in the LPS model of inflammatory pain. Capsazepine did not attenuate either the antiedematous (Fig. 8A) or antihyperalgesic (Fig. 8B) effects of URB597.

In the final experiment, we examined the effects of URB597 (10 mg/kg) on IL-1 β and TNF- α levels in paw homogenates 24 h after intraplantar LPS administration. Both URB597 and dexamethasone (positive control), a synthetic glucocorticoid hormone, significantly attenuated LPS-in-

Fig. 5. Evaluation of cannabinoid receptors in the FAAH(-/-) antiedema and antihyperalgesic phenotypes. A, pretreatment with the CB₂ SR144528 (SR2; 3 mg/kg i.p.), but not the CB₁ rimonabant (SR1; 3 mg/kg i.p.) administered 1 h before and 6 and 23 h after intraplantar LPS significantly prevented the FAAH anti-inflammatory phenotype in LPS-treated mice. B, SR1 and SR2 normalized hot-plate responses in FAAH(-/-) mice. *, $p < 0.05$ compared with LPS-treated wild-type mice; †, $p < 0.05$ versus FAAH(-/-) LPS-treated mice. $n =$ nine mice per group. Data are depicted as means $\pm$ S.E.M. Veh, vehicle.

duced increases in IL-1 β ($p < 0.0001$; Fig. 9A) and TNF- α ($p < 0.001$; Fig. 9B) levels.

Discussion

Increased sensitivity to noxious stimuli, or hyperalgesia, is a significant problem associated with a wide range of inflammatory states. The endogenous cannabinoid system possesses attractive targets to treat pain related to arthritis and other inflammatory conditions (Holt et al., 2005; Richardson et al., 2008) that include CB₁ and CB₂ and enzymes responsible for regulating the levels of endogenous cannabinoids. These enzymes include FAAH for anandamide and monoacylglycerol lipase for 2-AG (Pertwee, 2001; Ahn et al., 2008). In the present study, we investigated whether FAAH is a viable target for treating inflammatory pain by using an endotoxin model (Kanaan et al., 1996, 1997) in which mice were administered LPS into the plantar surface of paw. LPS also caused increased nociceptive responses to noxious thermal or mechanical stimuli in mice (Kanaan et al., 1996). Previous research has also demonstrated that a wide range of compounds, including dexamethasone, acetaminophen, indomethacin, and morphine, reduced LPS-induced hyperalgesia in rodents (Kanaan et al., 1996, 1997). In the present study, the efficacious mixed CB₁/CB₂ agonist WIN 55212-2 amelio-

Fig. 6. Effects of a single injection of the FAAH inhibitor URB597 (URB; intraperitoneally) on LPS-induced edema and hyperalgesia. URB or vehicle was administered 23 h after LPS. Paw thickness values and hot-plate latencies were assessed at 24 h. A single injection of URB did not reverse LPS-induced edema (A), but significantly attenuated LPS-induced hyperalgesia (B). *, $p < 0.05$; **, $p < 0.01$ compared with LPS-treated mice that received intraperitoneal vehicle. $n = 6$ to 10 mice per group. Data are depicted as means $\pm$ S.E.M.

rated LPS-induced paw edema, produced analgesia in the hot-plate test, and also elicited obvious cannabimimetic CNS effects (Compton et al., 1992). The beneficial effects of endocannabinoids on both endotoxin-induced inflammation and nociception were revealed by either the genetic deletion or the pharmacological inhibition of FAAH. It is noteworthy that these effects occurred in the absence of analgesia or any overt behavioral alterations.

In an effort to determine whether FAAs in neurons or non-neuronal peripheral tissue mediated the observed anti-edematous and hyperalgesic FAAH phenotypes, FAAH-NS mice, which express FAAH exclusively on neurons, were assessed in the LPS-induced paw edema model. FAAH-NS mice did not display a reduction in LPS-induced hyperalgesia, indicating that the elevation of FAAs in the central nervous system, peripheral nervous system, or a combination of both nervous systems could be driving the FAAH(−/−) antihyperalgesic phenotype. Conversely, the antiedematous phenotype was retained in FAAH-NS mice, suggesting that non-neuronal FAAs mediate this effect. The observation that URB597 reduced IL-1 β and TNF- α in LPS-injected paws is consistent with the notion that the elevation of local FAAs reduces the development of paw edema by dampening proinflammatory cytokine release. Concordant in vitro evidence supports the idea that cannabinoid receptor stimulation exerts anti-inflammatory effects by inhibiting proinflammatory cytokine release (Deutsch et al., 2001) and inhibiting antigen processing by macrophages (McCoy et al., 1999). Alternatively, other mechanisms of action are possible, including the involvement of the hypothalamic-pituitary-adrenal axis (Roche et al., 2006). Nonetheless, the present results suggest that blockade of FAAH could serve as a viable therapeutic approach for regulating the proinflammatory cytokine cascade resulting from insult.

Another objective of the present study was to elucidate the receptors mediating the antihyperalgesic and antiedematous effects caused by FAAH blockade. The observations that

Fig. 7. The antihyperalgesic and antiedematous effects of triple dosing of URB597 (URB) in the LPS model are mediated through respective CB₁ and CB₂ mechanisms of action. A, URB reduced LPS-induced paw edema in both CB₁(−/−) and CB₁(+/+) mice. B, URB reduced LPS-induced hyperalgesia in CB₁(+/+) mice, but not in CB₁(−/−) mice. C, URB reduced paw edema in CB₂(+/+) mice, but not in CB₂(−/−) mice. D, URB reduced hyperalgesia in both CB₂(+/+) and CB₂(−/−) mice. URB (10 mg/kg i.p.) or vehicle was administered 1 h before and 6 and 23 h after LPS. Paw thickness values and hot-plate latencies were assessed at 24 h. *, $p < 0.05$, compared with corresponding LPS control group; †, $p < 0.05$ versus LPS-treated CB₁(+/+) or CB₂(+/+) control mice. $n =$ six to eight mice per group. Data are depicted as means $\pm$ S.E.M.

Fig. 8. No apparent role of TRPV1 was found in the antihyperalgesic and antiedematous effects of URB597 (URB) in the LPS model. The TRPV1 antagonist capsazepine (CPZ) failed to prevent either the antiedematous (A) or the antihyperalgesic (B) effects of URB. Subjects were given an intraperitoneal injection of URB (10 mg/kg) 1 h before and 6 and 23 h after intraplantar LPS. The mean $\pm$ S.E.M. hot-plate latency before LPS administration was 13.4 ± 0.3 s. Subjects received CPZ (5 mg/kg i.p.) 10 min before each injection of URB597. *, $p < 0.05$; **, $p < 0.001$ versus vehicle-vehicle (Veh-Veh). $n = 10$ mice per group. Data are depicted as means $\pm$ S.E.M.

Fig. 9. Dexamethasone (DEX) and URB597 (URB) reduce proinflammatory cytokines. URB (10 mg/kg i.p.), DEX (2 mg/kg i.p.), or vehicle (VEH) was administered 1 h before and 6 and 23 h after LPS. The mice were humanely euthanized at 24 h, and paws were harvested. Pretreatment with URB or DEX significantly attenuated LPS-induced IL-1 β expression (A) and TNF- α (B). ***, $p < 0.0001$ compared with the vehicle plus saline (VEH+SAL) group. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with VEH+LPS group. $n = 12$ mice per group. Data are depicted as means $\pm$ S.E.M.

SR144528 completely blocked the antiedematous phenotype of FAAH(−/−) mice and CB₂(−/−) mice were resistant to the antiedematous effects of URB597 indicate that CB₂s play a necessary role in the observed FAAH-mediated reduction in paw edema. Indeed, a considerable body of literature suggests the importance of CB₂s in counteracting inflammation (Guindon and Hohmann, 2008). Conversely, rimonabant did not attenuate the antiedematous phenotype of FAAH(−/−) mice and URB597 continued to prevent the development of LPS-induced edema in CB₁(−/−) mice. In contrast, a different pattern emerged when examining the role of the cannabinoid receptors in the FAAH antihyperalgesic phenotype. Specifically, rimonabant completely blocked the antihyperalgesic phenotype of FAAH(−/−) mice, and URB597 did not elicit antihyperalgesic effects in CB₁(−/−) mice. Thus, although CB₁s are dispensable for the antiedematous actions caused by FAAH blockade, they are necessary for the FAAH antihyperalgesic phenotype. CB₁s located throughout the nervous system, including peripheral terminals of nociceptors, the dorsal horn of the spinal cord, and key brain areas associated with pain are known to contribute to cannabinoid-

induced antinociception (Lichtman et al., 1996; Nackley et al., 2003; Agarwal et al., 2007). Additional research is needed to elucidate which CB₁s contribute to the effects reported here.

In addition to activating CB₁s and CB₂s, anandamide is a full agonist at TRPV1 receptors, although with reduced affinity (Smart et al., 2000). Indeed, there is a wealth of recent pharmacological and neurochemical literature indicating close coupling of endocannabinoids and endovanilloids (Di Marzo and De Petrocellis, 2010). Accordingly, we evaluated whether the TRPV1 antagonist capsazepine would attenuate the antiedematous and antihyperalgesic caused by FAAH inhibition. However, capsazepine did not affect either of these pharmacological effects of URB597. Likewise, previous work failed to find a role of TRPV1 activity in the antiedematous effects of URB597 in the carrageenan model (Holt et al., 2005). Thus, the effects of URB597 in the LPS model do not seem to involve a necessary TRPV1 site of action.

It is noteworthy that FAAH catabolizes several other lipids, including *N*-palmitoylethanolamide (PEA), oleoylethanolamide (OEA), oleamide, and *N*-acyl taurines (Cravatt et al., 2001; Saghatelian et al., 2006). Moreover, FAAH(−/−) mice possess increased brain levels of anandamide and noncannabinoid lipid signaling molecules, any of which could contribute to this phenotype. In particular, PEA and OEA produce peripheral anti-inflammatory ef-

fects and central antihyperalgesic effects to carrageenan through the activation of peroxisome proliferator-activated receptor α (LoVerme et al., 2006; D'Agostino et al., 2009), raising the possibility that other substrates of FAAH could reduce the hyperalgesic and inflammatory actions of intraplantar LPS. The critical involvement of CB₁ and CB₂ in the respective antihyperalgesic and antiedematous phenotypes of FAAH-disrupted mice implicates anandamide as an important mediator of these effects, especially given that neither PEA nor OEA binds to cannabinoid receptors, although the possibility that other substrates of FAAH contribute to these actions cannot be ruled out.

The observation that FAAH can hydrolyze both anandamide and 2-AG at similar rates in vitro (Goparaju et al., 1998) raises the possibility that elevations in 2-AG may contribute to the results reported here and in other studies. However, the rate of 2-AG hydrolysis in brain and liver extracts is nearly 2 orders of magnitude greater than the rate of anandamide hydrolysis (Lichtman et al., 2002). Moreover, FAAH(-/-) mice (Lichtman et al., 2002) or URB597-treated wild-type mice (Kathuria et al., 2003) do not display alterations in either endogenous levels or hydrolysis rates of 2-AG. Thus, the results of these previous studies, taken together with the present data, further support the notion that elevations in endogenous anandamide or other unidentified endocannabinoids produce both antiedematous and antihyperalgesic effects.

In the absence of FAAH blockade, there was no apparent influence of endocannabinoid signaling over nociception (Fig. 5, B and D, and Supplemental Fig. 1b). Likewise, the lack of effects of CB₁ or CB₂ deactivation on inflammatory responses in FAAH-competent mice (Figs. 2D and 5, A and C, and Supplemental Fig. 1a) suggests that FAAH normally curtails endocannabinoid dampening of inflammatory responses. The results of studies examining the tonic involvement of endocannabinoids in pain have been mixed, with some reports showing that rimonabant produces hyperalgesia in wild-type animals (Calignano et al., 1998), whereas other reports found no difference (Beaulieu et al., 2000). On the other hand, exposure to prolonged foot shock elicits an endocannabinoid-mediated, stress-induced analgesia that is further augmented by inactivation of either FAAH or monoacylglycerol lipase, the enzyme responsible for 2-AG metabolism (Hohmann et al., 2005).

WIN 55212-2 and other direct-acting cannabinoid receptor agonists produce analgesia in a wide range of acute and chronic pain models; however, efforts to develop these agents to treat clinical pain have been greatly hampered by their CNS effects, which in mice include catalepsy, hypomotility, and hypothermia (Compton et al., 1992). Likewise, WIN 55212-2 produced a potent and fully efficacious analgesic response in the hot-plate test, but was only effective in blocking LPS-induced hyperalgesia without elevating the response latencies above normal baseline values of control mice at only a single dose (i.e., 0.3 mg/kg). In addition, mice treated with WIN 55212-2 at doses larger than 1 mg/kg displayed obvious changes in behavior, insofar as that they remained mostly immobile in their cages and would show an exaggerated startle response if touched. This narrow window between the antihyperalgesic effects and analgesic or CNS effects of WIN 55212-2 typifies limitations on developing

high-efficacy, synthetic cannabinoid receptor agonists to treat hyperalgesic states.

In conclusion, the present findings suggest that anandamide tone is normally curtailed by enzymatic degradation, but is unmasked by pharmacological inhibition or genetic deletion of FAAH. We found that FAAH-regulated endocannabinoids function at multiple anatomical levels, and at multiple receptors, to control inflammatory pain responses. This hierarchical regulation of pain and inflammation by a single enzyme holds promising therapeutic implications. Blockade of endocannabinoid metabolism could, however, have the added advantage in inflammatory states, such as rheumatoid arthritis, by producing dual anti-inflammatory and antihyperalgesic effects, albeit without the potential for abuse or dependence (Gobbi et al., 2005), as in the case of direct cannabinoid receptor agonists. Thus, FAAH represents a promising therapeutic target for treating acute and chronic inflammatory pain disorders.

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