Implication of the anti-inflammatory bioactive lipid prostaglandin D2-glycerol ester in the control of macrophage activation and inflammation by ABHD6

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Edited by Charles N. Serhan, Brigham and Women's Hospital /Harvard Medical School, Boston, MA, and accepted by the Editorial Board September 5, 2013 (received for review July 24, 2013)

Proinflammatory macrophages are key mediators in several pathologies; thus, controlling their activation is necessary. The endocannabinoid system is implicated in various inflammatory processes. Here we show that in macrophages, the newly characterized enzyme α/β-hydrolase domain 6 (ABHD6) controls 2-arachidonoylglycerol (2-AG) levels and thus its pharmacological effects. Furthermore, we characterize a unique pathway mediating the effects of 2-AG through its oxygenation by cyclooxygenase-2 to give rise to the anti-inflammatory prostaglandin D_2-glycerol ester (PGD_2-G). Pharmacological blockade of cyclooxygenase-2 or of prostaglandin D synthase prevented the effects of increasing 2-AG levels by ABHD6 inhibition in vitro, as well as the 2-AG–induced increase in PGD_2-G levels. Together, our data demonstrate the physiological relevance of the interaction between the endocannabinoid and prostanoid systems. Moreover, we show that ABHD6 inhibition in vivo allows for fine-tuning of 2-AG levels in mice, therefore reducing lipopolysaccharide-induced inflammation, without the characteristic central side effects of strong increases in 2-AG levels obtained following monoacylglycerol lipase inhibition. In addition, administration of PGD_2-G reduces lipopolysaccharide-induced inflammation in mice, thus confirming the biological relevance of this 2-AG metabolite. This points to ABHD6 as an interesting therapeutic target that should be relevant in treating inflammation-related conditions, and proposes PGD_2-G as a bioactive lipid with potential anti-inflammatory properties in vivo.

COX-2 | prostaglandin synthase | glyceryl prostaglandin | FAAH | anandamide

Macrophages are key players in innate and adaptive immune responses to bacterial infections or noxious agents. Their role during inflammatory processes is to eliminate the threat and protect the body (1, 2). Macrophages exhibiting an inflammatory phenotype secrete proinflammatory mediators and reactive oxygen and nitrogen species that influence the polarization of T-helper cells, further drive the inflammatory response, and activate various antimicrobial mechanisms (3). Under persistence of the proinflammatory phase, inflammation becomes chronic, thus deleterious (3). Macrophages are key mediators in the immunopathology of metabolic inflammation and autoimmune diseases such as inflammatory bowel disease (IBD) and rheumatoid arthritis (2, 4). Thus, in these pathologies, proinflammatory macrophage responses must be controlled and reduced.

The endocannabinoid 2-arachidonoylglycerol (2-AG) is involved in various (patho)physiological processes and exerts numerous beneficial actions (ranging from pain modulation to reduction of anxiety) (5–11). The activity of this bioactive lipid depends on its endogenous levels, tightly controlled by the 2-AG hydrolyzing enzymes (12). Monoacylglycerol lipase (MAGL) is thought to be the primary enzyme responsible for 2-AG metabolism. Although this has been proven in the brain, where MAGL controls around 80% of 2-AG hydrolysis, it remains unclear whether this occurs in other tissues (13, 14). Because of this control of brain 2-AG levels, inhibition of MAGL leads to increased central levels of 2-AG and therefore to undesirable psychotropic side effects due to activation of the CB_1 cannabinoid receptor (15). More recently, other enzymes have been implicated in 2-AG hydrolysis, such as the newly annotated enzyme α/β-hydrolase domain 6 (ABHD6) (16–19). Here, we demonstrate the anti-inflammatory effects of inhibiting ABHD6 and thus increasing 2-AG levels, without the side effects of MAGL inhibition. Classically, 2-AG binds to and activates two G-protein–coupled receptors, termed cannabinoid receptors 1 and 2 (CB_1 and CB_2); however, it may also activate the peroxisome proliferator-activated receptors (PPARs) (20). Alternatively, this endogenous lipid, like arachidonic acid, may also be oxidized by cyclooxygenase (COX) enzymes to produce prostaglandin glycerol esters (PG-Gs) (21). So far, the biological effects of these PG-Gs are not fully elucidated. In this study, we also demonstrate that following increased 2-AG levels in macrophages, COX-2–mediated production of PGD_2-G increases, resulting in the anti-inflammatory effects observed with 2-AG (Fig. S1). We also put forth, in vivo, the anti-inflammatory properties of PGD_2-G.

Significance

2-Arachidonoylglycerol (2-AG) is an endogenous bioactive lipid implicated in numerous (patho)physiological processes. 2-AG classically activates the cannabinoid receptors, and its activity is terminated by enzymatic hydrolysis. The main enzyme studied in this context is monoacylglycerol lipase (MAGL). Although its inhibition, to increase 2-AG levels, has proven beneficial, it is hindered by psychotropic side effects due to drastically elevated brain 2-AG. Here we show the anti-inflammatory effects of inhibition of another 2-AG hydrolase, α/β-hydrolase domain 6, without the side effects associated with MAGL inhibition. We also show that 2-AG decreases macrophage activation and this effect is not mediated by its classical receptors. Furthermore, we demonstrate that a cyclooxygenase-2–derived metabolite of 2-AG, prostaglandin D2-glycerol ester, is responsible for the documented effects.

Author contributions: M.A. and G.G.M. designed research; M.A., J.M., and G.G.M. performed research; G.G.M. contributed new reagents/analytic tools; D.M.L. and P.D.C. provided reagents; M.A., J.M., and G.G.M. analyzed data; and M.A., J.M., P.D.C., D.M.L., and G.G.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. C.N.S. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314017110/-/DCSupplemental.

Results

ABHD6, but Not MAGL, Inhibition Reduces Macrophage Activation. Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is a potent macrophage activator (22). Macrophages activated with LPS release several proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and produce nitric oxide (Fig. S2*A*) and prostaglandins (PGs).

To assess the implication of ABHD6 in 2-AG metabolism by macrophages, J774 macrophages incubated with increasing doses of the selective ABHD6 inhibitor WWL70 (17, 23, 24) were stimulated with LPS. WWL70 induced a dose-dependent increase in 2-AG levels (Fig. 1*A*), with an EC_{50} of 0.3 μM. Increasing exogenous or endogenous 2-AG levels by exposing the cells to increasing concentrations of 2-AG and WWL70 resulted in a dose-dependent decrease of IL-1β mRNA expression (Fig. 1*B*), with an IC_{50} of the same order of magnitude for the two compounds: 6 μM for 2-AG and 1 μM for WWL70. Additionally, 2-AG did not decrease macrophage activation when the cells were assessed at 24 h, whereas WWL70 retained its efficacy (Fig. S3*A*). This probably is a result of the lower stability of 2-AG in the medium or its hydrolysis by ABHD6 in the absence of the inhibitor, thus strengthening the rationale to use enzyme inhibitors to increase this bioactive lipid's levels and make use of its therapeutic potential. The effect of WWL70 and 2-AG was further confirmed when the compounds were added 2 h post activation of the cells with LPS (Fig. 1*C*), again with WWL70 retaining its efficacy at 24 h (Fig. S3*B*). The relevance of using mRNA expression of IL-1β to assess LPS-induced macrophage activation was supported by ELISA (Fig. S3*C*). Moreover, the ABHD6 inhibitor decreased the production of arachidonic acid-derived PGs (PGD_2, PGJ_2, and PGE_2) (Fig. 1*D*) as well as nitric oxide (Fig. 1*E*). Interestingly, the selective MAGL inhibitor JZL184 (15) (10 μM) had no effect in this setting (Fig. 1*F*), which might be a result of the fact that JZL184 did not increase 2-AG levels in J774 macrophages (Fig. 1*G*). However, the JZL184 concentration used is sufficient to inhibit MAGL and increase 2-AG levels, because when assessed in the melanoma cell line B16, JZL184, even at 1 μM, induced a fourfold increase in 2-AG levels (Fig. 1*G*). mRNA expression of MAGL and ABHD6 in J774 cells delivered an explanation for this lack of effect of the MAGL inhibitor, because there was practically no measurable MAGL mRNA in these cells, as shown by the corresponding amplification plots and melt curves (Fig. S4 *A–C* and *J*).

The results obtained in J774 cells were confirmed further in primary peritoneal macrophages, a more physiological model, as well as in other macrophage-like cell lines, such as RAW264.7 cells and the BV2 microglial cell line. Indeed, treatment of these cells with WWL70 significantly increased 2-AG levels (Fig. 1*H* and Fig. S5*A*), thus leading to a decrease in LPS-induced macrophage activation (Fig. 1 *I* and *J* and Fig. S5 *B* and *D*), as measured by mRNA expression of IL-1β as well as PG production. Quantitative PCR (qPCR) for mRNA expression of MAGL and ABHD6 confirmed the absence of MAGL in the RAW264.7 cells, as well as the presence of ABHD6 (Fig. S4 *D–F*).

The Effect of 2-AG and ABHD6 Inhibition Is Independent of Cannabinoid Receptors or PPARs. 2-AG is an agonist at both the CB_1 and CB_2 cannabinoid receptors (20). To determine whether the effect of 2-AG on LPS-induced macrophage activation is CB_1/CB_2 dependent, we used selective antagonists of these receptors, namely SLV319 (SLV, 1μM) and SR141716A (SR1, 1μM) for CB_1 and SR144528 (SR2, 1μM) for CB_2. Coincubation of these antagonists with either 2-AG or WWL70 in the J774 and RAW264.7 cell lines did not block their effect on macrophage activation (Figs. S3*D* and S5*E*). To further confirm that the decrease observed following ABHD6 inhibition is not cannabinoid receptor dependent, we used two potent CB_1/CB_2 agonists, HU210 (100 nM) and CP55,940 (100 nM) (20). Both agonists had no effect on LPS-induced expression of IL-1β (Fig. S3*E*). Moreover, fatty acid amide hydrolase inhibition with the highly selective inhibitor PF750 (10 μM) (25) did not result in a decrease of LPS-induced macrophage activation, despite an increase in levels of the other major endocannabinoid, anandamide (AEA; Fig. S3*F*). Of note, AEA activates both cannabinoid receptors, as well as the PPARs (20). 2-AG also has been shown to activate the PPAR receptors (26), which play important roles in macrophage activation and function (22, 27). Thus, we used MK886 (10 μM) and T0070907 (3 μM), selective antagonists of PPAR-α and PPAR-γ, respectively (28, 29), to determine whether the effect of 2-AG and WWL70 is PPAR dependent. In both cell lines, the antagonists did not block the 2-AG–induced decrease in macrophage activation (Figs. S3*G* and S5*F*).

The Effect of ABHD6 Inhibition Is COX-2 and PGD Synthase Dependent. Another feature of macrophage activation by LPS is induction of COX-2; indeed, COX-2 mRNA expression is induced by LPS in the macrophage cell lines studied (Figs. S5*C* and S6 *A* and *B*).

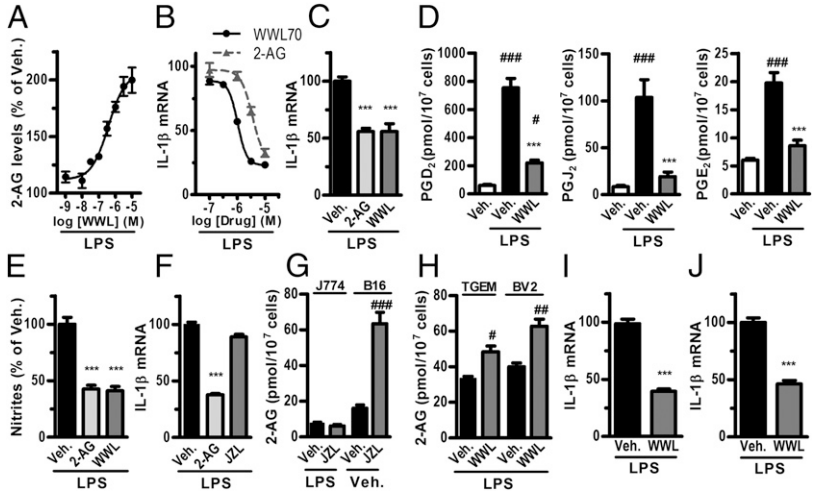

Fig. 1. ABHD6 but not MAGL inhibition increases 2-AG levels and reduces macrophage activation. Macrophages were activated by incubation with LPS (100 ng/mL) for 8 h. (*A*) The ABHD6 inhibitor WWL70 (WWL) dose-dependently increases 2-AG levels. (*B*) 2-AG and WWL70 dose-dependently decrease LPS-induced IL-1β mRNA expression. (*C*) 2-AG (10 μM) and WWL70 (10 μM), added 2 h post LPS, reduce IL-1β mRNA expression. (*D*) ABHD6 inhibition (WWL, 10 μM) decreases LPS-induced PG production in J774 cells. (*E*) Incubation with 2-AG (10 μM) or WWL (10 μM) decreases LPS-induced NO production by J774 cells. (*F*) The MAGL inhibitor JZL184 (JZL, 10 μM) does not reduce LPS-induced expression of IL-1β in J774 cells. (*G*) Following MAGL inhibition, 2-AG levels are not increased in J774 cells (JZL, 10 μM) but are increased in B16 melanoma cells (JZL, 1 μM). (*H–J*) ABHD6 inhibition (WWL, 10 μM) (*H*) increases 2-AG levels in thioglycolate-elicited peritoneal macrophages (TGEM) and BV2 microglial-like cells and (*I* and *J*) decreases IL-1β mRNA expression in LPS-stimulated (*I*) TGEM and (*J*) BV2 cells. Experiments were performed at least three times in triplicate. Compounds were added 1 h before LPS unless otherwise specified. Except for *D*, *G*, and *H*, the effect of LPS on DMSO-treated cells (Veh.) is set at 100%. Values are mean ± SEM. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ for treatments vs. Veh. in LPS-untreated control cells. ***, $P < 0.001$ for treatments vs. Veh. in the presence of LPS.

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2-AG, because of its arachidonic acid moiety, is also a substrate of this enzyme. When oxidized by COX-2, 2-AG is transformed *in fine* into PG-Gs (21). These PG-Gs are thought to have distinct receptors from the corresponding PGs, and their functions are not yet fully elucidated (21). We therefore sought to inhibit COX-2 to test whether it is involved in the effect of ABHD6 inhibition. The 2-arylpropionic acid derivatives or "profens" are COX inhibitors that inhibit arachidonic acid conversion into PGs. Profens are widely used anti-inflammatory agents and therefore cannot be used to block the anti-inflammatory effect of ABHD6 inhibition. However, only their *S* enantiomer is active at blocking arachidonic acid oxygenation, whereas recently it was shown that their *R* enantiomer selectively inhibits COX-2–mediated oxygenation of 2-AG without affecting arachidonic acid metabolism (30). Therefore, we used *R*-flurbiprofen to selectively block the oxygenation of 2-AG by COX-2. Here, we show that *R*-flurbiprofen, in a dose-dependent manner, blocks the effect of ABHD6 inhibition on LPS-induced expression of IL-1β by J774 macrophages (Fig. 2*A*) and thioglycolate-elicited peritoneal macrophages (Fig. 2*B*). Moreover, the fact that *R*-flurbiprofen increased 2-AG levels (Fig. 2*C*) while having no effect per se on macrophage activation (Fig. 2*A*) confirms that, in this model, the effect of 2-AG is mediated by its metabolism by COX-2 into PG-Gs.

Further confirming the implication of COX-2 is the requirement of an arachidonic acid moiety, because 1-arachidonoylglycerol also slightly reduced the LPS-induced expression of IL-1β, whereas other monoacylglycerols that can be hydrolyzed by ABHD6, such as oleoylglycerol and palmitoylglycerol, have no effect (Fig. 2*D*). Moreover, arachidonic acid (10 μM) had no effect on LPS-induced IL-1β expression (Fig. S6*E*), thus confirming that although COX-2 dependent, the effects of increased 2-AG levels are not the result of its hydrolysis, by another lipase, into arachidonic acid but rather the result of its oxygenation by COX-2.

Because the 2-AG–mediated reduction in macrophage activation is probably a result of its conversion to PG-Gs, we tested the effect of several PG-Gs, namely PGE_2-G, $PGF_{2\alpha}$-G, and PGD_2-G, in the same setting. PGE_2-G and $PGF_{2\alpha}$-G at 10 μM further increased LPS-induced expression of IL-1β (Fig. 2*F*), thus further inducing macrophage activation, in a dose-dependent manner (Fig. S6*F*). However, PGD_2-G (10 μM) profoundly decreased LPS-induced macrophage activation, as measured by proinflammatory cytokine production in the J774 (IC_{50} 3 μM) (Fig. 2*G* and Fig. S6*G*), RAW264.7, and BV2 cell lines (Fig. S6*H*), as well as nitric oxide production in J774 cells (Fig. S6*I*). This suggests that the effect of 2-AG and ABHD6 inhibition might be a result of the conversion of 2-AG into PGD_2-G. The effect of PGD_2-G was not a result of its conversion into PGD_2, because the latter had no effect on LPS-induced IL-1β expression in macrophages (Fig. S6*J*). BWA868c and CAY10471, antagonists of the DP1 and DP2 receptors, respectively (29), did not reverse the effect of WWL70 or PGD_2-G, consistent with the fact that PG-Gs bind to as-yet unknown receptors, distinct from the prostanoid receptors (Fig. 2*H* and Fig. S6*K*) (21, 31). To further confirm the implication of the PGD_2-G pathway we used an inhibitor of PGD synthase, HQL79, which completely blocked the LPS-induced production of PGD_2-G (Fig. 2*I*) and dose-dependently blocked the beneficial effect of ABHD6 inhibition and 2-AG on LPS-induced macrophage activation (Fig. 2*E*). PGE_2-G levels were not affected by HQL79, thus confirming its selectivity (Fig. 2*J*). Moreover, incubation of LPS-activated J774 cells with 2-AG further increased production of PGD_2-G, which was blocked by coincubation with *R*-flurbiprofen or HQL79 (Fig. 2*K*), thus strengthening the implication of PGD_2-G in the effects of 2-AG.

Anti-inflammatory Effects of ABHD6 Inhibition in Vivo and Potential Involvement of COX-2 and Cannabinoid Receptors. We next asked whether ABHD6 inhibition exerts anti-inflammatory actions in vivo. Mice were administered LPS (300 μg/kg, i.p.) and killed 4 h later. The effect of ABHD6 inhibition was assessed by measuring mRNA expression of proinflammatory cytokines. LPS administration, at a dose relevant to what is observed in metabolic endotoxemia (32) and colitis (5), resulted in a strong increase of the proinflammatory cytokines IL-1β, IL-6, and monocyte chemoattractant protein-1 in the cerebellum, liver, and lungs compared with mice receiving vehicle only (Fig. 3 *A* and *C* and Fig. S7 *A*–*C*). ABHD6 inhibition resulted in a significant reduction of these proinflammatory cytokines (Fig. 3 *A* and *C* and Fig. S7 *A*–*C*).

2-AG levels generally were unaltered following LPS administration and increased in the lung and liver following ABHD6 inhibition (Fig. 3*D* and Fig. S7*D*). It is noteworthy that 2-AG levels were not increased in the cerebellum (Fig. 3*B*), even

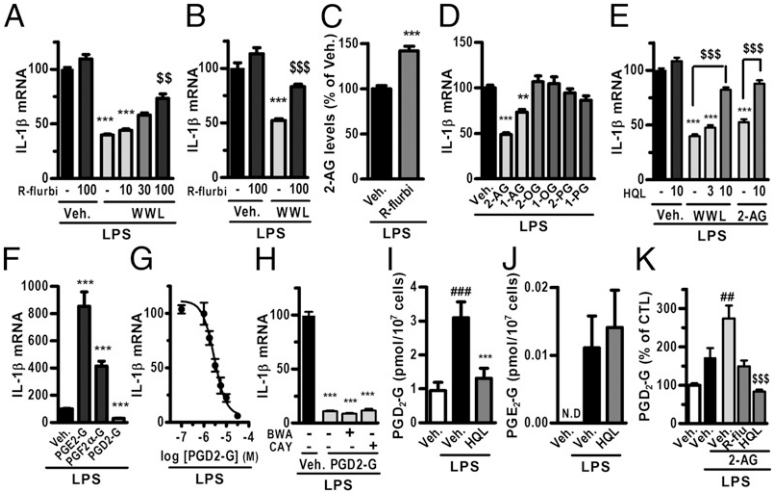

Fig. 2. The effect of ABHD6 inhibition on macrophage activation is COX-2 and PGD synthase dependent. Macrophages were activated by incubation with LPS (100 ng/mL) for 8 h. (*A* and *B*) *R*-flurbiprofen (*R*-flurbi; 10–100 μM) blocks the effect of ABHD6 inhibition (WWL, 10 μM) on LPS-induced IL-1β mRNA expression in (*A*) J774 macrophages and (*B*) thioglycolate-elicited peritoneal macrophages. (*C*) *R*-flurbiprofen (100 μM) increases 2-AG levels. (*D*) Only arachidonic acid-derived monoacylglycerols affect LPS-induced IL-1β expression. PG, palmitoylglycerol; OG, oleoylglycerol. (*E*) The PGD synthase inhibitor HQL79 (HQL, 3–10 μM) dose-dependently blocks the effect of WWL (10 μM) and 2-AG (10 μM) on IL-1β expression. (*F*) PGD_2-G (10 μM) decreases LPS-induced IL-1β, whereas PGE_2-G and $PGF_{2\alpha}$-G (10 μM) have the opposite effect in J774 cells. (*G*) Dose-dependent effect of PGD_2-G on LPS-induced IL-1β expression in J774 cells. (*H*) The effect of PGD_2-G on LPS-induced IL-1β mRNA expression in macrophages is not blocked by antagonists of the PGD_2 receptors: DP1 (BWA686c, 1 μM) and DP2 (CAY10471, 1 μM). (*I* and *J*) In J774 cells, LPS increases (*I*) PGD_2-G and (*J*) PGE_2-G levels (measured by HPLC-MS) compared with control cells. The PGD synthase inhibitor HQL79 (10 μM) (*I*) blocks PGD_2-G production and (*J*) has no effect on PGE_2-G production. (*K*) PGD_2-G production is increased further when 2-AG (0.1 μM) is added to the cells and decreased by coincubation with *R*-flurbiprofen (100 μM) or HQL79 (10 μM). Experiments were performed at least three times in triplicate. Compounds were added 1 h before LPS unless otherwise specified. Except for *I*–*K*, the effect of LPS on DMSO-treated cells (Veh.) is set at 100%. Values are mean ± SEM. ##, $P < 0.01$; ###, $P < 0.001$ for treatments vs. Veh. in LPS-untreated control cells. **, $P < 0.01$; ***, $P < 0.001$ for treatments vs. Veh. in the presence of LPS; $$, $P < 0.01$; $$$, $P < 0.001$ for antagonists/inhibitors vs. treatment (WWL or 2-AG).

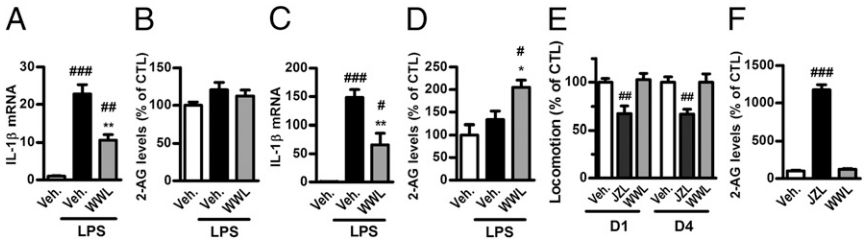

Fig. 3. ABHD6 inhibition in vivo reduces LPS-induced inflammation. (*A–D*) Inflammation was induced in C57BL/6 mice (seven mice per group) by i.p. administration of LPS (300 μg/kg) to mice treated with vehicle (Veh.) or WWL70 (WWL, 20 mg/kg) 2 h before LPS. Control mice (Veh.) received i.p. injections of the corresponding vehicles. (*A* and *C*) ABHD6 inhibition (WWL) reduced the LPS-induced increase in proinflammatory cytokines in the (*A*) cerebellum and (*C*) lung. (*B* and *D*) In the same experiment, ABHD6 inhibition increased 2-AG levels in (*D*) the lung but not in (*B*) the cerebellum. (*E* and *F*) C57BL/6 mice (10 mice per group) received i.p. injections of vehicle (Veh.), JZL184 (JZL, 20 mg/kg), or WWL70 (WWL, 20 mg/kg) once a day for 4 d. Locomotor activity was assessed 4 h after the injection on day 1 and on day 4. (*E*) MAGL inhibition (JZL) led to a decrease in locomotion compared with the control group (Veh.) on both days 1 and 4, whereas ABHD6 inhibition (WWL) had no effect. (*F*) 2-AG levels on day 4 were increased in the brain following MAGL inhibition (JZL), but not ABHD6 inhibition (WWL). Values are mean ± SEM. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ vs. control mice receiving only vehicle (Veh.) and no LPS. *, $P < 0.05$; **, $P < 0.01$ for treatments vs. Veh., both in the presence of LPS.

though a strong reduction in inflammation was observed in this tissue. Therefore, this anti-inflammatory effect in the cerebellum might be a consequence of the systemic anti-inflammatory effect of ABHD6 inhibition in the periphery. The lack of effect of ABHD6 inhibition on 2-AG levels in the cerebellum might be explained by the prevalent role exerted by MAGL on brain 2-AG hydrolysis (13, 14). Therefore, ABHD6 inhibition might be a way to increase peripheral 2-AG levels and, consequently, to exert an anti-inflammatory effect without the central side effects of MAGL inhibition. To verify this hypothesis, we evaluated the locomotor activity of mice receiving either WWL70 (20 mg/kg, i.p.) or JZL184 (20 mg/kg, i.p.). Locomotor activity was assessed on day 1, 4 h following administration of the enzyme inhibitors, and 4 d later, following repeated daily injections of the inhibitors. In both cases, mice receiving JZL184 exhibited significant hypomotility, whereas mice receiving WWL70 were not different from control mice (Fig. 3*E*). Here also, ABHD6 inhibition did not increase brain 2-AG levels, whereas MAGL inhibition induced a 10-fold increase (Fig. 3*F*), thus shedding light on the difference observed in locomotor activity. Thus, we confirm that ABHD6 controls 2-AG levels in vivo, leading to anti-inflammatory effects without the central side effects of MAGL inhibition, and therefore might be a more suitable therapeutic target for chronic inflammation.

In a second set of experiments, we sought to further dissect the mechanisms responsible for this anti-inflammatory effect of ABHD6 inhibition. Indeed, we found that in macrophages, the effects of 2-AG were largely the result of its COX-2 metabolite PGD_2-G. However, the story is likely more complex in vivo where the expression of receptors and enzymes is different between tissues, and where the actions of 2-AG could be mediated by several pathways. Mice receiving LPS were administered WWL70 (20 mg/kg) with or without the substrate-selective COX-2 inhibitor *R*-flurbiprofen (5 mg/kg), the CB_1 antagonist SR1 (3 mg/kg), and the CB_2 antagonist AM630 (10 mg/kg) (5). We found that the effects of ABHD6 inhibition on LPS-induced systemic inflammation were blocked in part by COX-2 inhibition in the spleen, liver, and cerebellum (Fig. 4 *A–D*) and in part by CB_1 antagonism in the liver (Fig. 4 *C* and *D*). This finding suggests that the effects of ABHD6 inhibition in vivo might be mediated, at least in part, by its COX-2 metabolite.

PGD_2-G Exerts Anti-inflammatory Effects in Vivo. In light of the in vitro effects of PGD_2-G and its possible involvement in the effects of 2-AG in vivo, we thought to assess its anti-inflammatory potential in vivo. PGD_2-G (20 mg/kg) administration reduced the LPS-induced increase in spleen weight and proinflammatory cytokine expression in the liver and, to a lesser extent, in the cerebellum (Fig. 4 *E–H*).

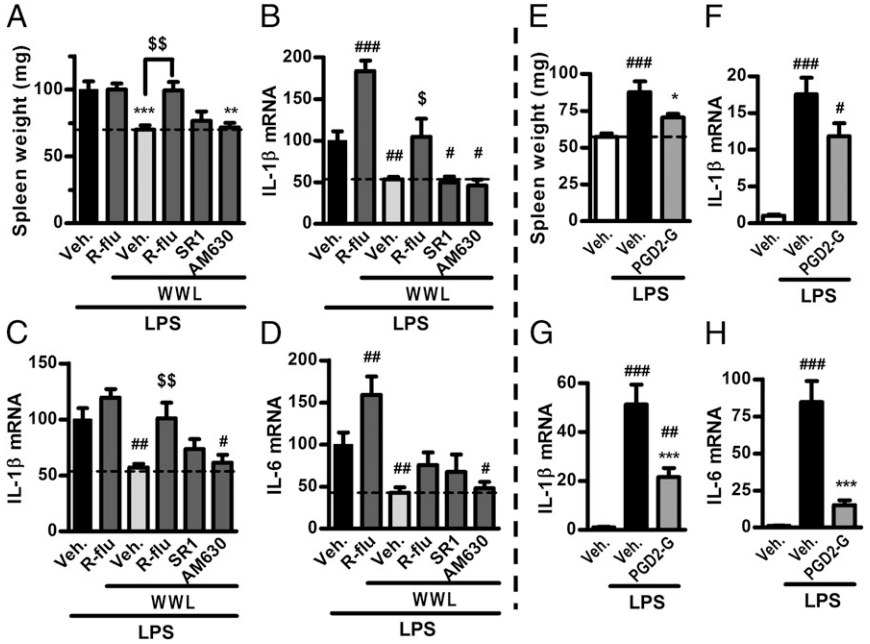

Fig. 4. PGD_2-G reduces LPS-induced inflammation in vivo. Inflammation was induced in C57BL/6 mice (seven mice per group) by i.p. administration of LPS (300 μg/kg). WWL70 (WWL, 20 mg/kg), the CB_1 antagonist SR1 (3 mg/kg), and the CB_2 antagonist AM630 (10 mg/kg) were administered 2 h before LPS. The substrate-selective COX-2 inhibitor *R*-flurbiprofen (R-flu, 5 mg/kg) and PGD_2-G (20 mg/kg) were administered 30 min before LPS. (*A*) Spleen weight is reduced by WWL administration to inflamed mice, and this effect is blocked by *R*-flurbiprofen but not CB_1 or CB_2 antagonism. (*B–D*) LPS-induced proinflammatory cytokine production in the (*B*) cerebellum and (*C* and *D*) liver is decreased by WWL. (*B*) *R*-flurbiprofen leads to a further increase in LPS-induced IL-1β expression in the cerebellum and blocks the effect of WWL. (*C* and *D*) COX-2 inhibition and CB_1 antagonism block, in part, the effects of WWL on LPS-induced (*C*) IL-1β and (*D*) IL-6 expression in the liver. (*E–H*) LPS administration leads to increased (*E*) spleen weight and proinflammatory cytokines expression in the (*F*) cerebellum and (*G* and *H*) liver, which are decreased by PGD_2-G. For *A–D*, the effect of LPS (Veh.) is set at 100%. Values are mean ± SEM. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ for treatments vs. Veh. in LPS-untreated control cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for treatments vs. Veh. in the presence of LPS. $, $P < 0.05$; $$, $P < 0.01$ for antagonists/inhibitors vs. WWL.

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Discussion

Proinflammatory macrophages are an essential component of the host defense mechanisms against bacteria or other microorganisms. However, when inflammation is sustained, proinflammatory cytokines, as well as reactive oxygen and nitrogen species, may cause extensive damage to the host (2, 3). Proinflammatory macrophages have been implicated in several autoimmune diseases, such as IBD, in which LPS, a potent macrophage activator, also plays a pathogenic role. The increased intestinal epithelial permeability in IBD leads to endotoxemia, which in turn is responsible for extra-intestinal manifestations of these diseases (5, 33). Moreover, in obesity, the switch from an alternatively activated phenotype to classically activated macrophages infiltrating the adipose tissue is associated with insulin resistance. In this context, chronic low-grade inflammation due to low circulating levels of LPS (32) and mediated by innate and adaptive immune cells constitutes a pathogenic link between obesity and chronic metabolic diseases, such as type 2 diabetes and coronary artery disease (4, 34). Thus, proinflammatory macrophages seem to be key mediators in several pathologies.

Our data uncover a role for ABHD6 in controlling 2-AG levels in vivo. The relevance of this enzyme is strengthened by the fact that its inhibition induces anti-inflammatory effects in mice. 2-AG exerts a plethora of effects in vivo, all of which were studied by increasing its levels through MAGL inhibition (5–9). Indeed, until recently, data pointed to MAGL as the primary enzyme responsible for 2-AG hydrolysis, at least in the brain (13, 14). The role of ABHD6 in 2-AG hydrolysis was first put forth in BV2 cells that did not express MAGL (16) and subsequently in neurons (17, 18). Here, we also show that the J774 and RAW264.7 macrophage cell lines do not express MAGL and that ABHD6 inhibition leads to increased 2-AG levels in these cells and reduces their activation. However, the role of ABHD6 is not restricted to cell lines or tissues that do not express MAGL. We found that primary peritoneal macrophages express both MAGL and ABHD6 (Fig. S4 *G–I*) and that ABHD6 inhibition in these freshly isolated macrophages led to increased 2-AG levels, thus strengthening the role of ABHD6 in the control of 2-AG levels and macrophage activation in a more physiological model compared with cell lines in culture. More interestingly, ABHD6 inhibition increased 2-AG levels in vivo in the liver and lung, two tissues in which MAGL is known to be expressed and to play an important role in 2-AG hydrolysis (35, 36). An important aspect of ABHD6 inhibition is the lack of increased 2-AG levels in the brain. Indeed, MAGL inhibition, although an efficient means to increase 2-AG levels in vivo, generally is hindered by two important side effects: the cannabinoid behavioral effects it provokes as well as functional antagonism of the endocannabinoid system following chronic MAGL inhibition (15, 37). Thus, fine-tuning 2-AG levels in vivo might be more interesting than completely inhibiting its hydrolysis. We propose here that ABHD6 constitutes the means to this end, because its inhibition increases 2-AG levels in vivo and leads to anti-inflammatory effects but no cannabinoid behavioral effects. The lack of increased 2-AG levels in the brain might be imputed to a lack of penetration of the ABHD6 inhibitor; however, we show that WWL70 indeed is present in the brain (Fig. S8). Moreover, when comparing the effect of ABHD6 and MAGL inhibition on 2-AG levels, ABHD6 inhibition leads to a lesser increase of 2-AG levels than MAGL inhibition, even in the periphery (Fig. S7*E*). Thus, using ABHD6 as a target to increase 2-AG levels allows for a fine-tuning of 2-AG levels.

Another important aspect of our study is the characterization of the effects of PG-Gs on macrophages. 2-AG is an endocannabinoid, and it classically exerts its effects through activation of the cannabinoid receptors, CB_1 and CB_2. However, it also is a known substrate for the COX and lipoxygenase enzymes (19, 21, 38). Some of the effects of 2-AG have been shown to be mediated by some of its metabolites, such as arachidonic acid and its PG derivatives, or through the action of 2-AG on the PPARs (10, 19, 39–41). Although it is known that PG-Gs constitute COX-derived 2-AG metabolites, their effects are not well documented. Here, we show that in our macrophage cell lines in culture, ABHD6 inhibition provides an untapped 2-AG pool for the LPS-induced COX-2. Thus, the effect of 2-AG on macrophage activation is caused by one of its COX-2 metabolites, namely PGD_2-G, which reduces IL-1β expression through as-yet unidentified receptors. We also show that PGD_2-G reduces LPS-induced inflammation in mice, thus implicating this bioactive lipid in an inflammatory setting in vivo. Furthermore, we show that two other PG-Gs, PGE_2-G and $PGF_{2\alpha}$-G, have the opposite effect on macrophage activation, thus leading to a further increase in IL-1β production. The latter effect seems consistent with a report that PGE_2-G induces hyperalgesia in vivo (31). The question arising from these observations is why the effects of 2-AG on macrophage activation are mediated by PGD_2-G and not one of the other PG-Gs. One possible explanation is that in these cell lines, the activity of the PGD synthase is higher than the activity of the PGE synthase. Indeed, we and others (42) show that these cell lines produce significantly more PGD_2 than PGE_2 in basal conditions and when stimulated with LPS (Fig. 1*D* and Fig. S5*D*). PG synthases compete for the same substrate (arachidonic acid-derived PGH_2) (43); therefore, because PGD_2 synthesis is significantly higher than PGE_2 synthesis, we can infer that there is more PGD synthase activity than PGE synthase activity in these cells upon activation by LPS. Accordingly, we found higher amounts of PGD_2-G than PGE_2-G (Fig. 2 *I* and *J*).

Although the picture seems simple in vitro, it is quite more complicated in vivo, where the expression of enzymes and receptors is different between tissues. The effect of ABHD6 inhibition in vivo might be the result of a direct action of 2-AG on the cannabinoid receptors or the PPARs, and/or to a metabolite of 2-AG. In preliminary studies, we show that the effects of ABHD6 inhibition might be mediated by COX-2 metabolism, CB_1 activation, or both, depending on the tissue. More studies are warranted to dissect these mechanisms further.

In conclusion, we put forth the relevance of ABHD6 in increasing 2-AG levels in vivo to exert potential therapeutic effects on inflammation, without the inconveniences of chronic MAGL inhibition, and we also uncover the anti-inflammatory effects of a COX-2 metabolite of 2-AG, PGD_2-G.

Methods

Cell Cultures. Murine macrophage cell lines were activated by incubation with LPS for 8 h. Unless specified otherwise, compounds were added 1 h before LPS and a control condition was performed.

Real-Time qPCR. Following total RNA extraction with TriPure reagent, cDNA was synthesized by using a reverse transcription kit (Promega) from 1 μg of total RNA, and qPCR was performed with a StepOnePlus instrument and software (Applied Biosystems) as previously described (5). Primers sequences are given in Table S1.

2-AG and AEA Quantification. Lipids were extracted and purified in the presence of deuterated standards, as previously described (16, 44). The resulting lipid fraction was analyzed by HPLC-MS (LTQ Orbitrap MS coupled to an Accela HPLC system) (45).

PG and PG-G Quantification. Lipids were extracted, directly (PG-Gs) or following acidification (PGs), in the presence of d_4-PGE_2 and d_5-2-AG. The lipid extracts were purified by solid phase extraction using silica and elution with a $CHCl_3$–MeOH (8:2) solution (46). The fractions were analyzed by HPLC-MS (LTQ Orbitrap MS coupled to an Accela HPLC system). PGs and PG-Gs were quantified by isotope dilution using d_4-PGE_2 and d_5-2-AG, respectively, as an internal standard. The ions used for PG-G detection are described in Fig. S9.

Cytokine Quantification by ELISA. Concentrations of IL-1β in supernatants were determined by using the Ready-Set-Go! Kit following the manufacturer's instructions (eBioscience).

Nitrite/Nitrate Measurement. Concentrations of nitric oxide in supernatants were determined by measuring nitrite/nitrate accumulation in the medium with a colorimetric assay kit (Cayman Chemical).

Animals. Mice were housed under standard conditions and supplied with water and food ad libitum. Protocols were approved by the animal committee of Université catholique de Louvain (UCL/MD/2009–010).

Primary Peritoneal Macrophage Isolation. Murine peritoneal macrophages were obtained by eliciting an acute peripheral inflammatory reaction with an i.p. injection of thioglycolate (47).

In Vivo Studies. In LPS experiments, compound or vehicle was administered i.p. once, 2 h (WWL70, SR1, AM630) or 30 min (PGD_2-G, *R*-flurbiprofen) before LPS administration (300 μg/kg, i.p.). Four hours after LPS, mice were killed by cervical dislocation. For locomotor activity assessment, mice received WWL70 (20 mg/kg), JZL184 (20 mg/kg), or vehicle, i.p. once daily for 4 d.

Statistical Analysis. Results are expressed as mean ± SEM. We determined significance between two groups by unpaired Student *t* test and comparisons among several groups by one-way or two-way ANOVA followed by the Bonferroni posttest, or the Kruskal–Wallis nonparametric test followed by the Dunn posttest.

ACKNOWLEDGMENTS. We thank T. Timmerman and B. Buisseret for their technical assistance. P.D.C. is a research associate of the Fonds de la Recherche Scientifique (FRS-FNRS), Belgium. G.G.M. is the recipient of research grants from the Université Catholique de Louvain (FSR) and FRS-FNRS, Belgium (Grants CC 1.5034.10F, FRFC 2.4555.08, and FRSM 3.4569.12).

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