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Inhibition of monoacylglycerol lipase (MAGL) attenuates NSAID-induced gastric hemorrhages in mice

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Running Title: Endocannabinoids block gastric hemorrhages

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Introduction: 740

Discussion: 1312

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Nonstandard Abbreviations

2-AG: 2-arachydonylglycerol

AEA: *N*-arachidonoyl ethanolamine, anandamide

CB_1, Cannabinoid receptor type 1

CB_2, Cannabinoid receptor type 2

COX: Cyclooxygenase

Dex: Dexamethasone, 9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one

Dic: Diclofenac sodium, 2-(2,6-dichloranilino) phenylacetic acid

eCB: Endocannabinoid

FAAH: Fatty acid amide hydrolase

G-CSF: Granulocyte colony stimulating factor

IL: Interleukin

JZL184: 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate

MAGL: Monoacylglycerol lipase

NSAID: Nonsteroidal anti-inflammatory drug

Omep: Omeprazole, 6-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl) methylsulfinyl)-1H-benzo[d]imidazole

PF-3845: N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)piperdine-1-carboxamide

PGD_2: Prostaglandin D_2

PGE_2: Prostaglandin E_2

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PPI: Proton pump inhibitor

Rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl;

SR144528, N-[(1S)-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide;

THC: Δ^9-Tetrahydrocannabinol

TNF-α: Tumor necrosis factor alpha

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used analgesics, but can cause gastric and esophageal hemorrhages, erosion, and ulceration. The endogenous cannabinoid (endocannabinoid; eCB) system possesses several potential targets to reduce gastric inflammatory states, including CB_1 and CB_2 receptors and enzymes that regulate the eCB ligands 2-arachidonoylglycerol (2-AG) and *N*-arachidonoyl ethanolamine (anandamide; AEA). In the presented study, we tested whether JZL184, a selective inhibitor of the primary catabolic enzyme of 2-AG, monoacylglycerol lipase (MAGL), would protect against NSAID-induced gastric damage. Food deprived mice administered the nonselective cyclooxygenase inhibitor, diclofenac sodium, displayed gastric hemorrhages and increases in proinflammatory cytokines. JZL184, the proton pump inhibitor omeprazole (positive control), or the primary constituent of marijuana, Δ^9-tetrahydrocannbinol (THC), significantly prevented diclofenac-induced gastric hemorrhages. JZL184 also increased stomach levels of 2-AG, but had no effect on AEA, as well as arachidonic acid or the prostaglandins PGE_2 and PGD_2. MAGL inhibition fully blocked diclofenac-induced increases in gastric levels of proinflammatory cytokines IL-1β, IL-6, TNF-α, and G-CSF, as well as IL-10. Pharmacological inhibition or genetic deletion of CB_1 or CB_2 receptors revealed that the gastroprotective effects of JZL184 and THC were mediated via the CB_1 receptor. The anti-hemorrhagic effects of JZL184 persisted with repeated administration, indicating a lack of tolerance. These data indicate that increasing 2-AG protects against gastric damage induced by NSAIDs, and its primary catabolic enzyme MAGL offers a promising target for the development of an analgesic therapeutic possessing

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gastroprotective properties.

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most commonly used classes of analgesics, are responsible for thousands of hospitalizations each year (Singh, 1998) because of their ulcerogenic side effects. To combat this damage to the stomach tissues, many chronic NSAID users are also prescribed proton pump inhibitors (PPIs), which reduce the production of stomach acid. Unfortunately, chronic use of PPIs is also associated with adverse side effects, including increased risk of gastric polyps and cancer, bone fractures, and bacterial infections, as well as vitamin deficiencies (Lodato et al., 2010). Although long-term prospective follow-up studies are lacking, investigation of alternative treatments for NSAID-induced gastrointestinal tissue damage is warranted.

Cannabis sativa and various cannabis extracts have been used for thousands of years to treat pain and other ailments (Kogan and Mechoulam, 2007). Empirical research of the analgesic effects of cannabis dates back to the 19th century (Dixon, 1899). Similar to NSAIDs, cannabinoids possess anti-inflammatory and analgesic properties. However, abuse and dependence potential, coupled with undesirable side effects including cognitive deficits, motor disturbances, and psychomimetic effects, dampen enthusiasm for their clinical development as therapeutic analgesics. On the other hand, a rapidly growing body of research focuses on targeting the endogenous cannabinoid (endocannabinoid; eCB) system for the development of new analgesics (Schlosburg et al., 2009).

Two cannabinoid receptors have been identified and cloned: CB_1 and CB_2. The CB_1 receptor is expressed throughout the body and is believed to be responsible for the

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central, psychoactive effects of cannabinoid agonists (Herkenham et al., 1991). The CB_2 receptor is expressed predominantly on immune cells in the periphery and appears to play a general anti-inflammatory role. Additionally, CB_2 receptors have been identified in primed microglia (Cabral et al., 2008), and labeled at low levels in neurons in the brainstem (Van Sickle et al., 2005). Several endogenous ligands of these receptors have been identified, and the best characterized are *N*-arachidonoyl ethanolamine (anandamide; AEA) (Devane et al., 1992) and 2-arachydonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). AEA and 2-AG are regulated *in vivo* by the catabolic enzymes fatty acid amide hydrolase (FAAH) (Cravatt et al., 2001) and monoacylglycerol lipase (MAGL) (Blankman et al., 2007), respectively. Injections of eCBs generally lack efficacy in pain and inflammation models, due to their rapid *in vivo* degradation. Alternatively, inhibiting FAAH and MAGL represents a viable strategy to elevate eCBs for therapeutic gain.

Although brain levels of 2-AG are more than 100-fold higher than those of AEA, the study of MAGL inhibition has lagged behind that of FAAH inhibition because of a lack of selective MAGL inhibitors, until the recent development of JZL184 (Long et al., 2009a; Long et al., 2009c), which is approximately 2,000 fold more selective in inhibiting MAGL than FAAH. Moreover, systemic administration of this selective MAGL inhibitor increased 2-AG, but not AEA, levels in whole brain and elicited CB_1 receptor-mediated analgesic, hypothermic, and locomotor suppressant effects (Long et al., 2009a).

FAAH (-/-) mice or wild type mice treated with the FAAH inhibitor, URB597, are protected from the ulcerogenic effects caused by high doses of the nonselective cyclooxygenase (COX) inhibitor diclofenac sodium (Naidu et al., 2009). However, it is

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unknown whether 2-AG possesses gastroprotective properties. Thus, the present study tested whether MAGL inhibition protects against NSAID-induced gastric lesions. Gastric hemorrhages were induced with the NSAID diclofenac sodium (Naidu et al., 2009). The anti-hemorrhagic effects of JZL184 were compared to the actions of the proton pump inhibitor, omeprazole, as well as Δ^9-tetrahydrocannabinol (THC), the primary constituent responsible for marijuana's cannabimimetic effects. While JZL184 elevates 2-AG in brain, it is unclear whether it also increases this eCB in stomach. Moreover, the observation that JZL184 reduces free arachidonic acid in brain, suggests the possibility that decreases in arachidonic acid metabolites, including prostaglandins, could contribute to the pharmacological effects of this MAGL inhibitor. Accordingly, we measured eCBs, free arachidonic acid, and prostaglandins from stomachs of mice that were treated with vehicle or JZL184 before receiving a high dose of diclofenac or vehicle. To test the anti-inflammatory effects of MAGL inhibition, we quantified cytokine levels in stomachs of mice treated with diclofenac in the presence and absence of JZL184. In addition, we used complementary genetic and pharmacological approaches to test whether CB_1 and CB_2 receptors mediate the gastroprotective effects of JZL184. Finally, to address further the possible therapeutic potential of endocannabinoid modulating compounds, we tested whether repeated administration of either JZL184 or the long-lasting FAAH inhibitor, PF-3845 (Ahn et al., 2009), would continue to provide gastroprotective effects.

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Materials and Methods

Animals

Subjects consisted of male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) that were approximately 10 weeks of age at the beginning of the study. In addition, male and female CB_1 (-/-) and CB_2 (-/-) mice, as well as respective CB_1 (+/+) and CB_2 (+/+) littermate control mice, were obtained from the Center Transgenic Colony at Virginia Commonwealth University. CB_1 (-/-) and CB_2 (-/-) mice were backcrossed onto a C57BL/6J background for 13 and 6 generations, respectively. Mice were housed in a temperature (20-22 °C) and humidity controlled, AAALAC-approved facility, with *ad libitum* access to food and water. Subjects weighed approximately 25 g, and were housed 4-6 per cage maintained on a 12:12 light:dark cycle. Mice were randomly assigned to treatment groups, although experiments using genetically altered mice also used a block design to evenly distribute male and female mice across conditions. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Induction of gastric lesions

The experimental procedure was based upon published reports (Sanchez et al., 2002; Naidu et al., 2009). Mice were weighed, then food deprived for 24 h with free access to water. Because mice are coprophagic, a wire mesh barrier was inserted into the cage to elevate the mice approximately 3 cm above the cage bedding. On the day of the hemorrhage induction, the mice were weighed, administered diclofenac (100 mg/kg, p.o.) or vehicle and returned to the cage. After 6 h, the mice were humanely euthanized

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via rapid cervical dislocation and stomachs were harvested, photographed, and snap frozen in LN2. Stomachs were stored at -80 °C until assayed for eCBs, prostaglandins, and cytokines as described below.

Gastric Hemorrhage Scoring

Gastric hemorrhages were quantified based on previously reported methods (Liu et al., 1998). Six hours after diclofenac treatment, stomachs were harvested, cut along the greater curvature, and rinsed with normal saline. The tissue was then placed on a lighted stage and photographed, along with a 1 mm reference, using a Canon EOS Rebel XS digital camera with a Canon 250D close-up lens (Adorama Inc., New York, NY). Images were renamed, to remove treatment information, and analyzed using Adobe Photoshop (v.7.0). The length of each hemorrhage was marked with a 1 pixel wide line, and compared to the reference, such that the total hemorrhage score in mm for each stomach = pixels (hemorrhage) / pixels(reference). The experimenter scoring gastric hemorrhages was blind to the treatment condition of each subject. This total gastric hemorrhage length measure has been reported to increase along with gastric myeloperoxidase (MPO) activity, a marker of neutrophil activation, as well as neutrophil elastase and decreases in gastric blood flow (Liu et al., 1998).

Endocannabinoid and Prostaglandin Quantification

Metabolite levels were measured as described previously (Long et al., 2009b) using an Agilent Triple Quadrupole LC/MS. Tissues were weighed and dounce homogenized in 6 ml of 1:1 v/v hexane: ethyl acetate and 2 ml of phosphate buffered saline containing

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internal standards for AEA, 2-AG, and a fatty acid (2 pmol *d*4-AEA, 0.5 nmol d5-2-AG, 10 nmol pentadecanoic acid). The mixture was vortexed and then centrifuged (1,400 x *g*, 10 min). The organic layer was removed, evaporated under a stream of nitrogen and resolubilized in 120 µL of chloroform. An aliquot of the extract (10 µL) was injected for analysis with an Agilent G6410B QQQ instrument. For LC separation, mobile phase A consisted of 95:5 water:methanol and mobile phase B consisted of 60:35:5 isopropanol:methanol:water. Formic acid (0.1 %) or ammonium hydroxide (0.1 %) was included to assist in ion formation in positive ionization and negative ionization modes, respectively. The flow rate for each run started at 0.1 mL/min with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 mL/min and increased linearly to 100% B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5 mL/min before equilibrating for 3 min at 0% B at 0.5 mL/min. The following MS parameters were used to measure the indicated metabolites (precursor ion, product ion, collision energy in V): AEA (348, 62, 11), *d*4-AEA (352, 66, 11), 2-AG (379, 287, 8), d5-2-AG (384, 287, 8), PGE_2 (351, 271, 10), PGD_2 (351, 271, 10). Arachidonic acid was measured by targeting the parent mass and quantified based on targeted measurements of pentadecanoic acid. PGE_2 and PGD_2 were quantified based on an external standard curve of the prostaglandin standards with pentadecanoid acid. MS analysis was performed with an electrospray ionization source. The drying gas temperature was 350°C, the drying gas flow rate was 11 L/min, and the nebulizer pressure was 35 psi. Lipids were quantified by measuring the area under the peak in comparison to the internal standards.

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Cytokine quantification (ELISA)

Cytokines were measured using the Mouse Inflammatory Cytokines Multi-Analyte ELISArray kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Whole stomachs were homogenized in phosphate-buffered saline in the presence of 1 x protease inhibitor cocktail (Roche, Indianapolis, IN). The supernatant from a 1000 x g centrifugation of the homogenate was then centrifuged at 100,000 x g for 40 min. The supernatant from this centrifugation (the mouse stomach soluble proteome) (100 ug) was used in the assay, per the manufacturer's instructions.

Drugs

The NSAID diclofenac sodium (100 mg/kg), the synthetic glucocorticoid dexamethasone (2 mg/kg), and the proton pump inhibitor omeprazole (20 mg/kg) were purchased from Sigma-Aldrich (St. Louis, MO). The CB_1 antagonist rimonabant (3 mg/kg), the CB_2 antagonist SR144528 (3 mg/kg), and Δ^9-tetrahydrocannbinol (THC; 10 mg/kg) were obtained from the National Institute on Drug Abuse (Bethesda, MD). The MAGL inhibitor JZL184 (Long et al., 2009a) and the FAAH inhibitor PF-3845 (Ahn et al., 2009) were synthesized in the Cravatt laboratory as described previously, and 2-Arachdonoylglycerol was synthesized as described previously (Han and Razdan, 1999). All drugs were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. All compounds were administered at a volume of 10 µl/g body mass, intraperitoneally (i.p.), with the exception of diclofenac, which was administered orally (p.o.). All solutions were warmed to room temperature prior to injection.

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Data Analyses

All data are reported as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA), with the exception of studies using genetic knockout mice, which were analyzed using two-way (genotype x drug treatment), between subjects ANOVA. Post hoc comparisons were made using the Student Newman-Keuls test, with the exception of the JZL184 dose response data, for which Dunnett's test was used to compare each dose to vehicle. All animals were included in analyses. Differences were considered statistically significant at $p < 0.05$.

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Results

The NSAID diclofenac induced gastric hemorrhages

As reported previously (Naidu et al., 2009), diclofenac (100 mg/kg, p.o.) administration to food deprived mice induced gastric inflammation, resulting in redness, spot ulcers, and hemorrhagic streaks 6 h after treatment. These gastric hemorrhagic streaks were quantified in each stomach. The plant-derived cannabinoid THC (10 mg/kg) significantly prevented diclofenac-induced hemorrhages [$F(4,51) = 7.82$; $p < 0.0001$; **Figure 1A**]. Pretreatment with the CB_1 antagonist rimonabant (3 mg/kg) reversed this effect, but the CB_2 antagonist SR144528 had no effect. Similarly, the proton pump inhibitor omeprazole (20 mg/kg) reduced the incidence of hemorrhages [$F(4,38) = 12.5$; $p < 0.0001$; **Figure 1B**]. Pretreatment with either antagonist had no effect on omeprazole treatment. An additional positive control, the synthetic glucocorticoid hormone dexamethasone (2 mg/kg) blocked diclofenac-induced hemorrhages [$F(1,27) = 7.7$; $p < 0.01$; **Figure 1C**].

MAGL inhibition blocked NSAID-induced hemorrhages and increased 2-AG in stomach

Pretreatment with JZL184 attenuated diclofenac-induced gastric hemorrhagic streaks. Various doses of JZL184 (ranging from 0.25 – 40 mg/kg, i.p.) were administered 2 h prior to diclofenac treatment. Overall, JZL184 significantly reduced the extent of diclofenac-induced hemorrhages [$F(6,48) = 3.48$; $p < 0.0001$; **Figure 2A**]. Post hoc analyses revealed that the gastroprotective effects of JZL184 occurred at doses of 4 mg/kg and higher. Exogenous 2-AG (50 mg/kg, i.p.) administered 2 h prior

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to diclofenac treatment neither induced nor blocked NSAID-induced gastric hemorrhages [$F(2,20) = 19.8$; $p < 0.0001$; **Figure 2B**].

MAGL inhibition increases CNS levels of 2-AG and decreases free arachidonic acid in brain, but does not affect brain AEA (Kinsey *et al.*, 2009; Long *et al.*, 2009a). To determine whether MAGL inhibition produces a similar profile in stomach tissue, we assessed the effects of JZL184 (4 mg/kg i.p.) on these biomarkers from either stomachs subjected to diclofenac-induced gastric hemorrhages or control stomachs. In whole stomach, JZL184 significantly increased levels of 2-AG [$F(1,12) = 57.3$; $p < 0.0001$; **Figure 4A**], but had no effect on AEA ($p = 0.31$; **Figure 4B**) or free arachidonic acid ($p = 0.41$; **Figure 4C**), a metabolite of 2-AG and AEA and a precursor of prostaglandin synthesis. JZL184 had no effect on stomach levels of PGE_2 ($p = 0.19$) or PGD_2 ($p = 0.16$). Conversely, diclofenac sodium treatment significantly reduced levels of prostaglandins PGE_2 [$F(1,12) = 46.8$; $p < 0.0001$; **Figure 4D**] and PGD_2 [$F(1,12) = 53.6$; $p < 0.0001$; **Figure 4E**], but had no effect on 2-AG ($p = 0.11$), AEA ($p = 0.92$), or AA ($p = 0.45$).

MAGL inhibition blocked NSAID-induced increases in stomach proinflammatory cytokines

Acute diclofenac treatment in food deprived mice caused statistically significant increases in levels of the proinflammatory cytokines IL-1β [$F(3,12) = 9.7$; $p < 0.01$; **Figure 5A**], IL-6 [$F(3,12) = 43.7$; $p < 0.0001$; **Figure 5B**], TNF-α [$F(3,12) = 20.5$; $p < 0.0001$; **Figure 5C**], and G-CSF [$F(3,12) = 10.9$; $p < 0.001$; **Figure 5D**], as well as the anti-inflammatory cytokine IL-10 [$F(3,12) = 15.5$; $p < 0.001$; **Figure 5E**]. Pretreatment

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with JZL184 significantly blocked diclofenac-induced elevations of each of these cytokines.

CB_1 receptors mediate the gastroprotective effects of JZL184

In the next series of experiments, we used complementary pharmacological and genetic approaches to determine whether cannabinoid receptors mediate the gastroprotective effects of JZL184. The CB_1 receptor antagonist rimonabant (3 mg/kg), or the CB_2 receptor antagonist SR144528 (3 mg/kg) was administered 10 min prior to JZL184 (4 mg/kg, i.p.). As with the above experiments, JZL184 significantly reduced diclofenac-induced gastric hemorrhages [$F(3,28) = 10.1$; $p < 0.0001$; **Figure 6A**]. Post hoc analyses revealed that rimonabant completely blocked the anti-hemorrhagic effects of JZL184, whereas SR144528 had no effect, indicating that CB_1, and not CB_2, mediated the gastroprotective effects of MAGL inhibition via JZL184. When administered alone, neither rimonabant (1.6 ± 0.4 mm) nor SR144528 (1.5 ± 0.3 mm) affected gastric hemorrhages.

Diclofenac induced gastric hemorrhages in both CB_1 (-/-) and CB_2 (-/-) mice with comparable severity to their wild type littermates (**Figure 6B**), indicating that endocannabinoids do not tonically modulate NSAID-induced hemorrhages. While JZL184 did not block hemorrhages in CB_1 (-/-) mice, it maintained efficacy in CB_2 (-/-) mice, indicating that the gastroprotective effects of JZL184 occur via a CB_1 receptor specific mechanism of action. In the CB_1 (-/-) experiment, there was a significant interaction between genotype and MAGL inhibition [$F(1,27) = 5.01$; $p < 0.05$], as well as a main effect of genotype [$F(1,27) = 4.44$; $p < 0.05$]. In the CB_2 (-/-) experiment, there

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was a main effect of MAGL inhibition [$F(1,25) = 17.3$; $p < 0.001$], but no interaction between genotype and MAGL inhibition ($p = 0.34$).

Anti-hemorrhagic effects of MAGL and FAAH inhibition persist after repeated treatment

In order to test whether prolonged blockade of MAGL leads to tolerance and loss of gastroprotective effects, we tested the effects of JZL184 inhibitor following chronic administration on diclofenac-induced hemorrhagic streaks. For comparison, we evaluated the impact of repeated administration of the FAAH inhibitor PF-3845 on diclofenac-induced ulcers. Mice were treated for five consecutive days with JZL184, PF-3845, or vehicle. On day 5, the mice were fasted for 22 h, then administered JZL184, PF-3845, or vehicle, followed 2 h later by diclofenac. Mice treated acutely with JZL184 or PF-3845 showed marked reductions in hemorrhagic streaks [$F(5,42) = 5.74$; $p < 0.001$; **Figure 7**]. Repeated administration of either PF-3845 or JZL184 did not reduce these gastroprotective effects, indicating a lack of tolerance to each enzyme inhibitor.

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Discussion

Since the 19th century, when Felix Hoffman first produced acetylsalicylic acid (aspirin), patients have complained about gastrointestinal irritation when taking NSAIDs (Lanas, 2009). Indeed, the primary undesirable side effect and source of noncompliance of NSAID use is dyspepsia. The role of the eCB system in modulating gut motility, secretion, and inflammation is a topic of a growing body of research in recent years (Izzo and Sharkey, 2010); however, less is known about their gastroprotective properties. AEA has been reported to have gastroprotective properties (Shujaa *et al.*, 2009) and blockade of its chief catabolic enzyme, FAAH, reduces NSAID-induced ulcers (Naidu et al., 2009). In the present study, we used the highly selective MAGL inhibitor JZL184 to investigate whether elevating 2-AG protects against the development of NSAID-induced gastric hemorrhages. JZL184, the phytocannabinoid THC, dexamethasone, and the proton pump inhibitor omeprazole significantly reduced the formation of gastric hemorrhagic streaks induced by the NSAID diclofenac sodium. Moreover, JZL184 completely prevented diclofenac-induced increases of proinflammatory cytokines in stomach. The gastroprotective effects of MAGL inhibition and THC were blocked by rimonabant pretreatment, whereas the CB_2 antagonist SR144528 had no effect, indicating a CB_1 receptor mechanism of action.

The results of the present study are consistent with the notion that JZL184 protects against NSAID-induced gastric lesions by elevating endogenous levels of 2-AG in the stomach through CB_1 receptor mediated actions. In brain, 2-AG levels are two orders of magnitude higher than those of AEA, and JZL184 increases 2-AG another 10-fold (Long et al., 2009a; Long et al., 2009c), but here we demonstrated that stomach 2-

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AG levels are almost five orders of magnitude higher than those of AEA, but JZL184 elevates gastric levels of 2-AG only about two-fold. Likewise, other studies have found that cannabinoids reduce stress- or NSAID-induced gastric lesions in rodents through a CB_1 receptor mechanism. For example, the synthetic cannabinoid agonist, WIN55,212-2 attenuates stress-induced gastric pathology in rats, but the inactive enantiomer WIN55,212-3 has no effect (Germano et al., 2001). The gastroprotective effects of WIN55,212-2 were blocked by rimonabant pretreatment, but not by SR144528, indicating a CB_1 receptor-mediated mechanism of action (Germano et al., 2001). The CB_1 receptor selective agonist ACEA attenuated aspirin-induced gastric ulcers in rats (Rutkowska and Fereniec-Goltbiewska, 2006), and the CB_1 receptor antagonist AM251 blocked the gastroprotective effects of exogenously administered AEA treatment in the rat water immersion with restraint model of stress-induced gastric lesions (Dembinski et al., 2006). These findings taken together suggest that CB_1 receptor activation is gastroprotective against NSAID-induced ulcers.

Although the mechanism underlying the gastroprotective effects are unknown, one possibility is decreased gastric acid secretion. Gastric acid secretion by parietal cells in the gastric fundus can be stimulated by neuronally or hormonally, by histamine (paracrine) or gastrin (endocrine) (Schubert, 2008). Although the synthetic cannabinoid agonists HU-210 and WIN55,212-2 had no effect on basal stomach acid production in rats, each compound blocked pentagastrin-induced acid secretion (Coruzzi et al., 1999; Adami et al., 2002). However, neither compound affected histamine-induced acid production, suggesting a systemic, endocrine mechanism of acid modulation, not direct tissue stimulation (Adami et al., 2002). Vagotomy blunted the acid-reducing effect of

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HU-210, indicating the involvement of peripheral cholinergic pathways and vagal afferents (Adami et al., 2002). These data are supported by a study in which intravenous (iv), but not intracerebroventricular (icv), administration of either HU-210 or WIN55,212-2 significantly reduced pentagastrin-induced acid production (Adami et al., 2004). Future studies will determine whether MAGL inhibition reduces basal or pentagastrin-induced gastric acid secretion as well as reduces NSAID-induced ulcers through this type of mechanism.

It is also possible that JZL184 produces its anti-ulcerogenic activity via one or more acid-independent pathways. For example, central (icv) or peripheral (iv) administration of WIN55,212-2, as well as anandamide and its biologically stable analog, methanandamide (Shujaa et al., 2009) attenuated acute ethanol-induced gastric mucosal damage, an acid-independent model (Glavin and Szabo, 1993). Alternatively, MAGL inhibition may increase blood flow in the stomach, thereby increasing oxygenation of gastric endothelial cells and decreasing infiltration of neutrophils. One mechanism through which this effect could occur is an increase in levels of heme oxygenase-1 (HO-1), which reduces oxidative stress in gastric endothelial cells. The PPI's omeprazole and lansoprazole upregulate HO-1, resulting in acid-independent gastroprotective effects (Becker et al., 2006). Similarly, the TRPV1 receptor agonist capsaicin causes vasodilatation in GI smooth muscle (Holzer et al., 1989). TRPV1 also binds the eCB anandamide, but not 2-AG (Schlosburg et al., 2009).

The anti-hemorrhagic effects of MAGL inhibition in the present studies occurred via CB_1 receptors, which are present in the CNS as well as in the stomach. For example, CB_1 is colocalized to cholinergic neurons innervating the gastric mucosal

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endothelium of the fundus, corpus, and antrum of the rat stomach (Adami et al., 2002). Similarly, CB_1 receptors have been identified in ganglia adjacent to the gastric endothelium along both the greater and lesser curvatures of the mouse stomach (Casu et al., 2003). Thus, it is possible that increased levels of 2-AG act centrally and/or locally in the stomach to attenuate diclofenac-induced gastric hemorrhages. Finally, in contrast to rodents, human gastric parietal cells express CB_1 receptors (Pazos et al., 2008). Thus, there may be species-specific mechanisms through which cannabinoids inhibit NSAID-induced gastric tissue damage.

NSAIDs damage the GI tract through multiple mechanisms, grossly grouped together as topical and systemic causes (Schubert, 2008). This drug class is believed to cause gastric pathology through the reduction of prostaglandin biosynthesis in stomach via COX inhibition, which leads to increased stomach acid production and decreased mucosal secretion (Musumba et al., 2009). As JZL184 also reduces free arachidonic acid levels in brain, another goal of the present study was to determine whether it produces the same effect in stomach as well as whether it reduces prostaglandins levels in stomach. Additionally, we evaluated gastric eCB levels to determine whether MAGL regulates 2-AG levels in stomach. The observation that JZL184 did not affect levels of free arachidonic acid, PGE_2, or PGD_2 in stomachs of mice treated with high dose diclofenac or vehicle suggests that MAGL inhibition protects against NSAID-induced gastric ulcers through a mechanism other than through the prostaglandins. Diclofenac-induced increases in proinflammatory cytokine levels were blocked by JZL184 pretreatment. However, as mentioned above, further studies measuring the effects of MAGL inhibition on gastric acid secretion *in vivo* are important

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for delineating the underlying mechanism(s) by which JZL184 produces gastroprotection.

As shown previously (Naidu et al., 2009), FAAH (-/-) mice or wild type mice treated with the FAAH inhibitor URB597 showed a significantly attenuated response to diclofenac-induced gastric hemorrhages. In addition to being more selective for FAAH than URB597, PF-3845 is longer lasting, inhibiting FAAH for at least 24 h (Ahn et al., 2009). Because repeated administration of high dose JZL184 leads to downregulation and desensitization of the CB_1 receptor, along with tolerance to its analgesic effects (Schlosburg *et al.*, 2010), we evaluated whether prolonged administration of FAAH or MAGL inhibitors would retain their anti-ulcerogenic actions . This apparent disparity between the present and previous (Schlosburg *et al.*, 2010) studies may be due to different end points measures (anti-ulcerogenic effects vs. antinociceptive effects) or due to the dose of JZL184 used. Whereas Schlosburg et al. (2010) used a 40 mg/kg dose, the present study administered a 4 mg/kg JZL184. It is noteworthy that, in both studies, tolerance did not develop to FAAH inhibition via PF-3845.

The present data indicate that inhibiting degradation of the endogenous cannabinoids AEA and 2-AG attenuates NSAID-induced gastric endothelial damage. These findings, coupled with the synergistic antinociceptive interactions between the NSAIDs and FAAH inhibition (Fowler et al., 2009; Naidu et al., 2009), support the idea that FAAH and MAGL are promising targets for the development of analgesic therapeutics that not only lack adverse gastrointestinal side effects, but also protect against NSAID-induced gastric ulcers.

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Authorship Contributions

Participated in research design: Kinsey, Lichtman, Grider, Nomura, Cravatt

Conducted experiments: Kinsey, Nomura, O'Neal

Contributed new reagents or analytic tools: Long, Mahadevan

Performed data analysis: Kinsey, Nomura

Wrote or contributed to the writing of the manuscript: Kinsey, Lichtman, Nomura, O'Neal

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Footnotes

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Figure Legends

Figure 1. Diclofenac induced gastric hemorrhages in mice. Mice were food deprived for 24 h, and then administered 100 mg/kg diclofenac sodium, p.o. Gastric hemorrhagic streaks were attenuated by a 2 h pretreatment with (**A**) the proton pump inhibitor omeprazole (Omp; 20 mg/kg), (**B**) the plant derived cannabinoid, Δ^9-tetrahydrocannabinol (THC;10 mg/kg), or (**C**) the synthetic glucocorticoid, dexamethasone (2 mg/kg). Pretreatment with the CB_1 antagonist rimonabant partially blocked the gastroprotective effects of THC, but the CB_2 antagonist SR144528 had no effect. Neither antagonist affected the gastroprotective effects of omeprazole. Data expressed as mean ± SEM (n = 8). ** $p < 0.01$, *$p < 0.05$ vs. vehicle treatment; ††$p < 0.01$ vs. diclofenac treatment.

Figure 2. Pretreatment with JZL184 prevented diclofenac-induced gastric hemorrhagic streaks, whereas exogenous 2-AG had no effect. (**A**) Various doses of JZL184, or (**B**) 2-AG (50 mg/kg), were administered i.p. 2 h prior to diclofenac treatment. Open circle/bar, vehicle treatment; filled circles/bars, diclofenac treatment. Data expressed as mean ± SEM (n = 7-8). **$p<0.01$ vs. vehicle treatment; ††$p < 0.01$ vs. diclofenac treatment.

Figure 3. Representative images of mouse stomachs. Mice were food deprived for 24 h, and then administered diclofenac sodium (100 mg/kg p.o.). Gastric hemorrhagic streaks were attenuated by pretreatment with the MAGL inhibitor JZL184 (4 mg/kg), the FAAH inhibitor PF-3845 (10 mg/kg), the phytocannabinoid THC (10 mg/kg), the proton

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pump inhibitor Omeprazole (20 mg/kg), or the synthetic glucocorticoid hormone dexamethasone (2 mg/kg).

Figure 4 . MAGL inhibition increased 2-AG levels, and diclofenac inhibited prostaglandin levels, in stomach. To assess possible changes in endocannabinoid levels in stomach tissue, mice were fasted for 24h, administered JZL184 (4 mg/kg i.p.) or vehicle, followed 2 h later by diclofenac (100 mg/kg, p.o.) or vehicle, and stomachs were harvested 6h later. (**A-C**) In whole stomach, JZL184 significantly increased levels of 2-AG but had no effect on levels of AEA, free arachidonic acid (AA), or (**D,E**) the prostaglandins PGE_2 and PGD_2. Conversely, diclofenac sodium significantly reduced levels of PGE_2 and PGD_2, but had no effect 2-AG, AEA, or AA. Open bars, vehicle treatment; filled bars, diclofenac. Data expressed as mean ± SEM (n = 4). *p<0.05, **p < 0.01 vs. vehicle/vehicle treatment.

Figure 5. MAGL inhibition blocked diclofenac-induced increases of proinflammatory cytokines in stomach. Mice were fasted for 24 h, administered JZL184 (4 mg/kg, i.p.) or vehicle, followed 2 h later by diclofenac (100 mg/kg, p.o.) or vehicle, and stomachs were harvested 6 h later. In whole stomach, diclofenac significantly increased levels of IL-1, IL-6, TNF-α, G-CSF, and IL-10. Pretreatment with JZL184 blocked this increase in cytokines. Open bars, vehicle treatment; filled bars, diclofenac. Data expressed as mean ± SEM standardized to mean levels of vehicle treated group (n = 4). **p < 0.01 vs. vehicle treatment; †† p < 0.01 vs. diclofenac treatment.

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Figure 6. JZL184 blocks diclofenac-induced hemorrhages via a CB_1 receptor mediated mechanism of action. Gastric hemorrhagic streaks were attenuated by a 2 h pretreatment with JZL184 (4 mg/kg). (**A**) This anti-hemorrhagic effect was blocked by the CB_1 receptor antagonist rimonabant (3 mg/kg), whereas the CB_2 receptor antagonist SR144528 (3 mg/kg) had no effect. Neither Rimonabant nor SR144528 affected hemorrhages on its own (n = 8). (**B**) Genetic deletion of the CB_1 receptor prevented the gastroprotective effects of MAGL inhibition with JZL184, whereas (**C**) genetic deletion of CB_2 had no effect. Diclofenac-induced gastric hemorrhages were assessed in CB_1 (-/-) vs. CB_1 (+/+) littermates (n = 5-8), and CB_2 (-/-) vs. CB_2 (+/+) littermates (n = 5-9). All mice received diclofenac sodium treatment (100 mg/kg). Open bars, vehicle treatment; filled bars, JZL184 treatment. *p<0.05, **p < 0.01, ***p < 0.001 vs. vehicle treatment; ††p < 0.01 vs. JZL184 treatment; †††p < 0.001 vs. CB_2 (+/+) diclofenac treatment.

Figure 7. The anti-hemorrhagic effects of MAGL or FAAH inhibition persist after repeated administration. (**A**) Repeated administration of either JZL184 (4 mg/kg) or PF-3845 (10 mg/kg) did not result in tolerance to the anti-hemorrhagic effects of either inhibitor. Open bar, vehicle treatment; filled bars, diclofenac. Data expressed as mean ± SEM (n = 8). **p < 0.01 vs. vehicle treatment. †p < 0.05, ††p < 0.01 vs. diclofenac treatment.

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Figure 1

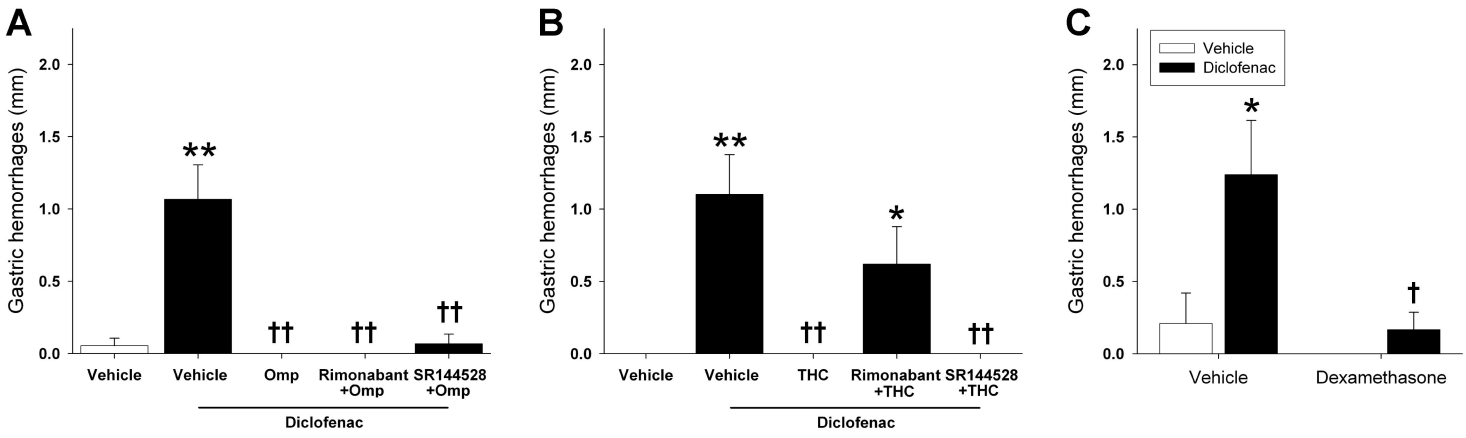

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Figure 2

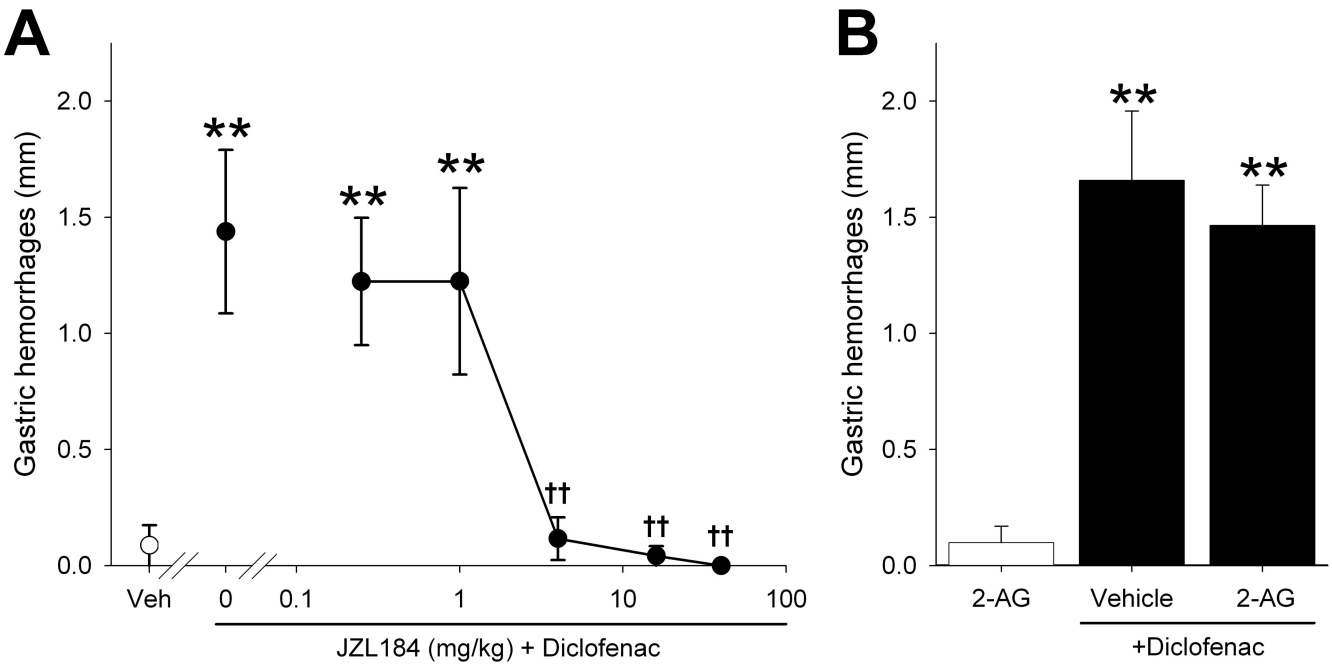

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Figure 3

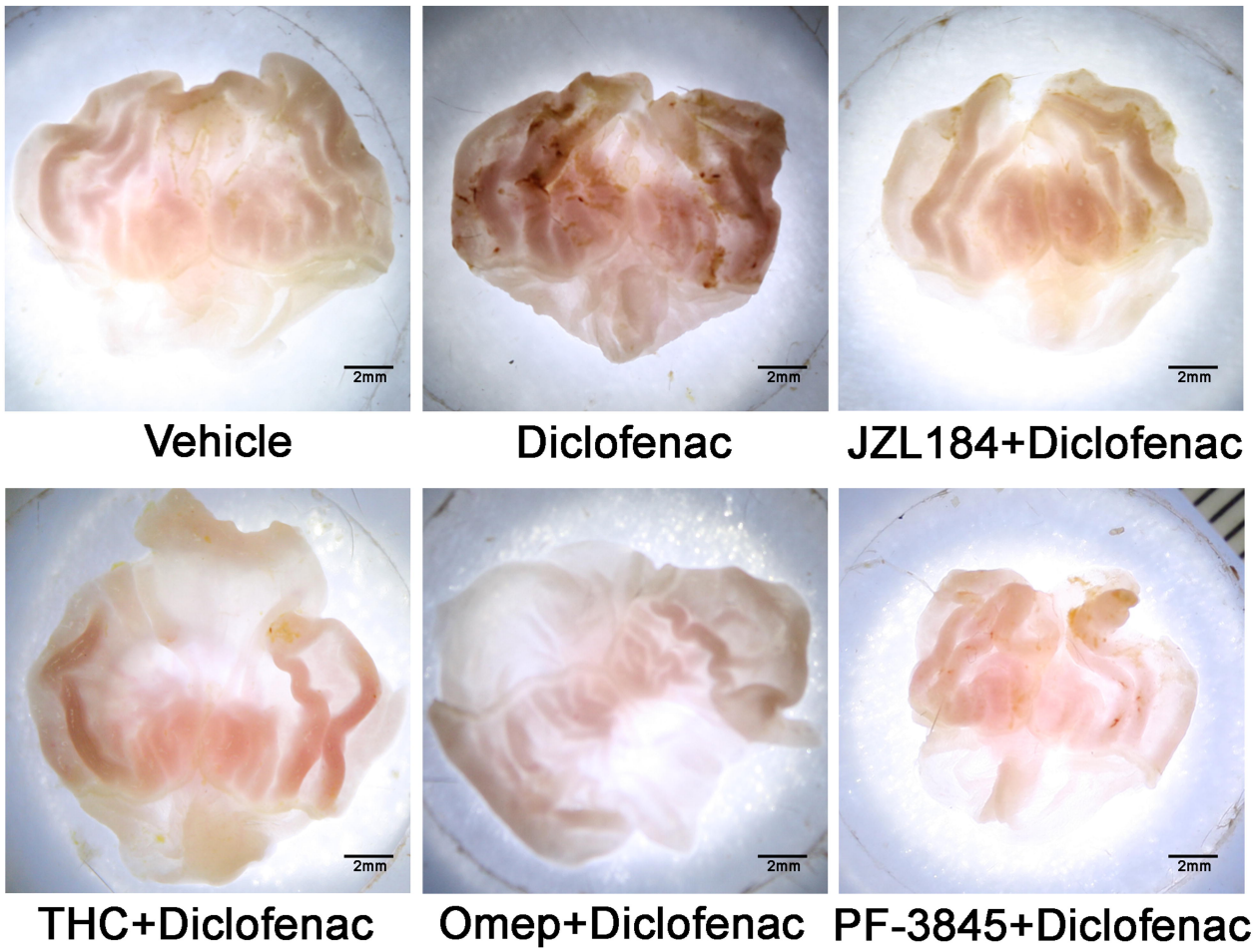

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Figure 4

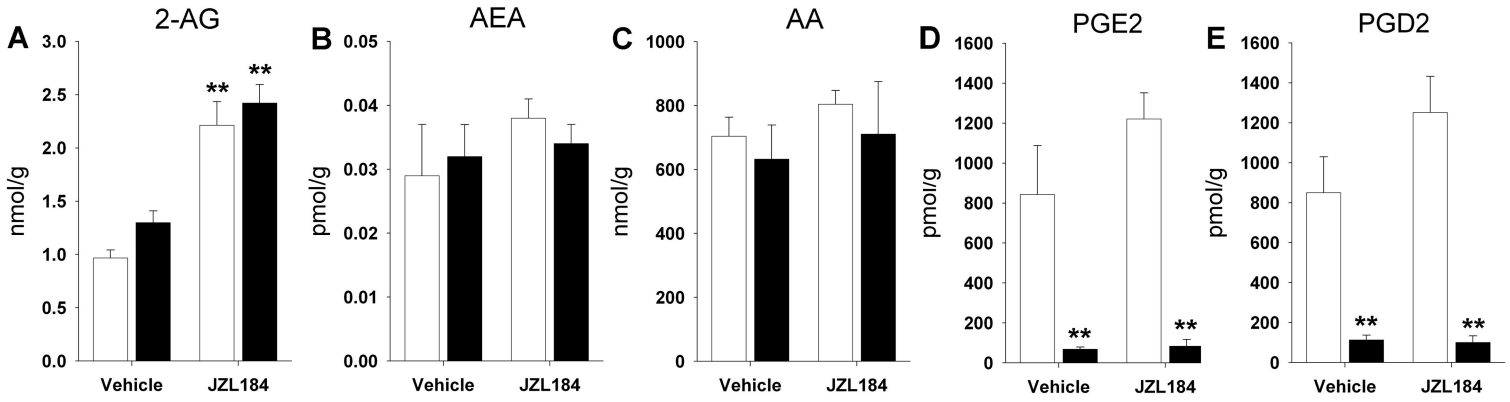

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Figure 5

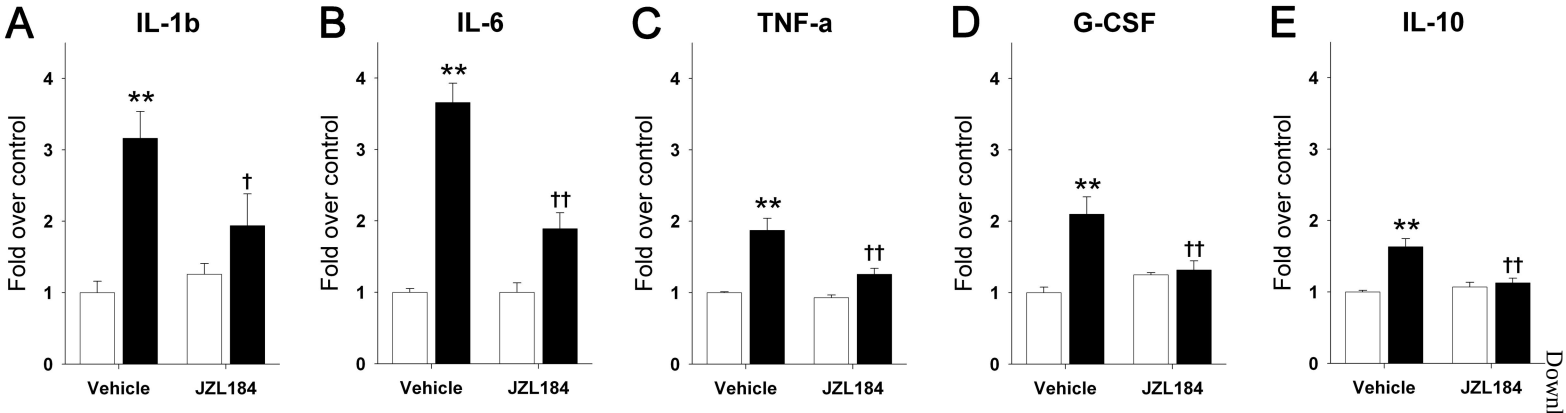

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Figure 6

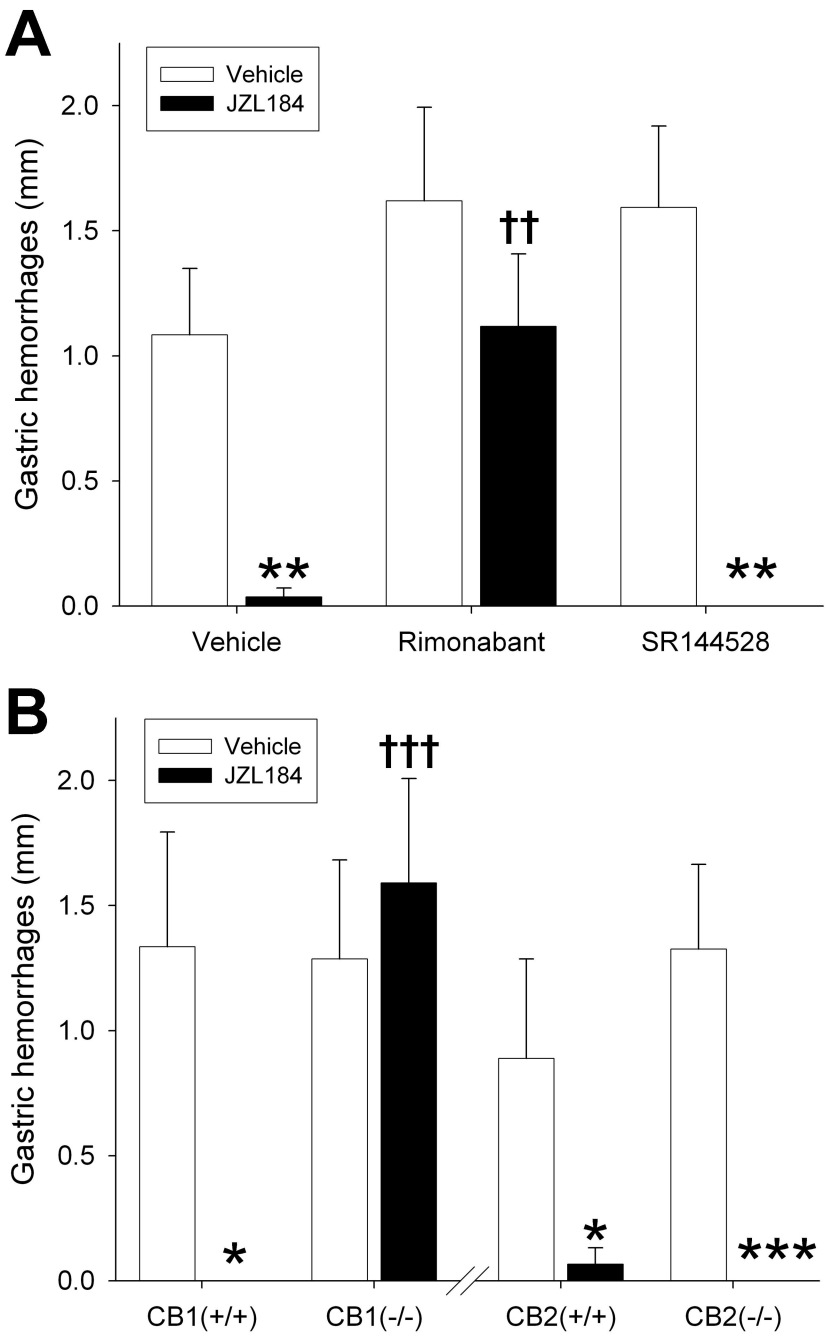

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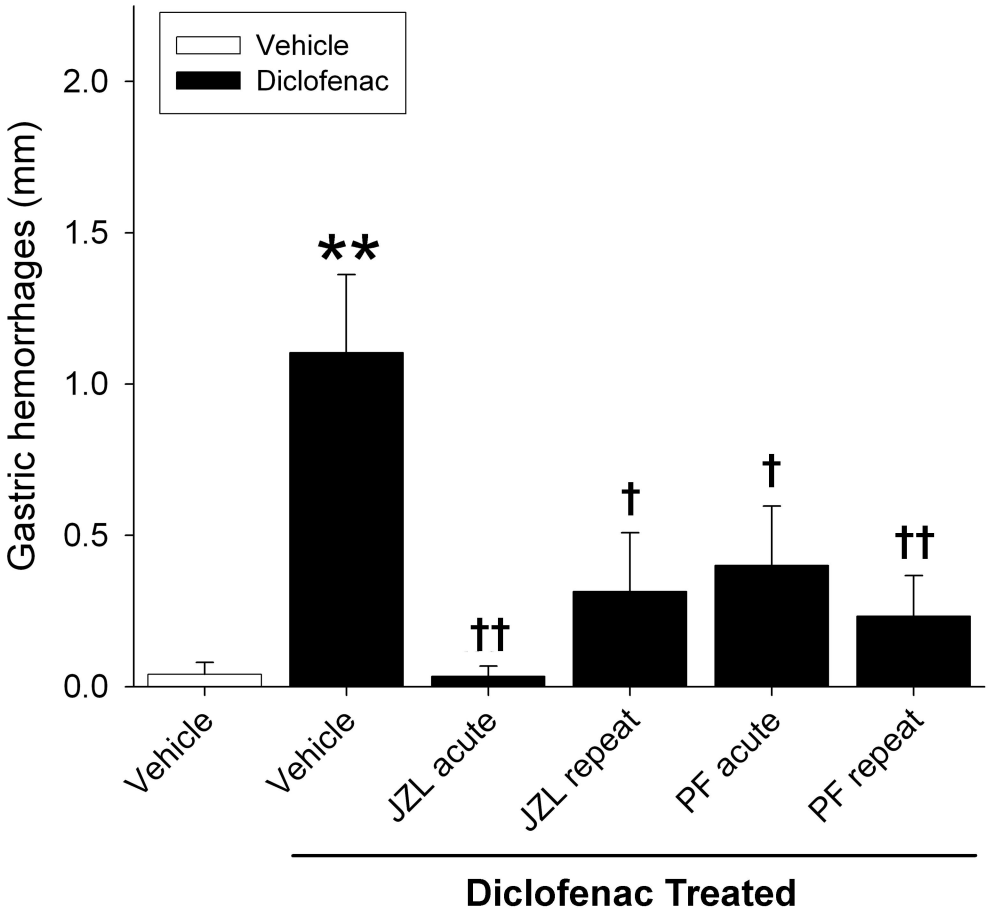
Figure 7



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