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Investigation of Diacylglycerol Lipase Alpha Inhibition in the Mouse Lipopolysaccharide Inflammatory Pain Model[S]

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ABSTRACT

Diacylglycerol lipase (DAGL) α and β, the major biosynthetic enzymes of the endogenous cannabinoid (endocannabinoid) 2-arachidonylglycerol (2-AG), are highly expressed in the nervous system and immune system, respectively. Genetic deletion or pharmacological inhibition of DAGL-β protects against lipopolysaccharide (LPS)-induced inflammatory responses in mouse peritoneal macrophages and reverses LPS-induced allodynia in mice. To gain insight into the contribution of DAGL-α in LPS-induced allodynia, we tested global knockout mice as well as DO34, a dual DAGL-α/β inhibitor. Intraperitoneal administration of DO34 (30 mg/kg) significantly decreased whole-brain levels of 2-AG (~83%), anandamide (~42%), and arachidonic acid (~58%). DO34 dose-dependently reversed mechanical and cold allodynia, and these antinociceptive effects did not undergo tolerance after 6 days of repeated administration. In contrast, DO34 lacked acute thermal antinociceptive, motor, and hypothermal pharmacological effects in naive mice. As previously reported, DAGL-β (−/−) mice displayed a protective phenotype from LPS-induced allodynia. However, DAGL-α (−/−) mice showed full allodynic responses, similar to their wild-type littermates. Interestingly, DO34 (30 mg/kg) fully reversed LPS-induced allodynia in DAGL-α (+/+) and (−/−) mice, but did not affect the antinociceptive phenotype of DAGL-β (−/−) mice in this model, indicating a DAGL-α–independent site of action. These findings suggest that DAGL-α and DAGL-β play distinct roles in LPS-induced nociception. Whereas DAGL-α appears to be dispensable for the development and expression of LPS-induced nociception, DAGL-β inhibition represents a promising strategy to treat inflammatory pain.

Introduction

Diacylglycerol lipase (DAGL)-α and DAGL-β (Bisogno et al., 2003; Gao et al., 2010; Tanimura et al., 2010) transform diacylglycerols into 2-arachidonoylglycerol (2-AG), the most highly expressed endocannabinoid in the central nervous system (Mechoulam et al., 1995; Sugiura et al., 1995). 2-AG plays critical roles in maintaining proper neuronal function (Goncalves et al., 2008; Tanimura et al., 2010), mediating neuronal axonal growth (Williams et al., 2003) and retrograde suppression of synaptic transmission (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Pan et al., 2009). These enzymes are differentially expressed within cells in the nervous system and peripheral tissue (Hsu et al., 2012). DAGL-α is expressed on postsynaptic neurons within various brain regions (Katona et al., 2006; Yoshida et al., 2006; Lafourcade et al., 2007; Uchigashima et al., 2007), and its genetic deletion results in marked decreases of 2-AG, anandamide (AEA), and arachidonic acid (AA) in the brain (Gao et al., 2010; Tanimura et al., 2010; Shonesy et al., 2014) and spinal cord (Gao et al., 2010). Accordingly, DAGL-α (−/−) mice display impaired depolarization-induced suppression of inhibition and excitation in the brain (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011). These mice also show an increased mortality rate (Sugaya et al., 2016), display increased spontaneous seizures in the kainate model of status epilepticus (Sugaya et al., 2016), and exhibit an anxiogenic phenotype (Shonesy et al., 2014). In contrast, DAGL-β is most highly expressed on macrophages, and, although its relative brain expression is sparse, it is highly expressed on microglia (Hsu et al., 2012). This distribution pattern suggests that DAGL-β activity contributes to inflammatory responses. Importantly, DAGL-β deletion does not affect endocannabinoid-mediated forms of retrograde synaptic suppression (Gao et al., 2010). However, DAGL-β blockade reduces lipopolysaccharide (LPS)-induced inflammatory responses in peritoneal macrophages from C57BL/6 mice by decreasing levels of 2-AG, AA, prostanoids, and proinflammatory cytokines (Hsu et al., 2012). Similarly, DAGL-α inhibition leads to protection from the neuroinflammatory effects of 20 mg/kg systemic LPS (Ogasawara et al., 2016).

A wide scope of evidence supports inflammatory as well as neuronal signaling contributions to many forms of pathologic pain. Immune cell signaling plays a critical role in the

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ABBREVIATIONS: AA, arachidonic acid; ABHD6, alpha beta hydrolase domain-containing protein 6; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variance; CI, confidence interval; DAGL, diacylglycerol lipase; LPS, lipopolysaccharide.

development and maintenance of neuropathic pain (Watkins et al., 2001; De Leo et al., 2006; Beggs and Salter, 2013). Likewise, increased neuronal signaling underlies pathologic inflammatory pain and can contribute to a positive pain feedback loop (De Leo et al., 2006; Chen et al., 2015). For example, in LPS-stimulated neurons, neuronal signaling leads to further inflammatory signaling and immune cell activation (Chen et al., 2015). Determining the antecedents of pathologic pain and the subsequent identification of potential therapeutic targets remain important areas of research. Accordingly, DAGL-α and DAGL-β represent provocative targets to treat pathologic pain conditions.

Complementary approaches of pharmacological agents and genetically modified mice demonstrate that DAGL-β blockade reduces nociceptive behavior in the LPS model of inflammatory pain (Wilkerson et al., 2016). The DAGL-β inhibitor KT109 reverses nociceptive behavior in models of neuropathic pain (Wilkerson et al., 2016). These findings strongly implicate inhibition of this enzyme as a viable approach to treat inflammatory and neuropathic pain. However, it remains to be determined whether DAGL-α inhibition or deletion produces antinociceptive effects in pathologic pain models. The present study attempted to investigate the role of this enzyme in LPS-induced allodynia, using the DAGL inhibitor DO34, which disrupts depolarization-induced suppression of excitation and depolarization-induced suppression of inhibition in the cerebellum and hippocampus and reduces LPS-induced anapyrexia in vitro responses (Ogasawara et al., 2016), providing a useful tool for in vitro and in vivo studies. Thus, the present study examined DAGL-α (−/−) and DAGL-β (−/−) mice in the LPS model of inflammatory pain.

In initial experiments, we quantified brain levels of endogenous cannabinoids and AA in mice administered vehicle or DO34 (30 mg/kg) and tested DO34 in assays of locomotor behavior, catalepsy, body temperature, and acute thermal antinociceptive responses. We then evaluated the dose-response relationship and time course of acute DO34 administration in attenuating LPS-induced mechanical and cold allodynia. In addition, we examined whether the antiallodynic effects would undergo tolerance after repeated DO34 administration. Finally, we tested DO34 in DAGL-α (−/−) and DAGL-β (−/−) mice, and respective wild-type littermates in the LPS model of inflammatory pain. Because DO34 also inhibits the serine hydrolase ABHD6 (Ogasawara et al., 2016), a 2-AG hydrolytic enzyme expressed on postsynaptic neurons (Blankman et al., 2007; Marrs et al., 2010), we used the selective ABHD6 inhibitor DO53, which lacks DAGL activity (Ogasawara et al., 2016), for comparison.

Materials and Methods

Animals. Adult male C57BL/6J and ICR mice (23–40 g; The Jackson Laboratory, Bar Harbor, ME) served as subjects in these experiments. DAGL-α (−/−) and DAGL-β (−/−) mice were generated in the Cravatt laboratory on a mixed C57BL/6J and 129/SvEv background, as previously described (Hsu et al., 2012), and breeding pairs were transferred to Virginia Commonwealth University. A total of 84 DAGL-α (−/−) and 36 DAGL-β (−/−) mice were used in these studies. Mice were housed four per cage in a temperature- (20–22°C), humidity- (55% ± 10%), and light-controlled (12-hour light/dark; lights on at 6:00 AM) Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD)-approved facility, with standard rodent chow and water available ad libitum.

All tests were conducted during the light phase. The sample size selected for each treatment group in each experiment was based on previous studies from our laboratory and complied with power analyses.

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). After testing was completed, mice were euthanized via CO_2 asphyxia, followed by rapid cervical dislocation.

Drugs. The DAGL inhibitor DO34 and the selective ABHD6 inhibitor DO53 were synthesized by the Cravatt laboratory according to previous methods (Ogasawara et al., 2016). All drugs were dissolved in a vehicle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9% NaCl) in a 1:1:18 ratio. All drugs were administered in an injection volume of 10 μl/g body mass. Each drug was given via the intraperitoneal route of administration. The dose range of DO34 was selected based on results reported by Ogasawara et al. (2016), indicating that acute administration of 30 mg/kg DO34 in mice treated with LPS was sufficient to produce inhibition of DAGL-α, as well as measured decreases in peritoneal macrophages of AA and proinflammatory cytokines.

Extraction and Quantification of Endocannabinoids by Liquid Chromatography-Tandem Mass Spectrometry. 2-AG, AA, and AEA levels were quantified from the whole brain of ICR mice, after acute administration of DO34 (30 mg/kg, i.p.) or 1:1:18 vehicle. Brains were collected and processed for quantification of 2-AG, AA, and AEA. Because equivalent doses of DO34 significantly attenuated allodynia associated with LPS at 2 hours after injection, mice were euthanized via rapid decapitation at this time point. Brains were rapidly harvested, snap frozen in dry ice, and stored at −80°C until the time of processing. Tissues were further processed according to methods described previously (Ignatowska-Jankowska et al., 2015). See Supplemental Material for details.

Evaluation of Acute Pharmacological Effects of DO34. Mice (counterbalanced Latin square within subject design) were housed individually overnight. The behavioral testing was conducted in the following order: bar test (catalepsy), tail withdrawal test, rectal temperature, and locomotor activity. Testing was performed according to previously described procedures (Long et al., 2009; Schlosburg et al., 2010). Catalepsy was assessed on a bar 0.7 cm in diameter placed 4.5 cm off of the ground. The mouse was placed with its front paws on the bar and a timer (Timer #1) was started. A second timer (Timer #2) was turned on only when the mouse was immobile on the bar, with the exception of respiratory movements. If the mouse moved off the bar, it was placed back on in the original position. The assay was stopped either when Timer #1 reached 60 seconds or after the fourth time the mouse moved off the bar, and the cataleptic time was scored as the amount of time on Timer #2. Nociception was then assessed in the tail immersion assay. The mouse was placed head first into a small bag fabricated from absorbent underpads (4 cm diameter, 11 cm length; VWR Scientific Products, Radnor, PA) with the tail out of the bag. Each mouse was hand held, and 1 cm of the tail was submerged in a 52°C water bath. The latency for the mouse to withdraw its tail within a 10-second cutoff time was scored. Rectal temperature was assessed by inserting a thermocouple probe 2 cm into the rectum, and temperature was determined by thermometer (BAT-10 Multipurpose Thermometer; Protech International Inc., Boerne, TX). Locomotor activity was assessed 120 minutes after treatment for a 60-minute period in a Plexiglas cage (42.7 × 21.0 × 20.4 cm) and Anymaze software (Stoelting, Wood Dale, IL) was used to determine the percentage of time spent immobile, mean speed, and distance traveled.

LPS Inflammatory Pain Model. Mice were given an injection of 2.5 μg of LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis, MO) in 20 μl of physiologic sterile saline (Hospira Inc., Lake Forest, IL) into the plantar surface of the right hind paw. As previously reported, this is the minimally effective dose of LPS that elicits mechanical allodynia, but not measurable increases in paw thickness (Booker et al., 2012). After LPS administration, mice were returned to their home cages. At 22 hours, mice were given the appropriate injection of drug or vehicle and tested at 24 hours for allodynia. In the time course study, allodynia was assessed at 40 minutes and 1, 3, 5, 8, and 24 hours after the intraperitoneal injection.

To determine whether repeated administration of DO34 would produce sustained antinociceptive effects, mice were given injections of vehicle or DO34 (30 mg/kg, i.p.) once a day for 5 days. On day 5, each mouse received its appropriate intraperitoneal injection of vehicle or DO34, and 2 hours later all mice were given an intraplantar injection of LPS. On day 6 (22 hours after LPS administration), each mouse received its final intraperitoneal injection. The vehicle-treated mice were divided into two groups. The first group received another injection of vehicle (vehicle control group), and the second group was given 30 mg/kg DO34 (the acute DO34 group). The mice that had been given repeated injections of drug received their final injection of DO34 (the repeated DO34 group). All mice were tested for mechanical and cold allodynia 2 hours after the final intraperitoneal injection.

Behavioral Assessment of Nociception. Baseline responses to light mechanical touch were assessed using the von Frey test after habituation to the testing environment, as described previously (Murphy et al., 1999). In brief, mice were placed atop a wire mesh screen, with spaces 0.5 mm apart and habituated for approximately 30 min/day for 4 days. Mice were unrestrained, and were singly placed under an inverted wire mesh basket to allow for unrestricted airflow. The von Frey test utilizes a series of calibrated monofilaments (2.83–4.31 log stimulus intensity; North Coast Medical, Morgan Hills, CA) applied randomly to the left and right plantar surface of the hind paw for 3 seconds. Lifting, licking, or shaking the paw was considered a response. After the completion of allodynia testing for LPS experiments, cold allodynia testing was performed with the application of acetone (Decosterd and Woolf, 2000). In this assay, 10 μl of acetone (99% high-performance liquid chromatography grade; Thermo Fisher Scientific, Waltham, MA) was projected via a 100-μl pipette (Rainin Instruments, Woburn, MA) onto the plantar surface of each hind paw. Acetone was propelled from below via air burst by expressing the pipette, thereby avoiding mechanical stimulation of the paw with the pipette. Total time lifting/clutching each hind paw was recorded with an arbitrary maximum cutoff time of 60 seconds. For all behavioral testing, threshold assessment was performed in a blinded fashion.

Data Analysis. Data were analyzed using Student's *t* test (evaluation of endocannabinoid and AA levels) or one-way or two-way analysis of variance (ANOVA). Tukey's test was used for post hoc analysis after a significant one-way ANOVA. Multiple comparisons after two-way ANOVA were conducted with a Bonferroni post hoc comparison. A P value of <0.05 was considered statistically significant. The computer program GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA) was used in all statistical analyses. All data are expressed as the mean ± S.E.M.

Results

DO34 (30 mg/kg) significantly decreased whole-brain levels of 2-AG ($P < 0.0001$) (Fig. 1A), AEA ($P < 0.05$) (Fig. 1B), and AA ($P < 0.0001$) (Fig. 1C).

To examine whether DO34 produces overt pharmacological effects, we assessed whether it affects spontaneous locomotor behavior, elicits cataleptic effects in the bar test, produces antinociception in the warm water tail withdrawal assay, or alters body temperature. Naive mice given vehicle or 1, 3, 10, 30, 50, or 100 mg/kg DO34. DO34 did not display differences of treatment in catalepsy (Fig. 2A), hypothermia ($P = 0.60$) (Fig. 2B), thermal antinociception ($P = 0.13$) (Fig. 2C), or locomotor alterations (defined as time spent immobile; $P = 0.57$) (Fig. 2D).

Having confirmed that the DAGL inhibitor DO34 significantly reduces endocannabinoids and AA in whole brain but does not affect overt motor or sensory behavior, the next set of experiments investigated this compound in the LPS model of inflammatory pain. The dose-response evaluation of the antiallodynic effects of DO34 (1, 3, 10, and 30 mg/kg) at 2 hours postinjection in the von Frey and acetone-induced flinching assays are shown in Fig. 3, A and B, respectively. DO34 dose-dependently reversed LPS-induced mechanical allodynia [$F(3,20) = 14.12$; $P < 0.0001$] and cold allodynia, [$F(3,20) = 15.99$; $P < 0.0001$]. The respective ED_{50} values [95% confidence interval (CI)] of DO34 in reversing LPS-induced mechanical allodynia and cold allodynia were 3.8 mg/kg (95% CI, 2.8–5.3 mg/kg) and 6.0 mg/kg (95% CI, 4.0–9.0 mg/kg). The potency ratio (95% CI) of DO34 for mechanical versus cold

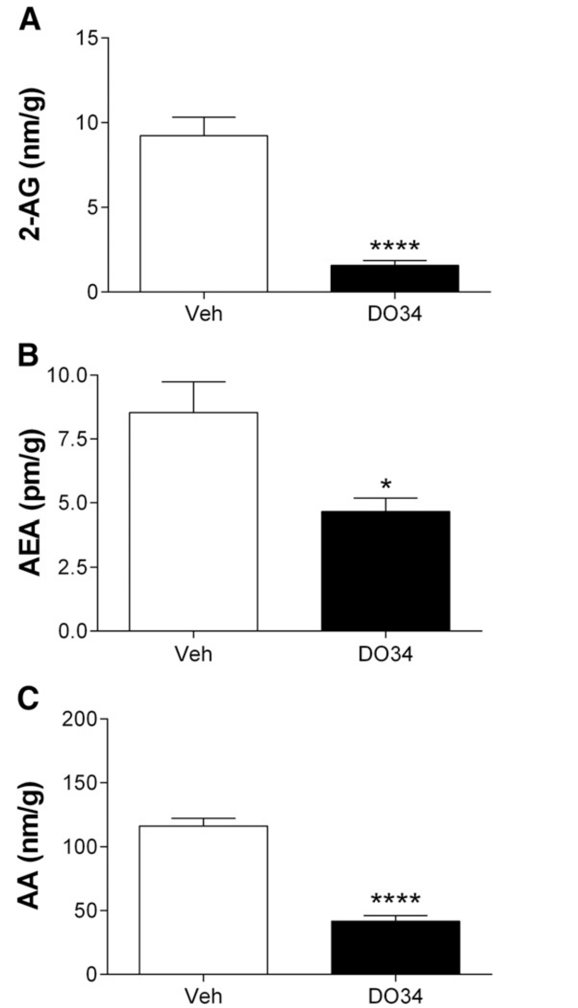

Fig. 1. Endocannabinoid levels in whole brain mouse tissue are altered 2 hours after 30 mg/kg DO34. DO34 decreases 2-AG (A), AEA (B), and AA (C) compared with vehicle (Veh). ****$P < 0.0001$; *$P < 0.05$ vs. vehicle. Data reflect the mean ± S.E.M. $n = 12$ mice/group.

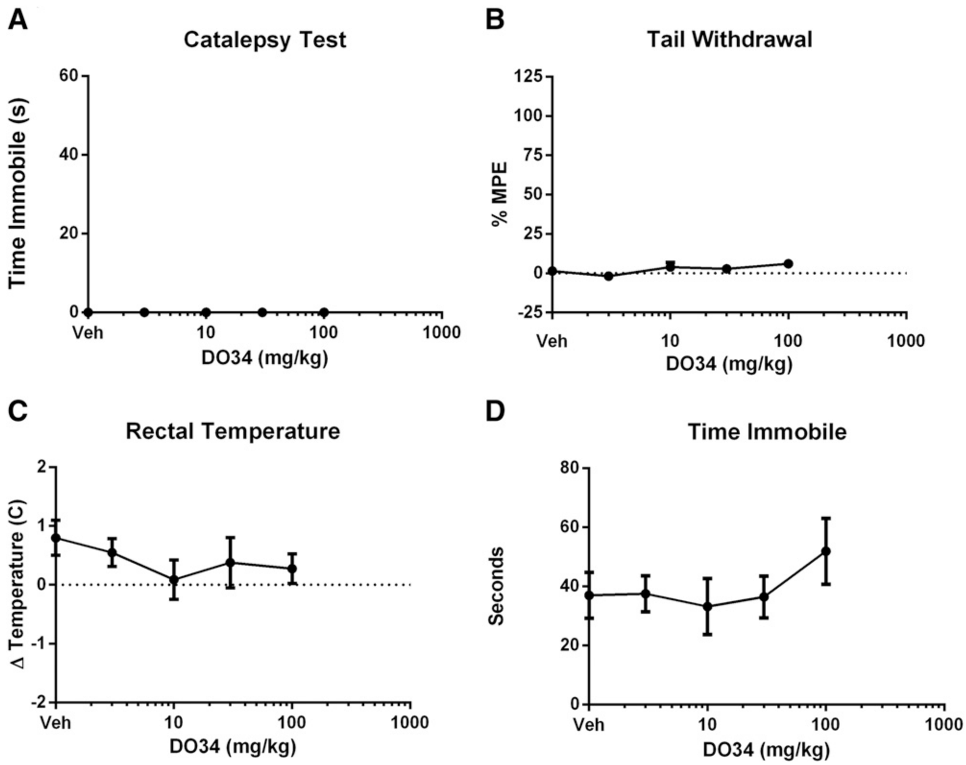

Fig. 2. Assessment of behavior reveals DO34 treatment does not produce common cannabimimetic effects in naive mice. DO34 does not produce catalepsy (A), antinociception (B), body temperature (C), or change in locomotion (D). Data reflect the mean ± S.E.M. n = 6 mice/group.

allodynia was 1.6 (95% CI, 1.0–2.6), indicating equipotence in the two nociceptive assays. As depicted in Fig. 3C, DO34 significantly reversed allodynia within 30 minutes of intraperitoneal administration, and the antinociceptive effect of 30 mg/kg DO34 persisted for at least 8 hours [interaction between treatment and time: $F(20,125) = 3.58$; $P < 0.0001$].

To evaluate whether the antinociceptive effects of DO34 undergo tolerance, we evaluated von Frey thresholds and acetone-induced flinching in mice that received vehicle or DO34 (30 mg/kg) after one injection or 6 days of repeated administration. As shown in Fig. 4, DO34 retained its antiallodynic effects after 6 days of repeated administration in the von Frey assay [$F(1,20) = 22.55$; $P < 0.0001$] (Fig. 4A), as well as in the acetone-induced flinching assay [$F(1,20) = 46.34$; $P < 0.0001$] (Fig. 4B).

In the next series of experiments, we evaluated LPS-induced allodynia in DAGL-α (−/−) mice or DAGL-β (−/−) mice given an intraperitoneal injection of vehicle, DO34 (30 mg/kg), or DO53 (30 mg/kg), a selective ABHD6 inhibitor that served as a control for this off-target of DO34. As previously reported (Wilkerson et al., 2016), DAGL-β (−/−) mice displayed an antiallodynic phenotype in the von Frey assay [$F(1,10) = 27.9$; $P < 0.001$] (Fig. 5A). In addition, these mice showed a reduction in acetone-induced flinching [$F(1,10) = 225$; $P < 0.0001$] (Fig. 5B) 24 hours after intraplantar LPS administration. DO34 (30 mg/kg, i.p.) administered at 22 hours after LPS injection reversed mechanical ($P < 0.001$) (Fig. 5A) and cold ($P < 0.0001$) (Fig. 5B) allodynia in DAGL-β (+/+) mice but did not alter the antiallodynic phenotypes of the DAGL-β (−/−) mice. DO53 (30 mg/kg, i.p.) did not produce significant effects in either mechanical ($P = 0.97$) or cold ($P = 0.49$) allodynia and did not alter the DAGL-β (−/−) antiallodynic phenotype.

In the final experiment, we examined the dose-response relationship of DO34 (0, 1, 3, 10, or 30 mg/kg) in LPS-treated DAGL-α (−/−) and (+/+) mice. LPS elicited similar magnitudes of mechanical (Fig. 6A) and cold (Fig. 6B) allodynia regardless of genotype. DO34 dose-relatedly reversed LPS-induced mechanical allodynia in DAGL-α (+/+) mice [$F(3,20) = 29.42$; $P < 0.0001$] and DAGL-α (−/−) mice [$F(3,20) = 4.45$; $P < 0.05$] (Fig. 6A). The respective ED_{50} (95% CI) values of DO34 in reversing LPS-induced mechanical allodynia in DAGL-α (+/+) and (−/−) mice were 8.6 mg/kg (95% CI, 6.4–11.5 mg/kg) and 5.9 mg/kg (95% CI, 3.9–8.8 mg/kg). The potency ratio of DAGL-α (+/+) to DAGL-α (−/−) for mechanical allodynia was 1.4 (95% CI, 0.8–2.5). Likewise, DO34 reversed cold allodynia in DAGL-α (+/+) [$F(3,20) = 48.47$; $P < 0.0001$] and DAGL-α (−/−) mice [$F(3,20) = 30.99$; $P < 0.0001$] (Fig. 6B). The respective ED_{50} values (95% CI) of DO34 in reversing LPS-induced cold allodynia in DAGL-α (+/+) and (−/−) mice were 6.1 mg/kg (95% CI, 4.7–7.9 mg/kg) and 4.5 mg/kg (95% CI, 3.4–6.0 mg/kg). The potency ratio for DAGL-α (+/+) to DAGL-α (−/−) for cold allodynia was 1.35 (95% CI, 0.9–1.9). Finally, DO53 (30 mg/kg) administered at 22 hours after LPS injection did not produce antinociceptive effects in either genotype (Supplemental Fig. 1).

Discussion

The present study used complementary pharmacological and genetic approaches to test whether blockade of the 2-AG biosynthetic enzymes DAGL-β and DAGL-α produces antinociceptive effects in the LPS model of inflammatory pain. As

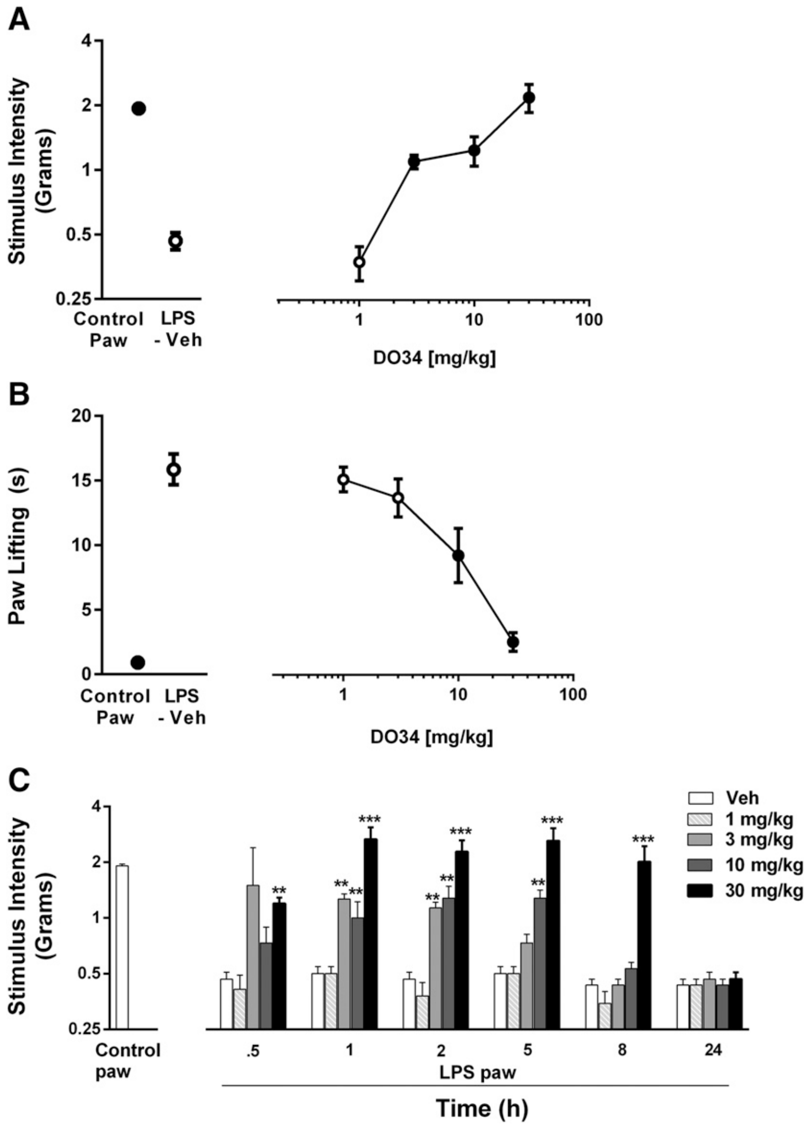

Fig. 3. Pharmacological inhibition of DAGL with DO34 reverses LPS-induced mechanical and cold allodynia. (A) DO34 reverses LPS-induced mechanical allodynia in a dose- and time-dependent manner. (B) DO34 reverses LPS-induced cold allodynia in a dose-dependent manner 2 hours after intraperitoneal administration. (C) DO34 significantly reversed allodynia in a time- and dose-dependent manner, within reversal onset at 30 minutes, lasting beyond 8 hours, after intraperitoneal administration. Data reflect the mean ± S.E.M. n = 6 mice/group. ***$P < 0.0001$; **$P < 0.001$ vs. LPS + vehicle. Filled circles = $P < 0.05$ vs. LPS + vehicle. Veh, vehicle.

previously reported (Wilkerson et al., 2016), DAGL-β (−/−) mice were resistant to the development of LPS-induced mechanical allodynia. Moreover these mice were also resistant to the development of LPS-induced cold allodynia. In contrast, DAGL-α (−/−) mice displayed full development of LPS-induced mechanical and cold allodynia. These findings suggest that DAGL-α and DAGL-β play differential roles in the development of LPS-induced hyperalgesic states. Thus, whereas DAGL-α is dispensible for the development of LPS-induced allodynia, DAGL-β plays a necessary role in the increased nociceptive behavior after endotoxin treatment.

The disparate roles that DAGL-α and DAGL-β play in LPS-induced allodynia are consistent with the differential expression of these enzymes on cells in the nervous system and peripheral tissue (Hsu et al., 2012). Specifically, DAGL-α is expressed on postsynaptic neurons within the hippocampus, cerebellum, prefrontal cortex, and striatum (Katona et al., 2006; Yoshida et al., 2006; Lafourcade et al., 2007; Uchigashima et al., 2007), and its genetic deletion results in marked decreases in 2-AG, AEA, and AA in the brain (Gao et al., 2010; Tanimura et al., 2010; Shonesy et al., 2014) and spinal cord (Gao et al., 2010). In contrast, the relative expression of DAGL-β throughout the brain is generally sparse. DAGL-β (−/−) mice express wild-type levels of 2-AG in the whole brain (Hsu et al., 2012), and endocannabinoid-mediated forms of retrograde synaptic suppression in these mice are spared (Gao et al., 2010). However, the high expression of DAGL-β on microglia in the central nervous system (Viader et al., 2016) and on macrophages in the periphery (Hsu et al., 2012) is consistent with its role within the innate immune system. Specifically, pharmacological inhibition or genetic deletion of DAGL-β leads to decreased levels of endocannabinoids, AA, prostanoids, and proinflammatory cytokines in LPS-treated peritoneal macrophage cell cultures from C57BL/6 mice (Hsu et al., 2012).

Here we show that acute administration of DO34, at a dose that produced reversal of mechanical and cold allodynia (30 mg/kg), produced significant reductions of 2-AG (~83%), AEA (~42%), and AA (~58%) in naive mice. These findings are in agreement with previous work showing that DO34 reduces 2-AG, AEA, and AA levels in mouse whole brains (Ogasawara et al., 2016). However, the use of whole brain precludes insight of whether DO34 differentially affects lipid levels in discrete brain regions.

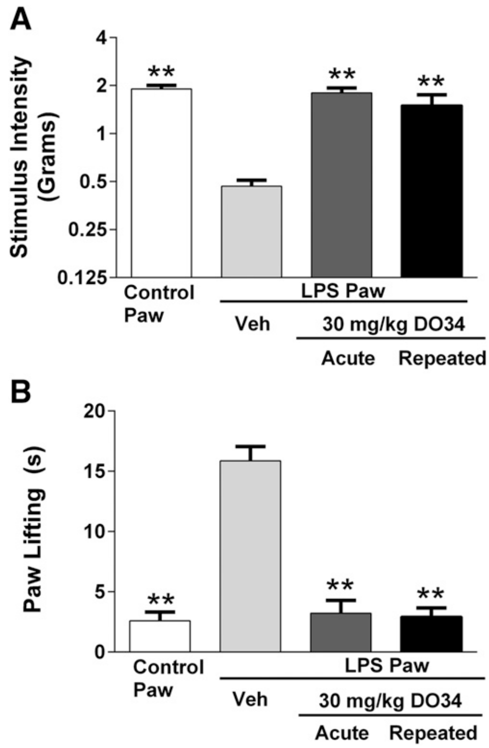

Fig. 4. Repeated administration of DO34 prevents LPS-induced mechanical and cold allodynia. (A) Acute or repeated administration of DO34 (30 mg/kg) prevents the expression of LPS-induced mechanical allodynia. (B) Acute or repeated administration of DO34 (30 mg/kg) prevents the expression of LPS-induced cold allodynia. Data reflect the mean ± S.E.M. n = 6 mice/group. $^{**}P < 0.001$ vs. LPS + vehicle. Veh, vehicle.

Here, we also report that the DAGL inhibitor DO34 dose-dependently reversed LPS-induced mechanical allodynia and cold allodynia. These antinociceptive effects are DAGL-α dispensible, as genetic deletion of this enzyme did not alter the dose-response curves of DO34 for both measures. DO34 also did not alter the antinociceptive phenotype DAGL-β (−/−) mouse response but completely reversed the LPS-induced allodynic responses in DAGL-β (+/+) mice. Additionally, the DO34 time course experiment demonstrates that its antiallodynic effects persist for at least 8 hours, which is consistent with its long duration of action in inhibiting DAGL-β activity (Ogasawara et al., 2016). Besides its actions in DAGL-α and DAGL-β, DO34 also inhibits ABHD6 (Ogasawara et al., 2016), a serine hydrolase that hydrolyzes 2-AG, but to a much lesser extent than monoacylglycerol lipase (Blankman et al., 2007; Marrs et al., 2010). Thus, we tested DO53, a structurally similar compound that inhibits ABHD6 without actions at either DAGL-β or DAGL-α (Ogasawara et al., 2016) in the LPS model of inflammatory pain. DO53 did not reverse LPS-induced allodynia in either DAGL-α (−/−) or (+/+) mice, suggesting that ABHD6 inhibition alone or in combination with DAGL-α inhibition does not elicit antinociceptive effects in this assay. The observation that another ABHD6 inhibitor, KT195, lacked efficacy in reversing LPS-induced mechanical allodynia (Wilkerson et al., 2016) further excludes consideration of the involvement of this enzyme in the results reported here. Although the indirect evidence offered here is consistent with the idea that DAGL-β mediates the antiallodynic effects of DO34, it does not rule out the possibility of another target.

Another relevant finding in the present study is that repeated administration of DO34 (30 mg/kg) for 6 days continued to prevent the expression of LPS-induced allodynia. Similarly, the antiallodynic effects of the preferential DAGL-β inhibitor KT109 in the LPS model of inflammatory pain did not undergo tolerance. This apparent lack of tolerance is consistent with the observation that DAGL-β (−/−) mice displayed an antiallodynic phenotype in the LPS model of inflammatory pain. It will be important in future studies to ascertain whether repeated administration DAGL inhibitors also reverses nociceptive behavior in chronic models of inflammatory or neuropathic pain. Additionally, given the previously mentioned caveats of target selectivity, there is a need for more selective inhibitors for DAGL-α and DAGL-β, and further studies with these inhibitors are needed to verify our proposed mechanism of action.

The underlying mechanisms for the antinociceptive effects of DO34 remain to be determined but may be related to a reduction of AA and its bioactive metabolites in macrophages expressed in the LPS-treated paw. In particular, KT109 as well as DAGL-β deletion resulted in decreased levels of a variety of proinflammatory lipids and proteins in LPS-stimulated peritoneal macrophage (Hsu et al., 2012). Specifically, prostaglandins are crucial for the development and maintenance of inflammatory pain (Ulmann et al., 2010; Endo et al., 2014; Sugita et al., 2016). DAGL-β inhibition is also protective from microglial activation in the brains of mice repeatedly administered LPS (Viader et al., 2016) and specifically produces reversal of LPS-stimulated proinflammatory cytokine release (Hsu et al., 2012) as well as reverses allodynia and thermal sensitivity in inflammatory, chronic constriction injury of the sciatic nerve, and chemotherapy-induced peripheral neuropathy pain models (Wilkerson et al., 2016). In these models of neuropathic pain, although these analgesic effects appear to be due to DAGL-β inhibition, the relative contribution of additional modulation of AA metabolites remains unclear. However, AA can act as a direct modulator of neuronal activity through its mechanosensory mediatory effects on lipid-sensitive ion channels (Meves, 2008; Brohawn et al., 2014).

Given that these studies represent one of the first in vivo evaluations of DO34, we assessed whether it would produce overt behavioral effects. Accordingly, we examined whether DO34 would produce changes in spontaneous activity or body temperature as well as assess whether it would elicit acute cataleptic or thermal antinociceptive effects. DO34 did not alter spontaneous activity or body temperature and was inactive in a warm water tail withdrawal test for acute thermal antinociception and in the bar test for catalepsy. Other studies have shown that DO34 decreases food intake (Deng et al., 2017). It is important to note here that DAGL-α (−/−) mice display increased anxiogenic behavior in multiple assays used to infer anxiety (i.e., the open-field, light/dark box, and novelty-induced hypophagia test) (Shonesy et al., 2014). Thus, it will be important to assess the effects of DO34 in fear and anxiety assays in future studies.

The results of the present study provide proof of principle that DAGL-β plays a necessary role in the expression of

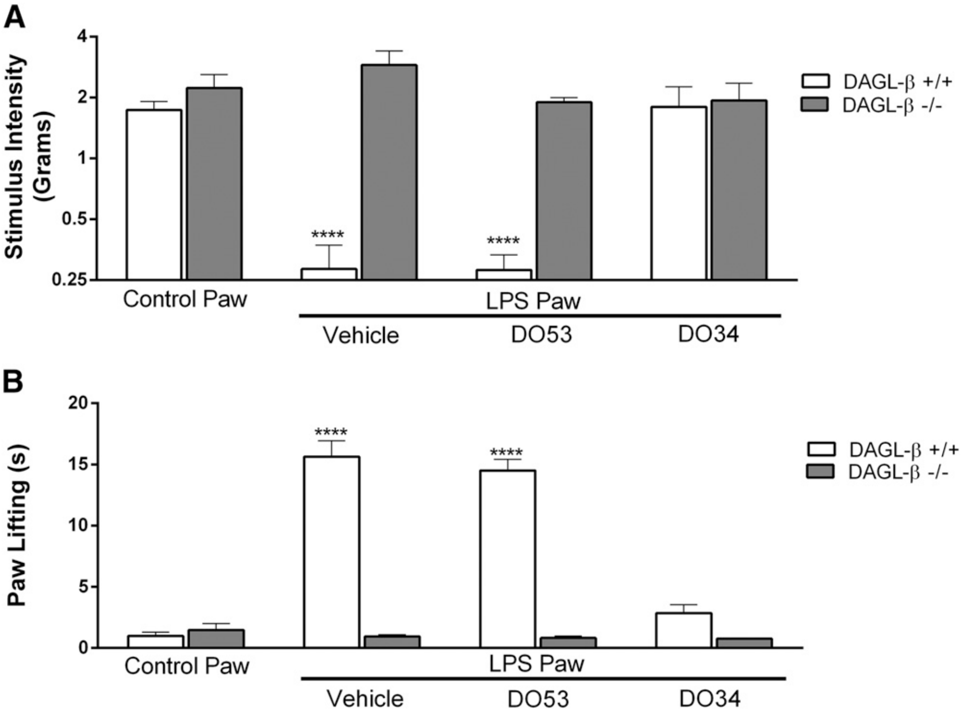

Fig. 5. Genetic inhibition of DAGL-β in the mediation of LPS-induced mechanical and cold allodynia. (A) LPS-treated DAGL-β (−/−) mice do not develop mechanical allodynia. The DAGL-α and DAGL-β inhibitor DO34 (30 mg/kg) reverses LPS-induced mechanical allodynia in DAGL-β (+/+) mice and does not further alter the antinociceptive effects in DAGL-β (−/−) mice. The ABHD6 inhibitor DO53 did not produce reversal of mechanical allodynia in DAGL-β (+/+) mice. (B) LPS-treated DAGL-β (−/−) mice do not develop cold allodynia. DO34 (30 mg/kg) reverses LPS-induced cold allodynia in DAGL-β (+/+) mice and does not further alter the antinociceptive effects in DAGL-β (−/−) mice. The ABHD6 inhibitor DO53 did not produce reversal of cold allodynia in DAGL-β (+/+) mice. N = 6 mice/group. Data reflect the mean ± S.E.M. ****$P <$ 0.0001 vs. respective genotype control paw.

nociceptive behavior in the LPS model of inflammatory pain. The observation that DAGL-α (−/−) mice display LPS-induced allodynia indicates that this enzyme is dispensable for these effects. Moreover, these null mice served as a useful tool in showing that the antiallodynic effects of DO34 are independent of its actions on DAGL-β. Taken together, the present study and our previous work (Wilkerson et al., 2016) suggest that DAGL-β represents a potential therapeutic target to relieve pain elicited by the activation of proinflammatory events.

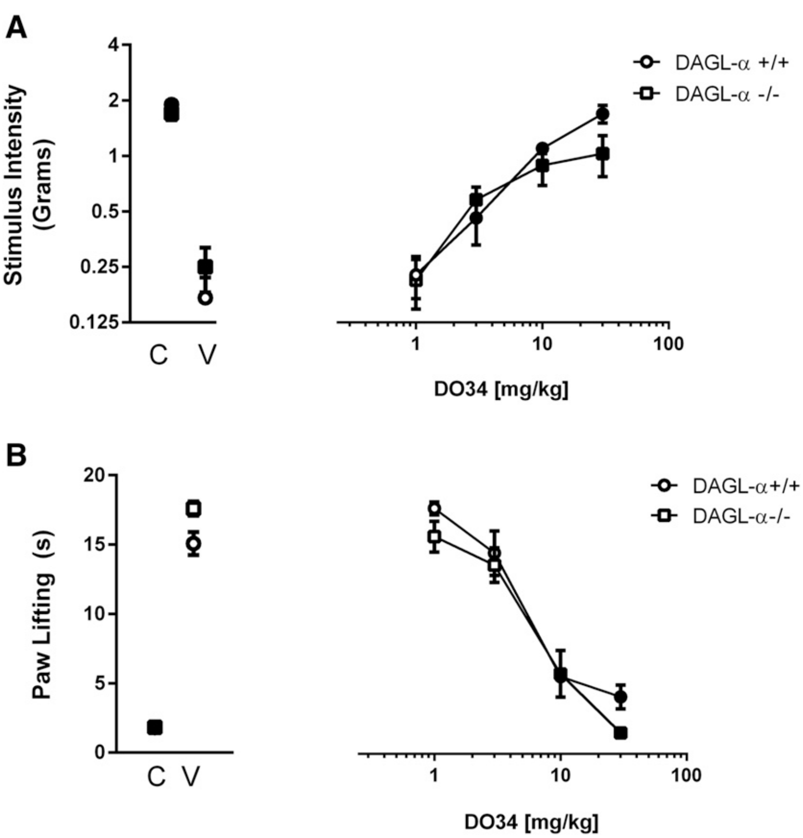

Fig. 6. The role of DAGL-α inhibition in the mediation of DO34 reversal of LPS-induced mechanical and cold allodynia. (A) LPS-treated DAGL-α (−/−) mice normally develop mechanical allodynia. DO34 (30 mg/kg) reverses LPS-induced mechanical allodynia in DAGL-α (+/+) and (−/−) mice. There is no significant shift in the dose-response curve due to genotype. (B) LPS-treated DAGL-α (−/−) mice develop cold allodynia. DO34 (30 mg/kg) reverses LPS-induced cold allodynia in DAGL-α (+/+) and (−/−) mice. There is no significant shift in the dose-response curve due to genotype. C = Non-LPS injected control paw, V = LPS-injected paw with vehicle treatment. N = 6 mice/group. Data reflect the mean ± S.E.M. Filled circles = $P < 0.05$ vs. LPS + vehicle in the respective genotype paw.

Authorship Contributions

Participated in research design: Wilkerson, Donvito, Grim, and Lichtman.

Conducted experiments: Wilkerson, Grim, and Abdullah.

Contributed new reagents or analytic tools: Ogasawara and Cravatt.

Performed data analysis: Wilkerson, Grim, and Abdullah.

Wrote or contributed to the writing of the manuscript: Wilkerson and Lichtman.

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