Inhibition of DAGLβ as a therapeutic target for pain in sickle cell disease

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Received: December 13, 2021.
Accepted: April 28, 2022.
Early view: May 26, 2022.

https://doi.org/10.3324/haematol.2021.280460

Abstract

Sickle cell disease (SCD) is the most common inherited disease. Pain is a key morbidity of SCD and opioids are the main treatment but their side effects emphasize the need for new analgesic approaches. Humanized transgenic mouse models have been instructive in understanding the pathobiology of SCD and mechanisms of pain. Homozygous (HbSS) Berkley mice express >99% human sickle hemoglobin and several features of clinical SCD including hyperalgesia. Previously, we reported that the endocannabinoid 2-arachidonoylglycerol (2-AG) is a precursor of the pro-nociceptive mediator prostaglandin E2-glyceryl ester (PGE_2-G) which contributes to hyperalgesia in SCD. We now demonstrate the causal role of 2-AG in hyperalgesia in sickle mice. Hyperalgesia in HbSS mice correlated with elevated levels of 2-AG in plasma, its synthesizing enzyme diacylglycerol lipase β (DAGLβ) in blood cells, and with elevated levels of PGE_2 and PGE_2-G, pro-nociceptive derivatives of 2-AG. A single intravenous injection of 2-AG produced hyperalgesia in non-hyperalgesic HbSS mice, but not in control (HbAA) mice expressing normal human HbA. JZL184, an inhibitor of 2-AG hydrolysis, also produced hyperalgesia in non-hyperalgesic HbSS or hemizygous (HbAS) mice, but did not influence hyperalgesia in hyperalgesic HbSS mice. Systemic and intraplantar administration of KT109, an inhibitor of DAGLβ, decreased mechanical and heat hyperalgesia in HbSS mice. The decrease in hyperalgesia was accompanied by reductions in 2-AG, PGE_2 and PGE_2-G in the blood. These results indicate that maintaining the physiological level of 2-AG in the blood by targeting DAGLβ may be a novel and effective approach to treat pain in SCD.

Introduction

Pain is a characteristic feature of sickle cell disease (SCD).[1,2] Patients experience acute and chronic pain which may be associated with hemolysis, vaso-occlusion, vasculopathy, ischemia-reperfusion injury, organ damage, neuropathy and persistent inflammation.[3-5] Opioids are typically used to treat pain in SCD[6] but are associated with increased symptom burden, depression and utilization of healthcare.[7,8] New, effective and safe treatments are needed to manage pain in SCD.

Transgenic Berkley (BERK) and Townes mouse models of SCD expressing >99% human sickle hemoglobin exhibit hyperalgesia and have provided valuable information on mechanisms underlying pain in SCD.[9,10] Inflammatory mediators such as prostaglandins, cytokines, interleukins and nerve growth factor are released from immune cells and endothelial cells[11-13] and contribute to hyperalgesia by exciting and sensitizing primary afferent nociceptors.[12,14] Importantly, many of these and other inflammatory mediators are increased in the blood of patients with SCD[15] and in murine models of SCD.[9,16,17] Targeting peripheral mechanisms that underlie nociceptor sensitization in SCD may provide a safe and effective approach for managing pain in these patients without the undesirable side effects of opiates.

The endocannabinoid 2-arachidonoylglycerol (2-AG) is an important pain modulator that has both anti- and pro-nociceptive effects.[18,19] The reduction in pain has been attributed to suppression of inflammation as well as direct effects on nociceptors and targets within the central nervous system.[20] In some pathological conditions, inhibitors of monoacylglyerol lipase, the enzyme that hydrolyzes 2-AG to arachidonic acid and glycerol, increased the level of 2-AG and reduced hyperalgesia through mechanisms dependent on the cannaboid CB1 and CB2 receptors.[21-25] Although increasing endogenous 2-AG may seem attractive

as a strategy for managing pathological pain, 2-AG is also an intermediate in the production of pro-nociceptive lipids. 2-AG is a substrate for cyclooxygenase-2 (COX-2). This enzyme is induced in certain inflammatory conditions,[26] including SCD.[17] Oxidation of 2-AG produces prostaglandin E2-glycerol (PGE_2-G), a highly potent pro-nociceptive lipid.[17,27] Hydrolysis of 2-AG by monoacylglyerol lipase also contributes to the metabolic pool of arachidonic acid, a precursor of multiple prostaglandins.

In the present study, we used a humanized transgenic murine model of SCD, the homozygous BERK mouse, to investigate whether hyperalgesia in SCD is associated with increased levels of circulating 2-AG and the enzyme most closely associated with its generation. In the periphery, 2-AG is synthesized from diacyglycerides by the β-isoform of diacylglycerol lipase (DAGLβ).[28-30] Because DAGLβ is upstream from monoacylglyerol lipase and COX-2 in the production of PGE_2 and PGE_2-G, we determined whether inhibition of DAGLβ reduces hyperalgesia in HbSS mice and whether the decrease is associated with a reduction of 2-AG and its related metabolites, PGE_2 and PGE_2-G. Our results show that 2-AG is an important intermediate in the synthesis of PGE_2 and PGE_2-G. The accumulation of 2-AG as a result of increased synthesis leads to an increase in the levels of pro-nociceptive lipids involved in the sensitization of nociceptors and pain in SCD. Targeting 2-AG synthesis may block pain at its source, thus contributing to prevention of hyperalgesia.

Methods

Mice

Male (5-9 months old), homozygous HbSS-BERK, HbAA-BERK and hemizygous HbAS mice were used (*Online Supplement*). All protocols were approved by the Institutional Animal Care and Use Committee.

Drugs

2-AG, anandamide (AEA), PGE_2-G, PGE_2, and their deuterated analogs 2-AG-*d5*, AEA-*d8*, PGE_2-G-*d5*, and PGE_2-*d4* were purchased from Cayman Chemical; stock solutions were prepared in ethanol (10 mg/mL). JZL184, a selective inhibitor of monoacylglyerol lipase, and KT182, an inhibitor of ABHD6,[31] were purchased from Cayman Chemical. KT109, an inhibitor of DAGLβ,[29,30] and KT195, an inhibitor of serine hydrolase ABHD6,[29,30] were purchased from Sigma-Aldrich. Stock solutions of enzyme inhibitors were prepared in dimethyl sulfoxide (DMSO, 10 mg/mL) and diluted to their final concentration in sterile saline with Tween 80.

Blood collection and analysis

Whole blood (0.5 mL) was collected into MiniCollect® EDTA Tubes (Greiner Bio-One). Blood cells were isolated from plasma by centrifugation for 10 min at 2,000 x g at 4°C. The pellet containing blood cells was used for western blot; the supernatant (i.e., plasma) was used for measurement of lipids. Samples were frozen in liquid nitrogen and stored at −80°C until processing. The amount of DAGLβ and COX-2 proteins in blood cell lysates was determined by western blot. Levels of 2-AG, AEA, PGE_2-G and PGE_2 were analyzed by liquid chromatography-nanoelectrospray tandem mass spectrometry. The specificity of the DAGLβ antibody was tested by knocking down DAGLβ in mouse fibrosarcoma cell clone NCTC 247237 with small interfering RNA (siRNA) specific for the DAGLβ gene. The specificity of the COX-2 antibody was tested by pre-incubation of the antibody with nickel resin (GE Healthcare) coated with a 10-fold molar excess of COX-2 His-tag protein purchased from R&D systems (*Online Supplement*).

Behavioral measures of hyperalgesia

Mechanical hyperalgesia was defined as a decrease in paw withdrawal threshold measured by the up-down method[32] or an increase in the frequency of paw withdrawal evoked by ten stimulations with a von Frey monofilament (Stoelting) with a bending force of 3.9 mN applied to each plantar hind paw (*Online Supplement*).[33,34] Heat hyperalgesia was defined as a decrease in the latency of paw withdrawal from radiant heat applied to each plantar hind paw.[35] Baseline measurements were taken over 3 days prior to each experiment. The withdrawal threshold, frequency of withdrawal responses and latency were averaged for both paws.

Statistical analyses

Data are presented as the mean ± standard error of the mean and were analyzed by one- and two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni *t* tests when normally distributed. Data are presented as the median with 95% confidence interval and compared using a nonparametric test when they did not meet the requirement of normality. The effective dose for 50% of the population (ED_{50}) was determined by non-linear regression analysis in Prism (GraphPad Software). Behavioral dose response data were initially converted to the percent of maximum possible effect (%MPE), which was calculated using the average response in the vehicle (V)-treated mice and the post-drug (PD) response in each KT109-treated HbSS mouse according to the equation:

$$\%MPE = (V\ HbSS - PD\ HbSS) / (V\ HbSS - V\ HbAA) \times 100\%$$

Results

Hyperalgesia in HbSS mice was accompanied by an increase in 2-AG in plasma

Consistent with previous reports,[9-11,17,20,36-38] the majority of

HbSS mice exhibited robust mechanical and heat hyperalgesia, and this was accompanied by an increase of 2-AG in plasma (Figure 1A-C). HbAS or HbSS-BERK sickle mice that exhibited baseline withdrawal frequencies less than 50% and withdrawal latencies to heat less than or equal to the mean minus two standard deviations for the HbAA group, were considered non-hyperalgesic (~15%). The plasma level of 2-AG in non-hyperalgesic HbSS mice was similar to that of HbAA mice.

To determine whether 2-AG contributes directly to hyperalgesia in SCD, 2-AG (18 μg/100 μL) was administered intravenously into the lateral tail vein of non-hyperalgesic HbSS mice in a vehicle of ethanol:saline (20:80, v:v). Mechanical hyperalgesia developed rapidly following a single injection of 2-AG in non-hyperalgesic HbSS mice and persisted for 24 h. No effect was observed in response to the vehicle in non-hyperalgesic HbSS mice, and 2-AG had no effect in HbAA mice (Figure 2A). Importantly, this dose of 2-AG administered by the intraplantar route suppressed mechanical hyperalgesia by ~68% in a mouse model of bone cancer pain and had no effect on naïve mice.[24]

JZL184, an inhibitor of 2-AG hydrolysis, increased 2-AG and decreased hyperalgesia in models of neuropathic and bone cancer pain.[25] Therefore, a single intraperitoneal injection of JZL184 (0.33 mg/kg) or vehicle consisting of DMSO:Tween-80:saline (12:1:87, v:v:v) were used to test the effect of elevating the level of endogenous 2-AG in non-hyperalgesic HbSS mice. A single injection of JZL184 transformed the silent state of non-hyperalgesic hemizygous mice (HbAS), causing mechanical hyperalgesia in these mice and inducing heat hyperalgesia in non-hyperalgesic HbSS mice (Figure 2B, C). Injection of the vehicle in both cases had no effect. Administration of the same dose of JZL184 to hyperalgesic HbSS mice did not increase the hyperalgesia, which most likely reflects maximum hyperalgesia in these mice. JZL184 had no effect in HbAA mice.

Although DAGLβ is upstream of COX-2 in the synthesis of nociceptive derivatives of 2-AG, elevated levels of COX-2 in tissues from SCD contribute to systemic increases in pronociceptive products of 2-AG. COX-2 protein was significantly elevated in blood cells of both hyperalgesic and non-hyperalgesic HbSS mice compared to samples from HbAA mice (Figure 3).

KT109 reduced mechanical and heat hyperalgesia in HbSS mice

The higher level of 2-AG in plasma of hyperalgesic HbSS may reflect an increase in 2-AG synthesis or a decrease in its hydrolysis. Initially we determined whether the increase in 2-AG in plasma was associated with an increase in its biosynthesis. The β-isoform of DAGL contributes to the synthesis of 2-AG in the periphery. Indeed, hyperalgesia in HbSS mice was accompanied by an increase in DAGLβ protein in blood cells (Figure 4). It is noteworthy that the amount of DAGLβ protein in blood cells of non-hyperalgesic HbSS mice did not differ from that of HbAA mice.

We next determined if inhibition of DAGLβ would reduce hyperalgesia in HbSS mice. KT109, an inhibitor of DAGLβ with no activity against DAGLα,[29] and KT195, a control for the inhibition of serine hydrolase ABHD6 by KT109[29] were used to selectively inhibit DAGLβ. Mice were injected intraperitoneally with 50 μL of KT109 or the vehicle for the highest dose of KT109 (100 μg in dimethylsulfoxide [DMSO]:Tween 80:saline in a 30:1:69 v:v:v ratio). Systemic (intraperitoneal) administration of KT109 reduced mechanical hyperalgesia (Figure 5A). A dose of 30 μg eliminated

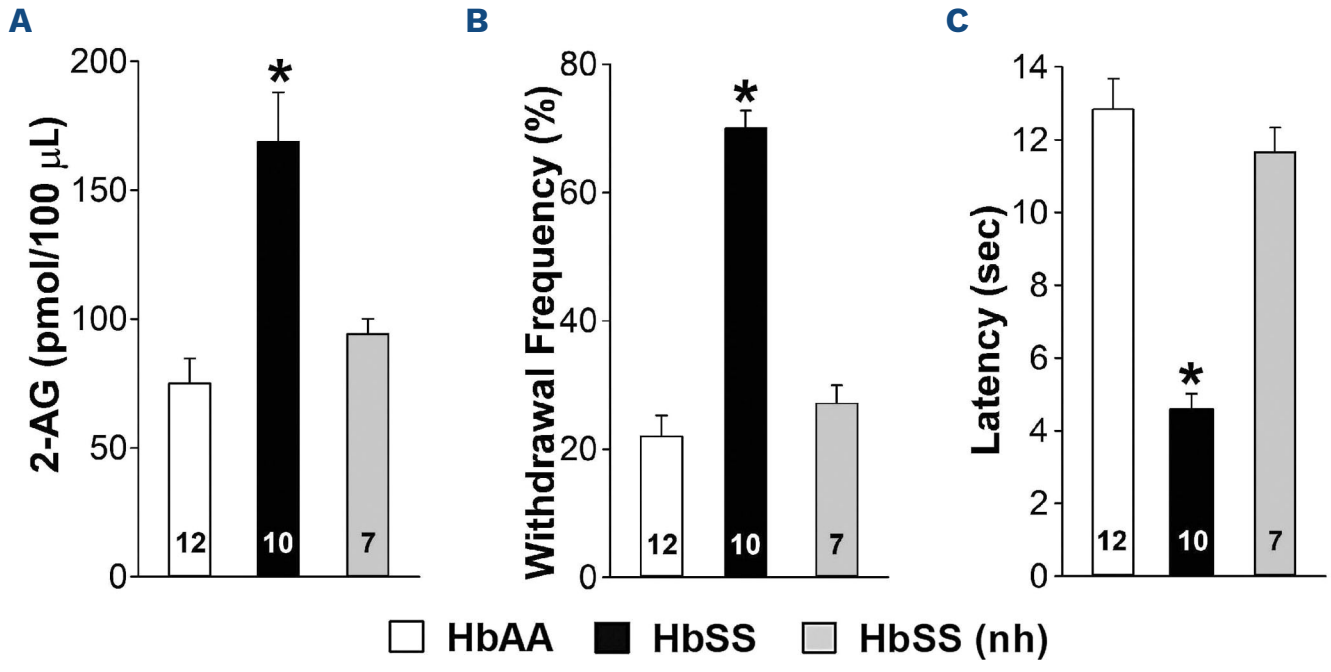

Figure 1. Increased 2-AG in plasma is associated with hyperalgesia in HbSS-BERK mice. (A) The level of 2-AG was higher in plasma of HbSS mice compared to HbAA mice and non-hyperalgesic (nh) HbSS mice. *Different from HbAA and HbSS (nh) mice at *P*=0.004, one-way analysis of variance (ANOVA) with Bonferroni *t* test. Unlike HbAA-BERK and non-hyperalgesic HbSS-BERK mice, hyperalgesic HbSS-BERK mice showed strong mechanical (B) and thermal (C) hyperalgesia. *Different from HbAA and HbSS (nh) mice at *P*<0.001, one-way ANOVA with Bonferroni *t* test. Numbers inside bars indicate group size.

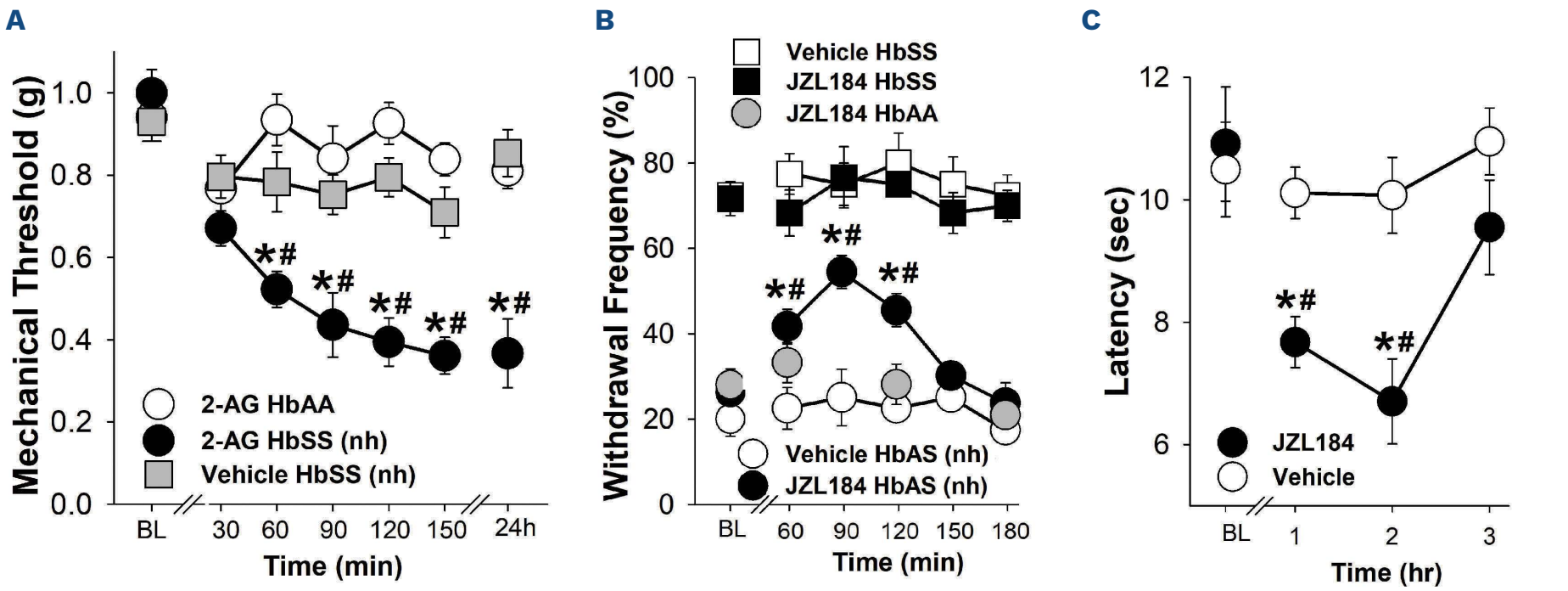

Figure 2. An increase in systemic 2-AG produced hyperalgesia. (A) 2-AG (18 μg/100 μL) was administered by intravenous injection to HbAA and non-hyperalgesic HbSS mice. Unlike HbAA mice, non-hyperalgesic HbSS mice developed hyperalgesia 60 min after injection; hyperalgesia persisted for 24 hours. The vehicle was ethanol in saline (20:80, v:v). *Different from HbAA mice and #different from vehicle at $P<0.001$ ($F[6,54]=5.52$, 2-way repeated measures analysis of variance [ANOVA] with Bonferroni *t* test, n=5-6 mice/group). (B) Intraperitoneal injection of JZL184 (0.33 mg/kg), an inhibitor of 2-AG hydrolysis, did not reduce hyperalgesia in HbSS mice (2-way ANOVA, $P=0.913$, n=6 mice/group). When injected into non-hyperalgesic HbAS mice, JZL184 (0.33 mg/kg, intraperitoneal) generated mechanical hyperalgesia in comparison to the vehicle (DMSO:Tween-80:saline, 12:1:87 v:v:v). *Different from baseline and #different from vehicle at $P=0.005$ ($F[5,50]=3.88$, 2-way repeated measures ANOVA with Bonferroni *t* test, n=6-5 mice/group). (C) JZL184 (0.33 mg/kg, i.p.) evoked thermal hyperalgesia in non-hyperalgesic HbSS mice. *Different from baseline and #different from vehicle at $P<0.05$ ($F[3,24]=2.06$, 2-way repeated measures ANOVA with Bonferroni *t* test, n=5 mice/group). BL: baseline; nh: non-hyperalgesic.

mechanical hyperalgesia in HbSS mice by 60 min after injection; the frequency of withdrawal from the mechanical stimulus was not different from that of HbAA mice treated with vehicle at this time point (31.4±4.7% and 27.5±5.2%, respectively). Although the reduction in hyperalgesia in HbSS mice treated with KT109 persisted at 3 h after administration compared to that in HbSS mice treated with vehicle, the effect of the drug was diminished: after 3 h the withdrawal frequency in HbSS mice treated with KT109 was greater than that of HbAA mice treated with vehicle at that time point. The responses of HbAA mice treated with KT109 were not different from baseline or from those given the vehicle through the 3 h testing period ($F[1,50]= 0.59$, $P=0.46$ for treatment, n=4-6 mice/group, 2-way repeated measures ANOVA). Because the time course for higher doses was similar, the anti-hyperalgesic effect of doses ranging from 3-300 μg are shown at 90 min after injection (Figure 5B). A dose-response effect was determined on the percent of the maximum possible effect (%MPE). The minimally effective dose was 30 μg and the ED_{50} was 13.1 μg (95% confidence interval: 0.61-283 μg) (GraphPad Prism).

In order to determine whether the systemic effect of KT109 on mechanical hyperalgesia was due to a peripheral site of action, mice received one intraplantar injection of vehicle (DMSO:Tween 80:saline, 13:05:86.5 v:v:v) or KT109 at doses of 1, 3 and 10 μg into one hind paw (10 μL). Following injection of vehicle, HbSS mice exhibited mechanical hyperalgesia compared to vehicle-treated HbAA mice throughout the 48 h testing period (Figure 5C). Whereas 3 μg KT109 by intraplantar injection had no effect in HbAA mice, this dose blocked mechanical hyperalgesia in HbSS mice from 30 min through 24 h after injection. Importantly, responses to the mechanical stimulus were also inhibited in the contralateral paw following injection of 3 μg KT109 (Figure 5D). The reduction in mechanical hyperalgesia in the contralateral paw did not occur until 90 min after injection, and mechanical hyperalgesia was blocked on both hind paws through 24 h after injection. Administration of 1, 3 and 10 μg (intraplantar) KT109 confirmed that 3 μg was the minimally effective dose to reduce mechanical hyperalgesia in the paw ipsilateral to the injection in HbSS mice (Figure 5E).

KT109 also inhibits ABHD6,[29] but no other serine hydrolases. KT195 is a structural analog of KT109 and a more potent inhibitor of ABHD6 but is inactive against DAGLβ and other serine hydrolases.[29] Therefore, we tested the effect of KT195 in HbSS mice (Figure 5F). Consistent with the previous experiment, KT109 (3 μg, intraplantar) reduced mechanical hyperalgesia through the 3 h observation period after treatment. In contrast, intraplantar injection of KT195 did not alter mechanical sensitivity at any time nor did it have an effect in HbAA mice ($P=0.34$, 1-way repeated measures ANOVA, n=4 mice/group; *data not shown*). A more potent derivative of KT195, KT182, at the same dose was also with-

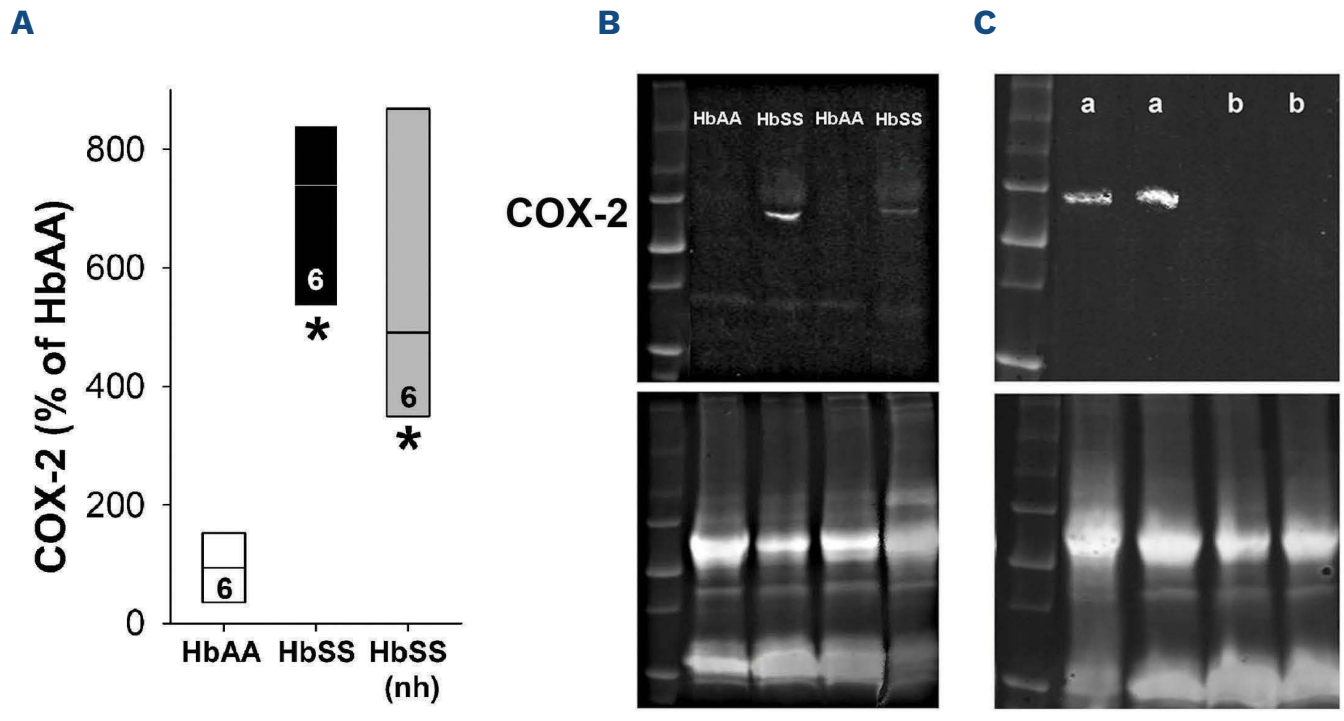

Figure 3. Levels of COX-2 were higher in blood cells from HbSS mice than in HbAA mice. (A) There was no difference in the level of COX-2 between hyperalgesic and non-hyperalgesic (nh) HbSS mice. In both groups the level of COX-2 was higher than that in HbAA mice. COX-2 was detected with rabbit anti-COX-2 (1:500, ABclonal). The secondary antibody was IRDye 800CW goat anti-rabbit (1:15,000; LI-COR). Numbers inside bars indicate group size. *Different from HbSS and HbSS (nh) at *P*=0.008 (one-way analysis of variance with the Student-Newman-Keuls test). (B) Representative images of western blot immunoreactive bands corresponding to COX-2 protein isolated from blood cells (top) and the total protein stain for loading control (bottom). A prominent band corresponding to the ~72 kDa protein was identified as COX-2. (C) The specificity of the COX-2 antibody was tested by pre-incubation of the antibody with nickel resin coated with a 10-fold molar excess of COX-2 His-tag protein (b). The negative control (a) included incubation of COX-2 antibody with nickel resin without protein coating (*Online Supplement*).

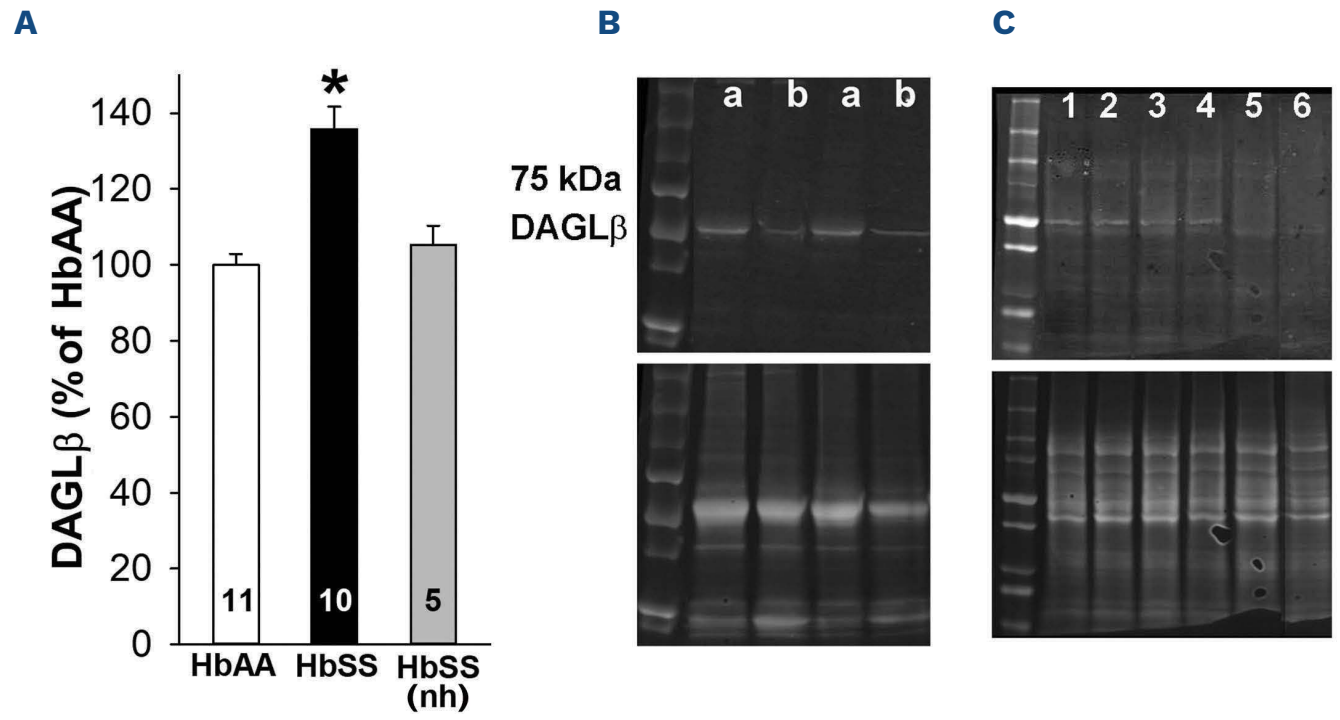

Figure 4. An increase in the amount of DAGLβ in blood cells contributes to the accumulation of 2-AG in plasma. (A) The relative level of DAGLβ protein was defined as the amount of HbSS immunoreactivity in the sample/average amount of HbAA immunoreactivity in the sample/average amount of HbAA immunoreactivity x 100. DAGLβ was detected with rabbit anti-DAGLβ (1:500, Abcam). The secondary antibody was IRDye 800CW goat anti-rabbit (1:15,000; LI-COR). The amount of DAGLβ protein in hyperalgesic HbSS mice was greater than that of non-hyperalgesic (nh) HbSS and HbAA mice. Numbers inside bars indicate group size. *Different from HbSS (nh) and HbAA mice at *P*=0.002, one-way analysis of variance with Bonferroni *t* test. (B) Representative images of immunoreactive bands corresponding to DAGLβ isolated from blood cells [top, HbSS mice (a) and HbAA mice (b)] and the total protein stain for loading control (bottom). (C) The specificity of the rabbit anti-DAGLβ antibody was tested by knocking down the DAGLβ gene with siRNA in cultured fibrosarcoma cells. Western blot analysis was performed on 45 μg of protein (top) and verified by Revert™ 700 Total Protein Stain in each well (bottom). The digits represent positive controls (1, 2), negative controls of scrambled siRNA sequence (3) and GAPDH siRNA (4), and DAGLβ siRNA s107015 (5) and s107016 (6). A prominent band corresponding to the ~68 kDa protein was identified as DAGLβ. This band was missing in DAGL$^{-/-}$ cells (*Online Supplement*).

out effect (*P*=1.0 for KT182 compared to vehicle, 2-way repeated measures ANOVA with the Bonferroni *t* test; *data not shown*). Together these data support the conclusion that the effect of KT109 was specific to the inhibition of DAGLβ.

Intraplantar administration of KT109 (3 μg) also reduced sensitivity to noxious heat (Figure 6A), but the effect had a longer latency and a shorter duration compared to the change in mechanical sensitivity. KT109 did not reduce the level of heat hyperalgesia in HbSS mice until 120 min after injection and the effect was no longer present at 24 h. Similar to the data for mechanical hyperalgesia, the effect of intraplantar injection of KT109 on the paw contralateral to the injection was consistent with its effect on the paw ipsilateral to the injection (Figure 6B). Neither KT109 nor its vehicle had an effect in HbAA mice. Administration (intraplantar) of 1, 3 and 10 μg KT109 confirmed that 3 μg was the minimally effective dose to reduce thermal hyperalgesia in the paw ipsilateral to the injection in HbSS mice (Figure 6C).

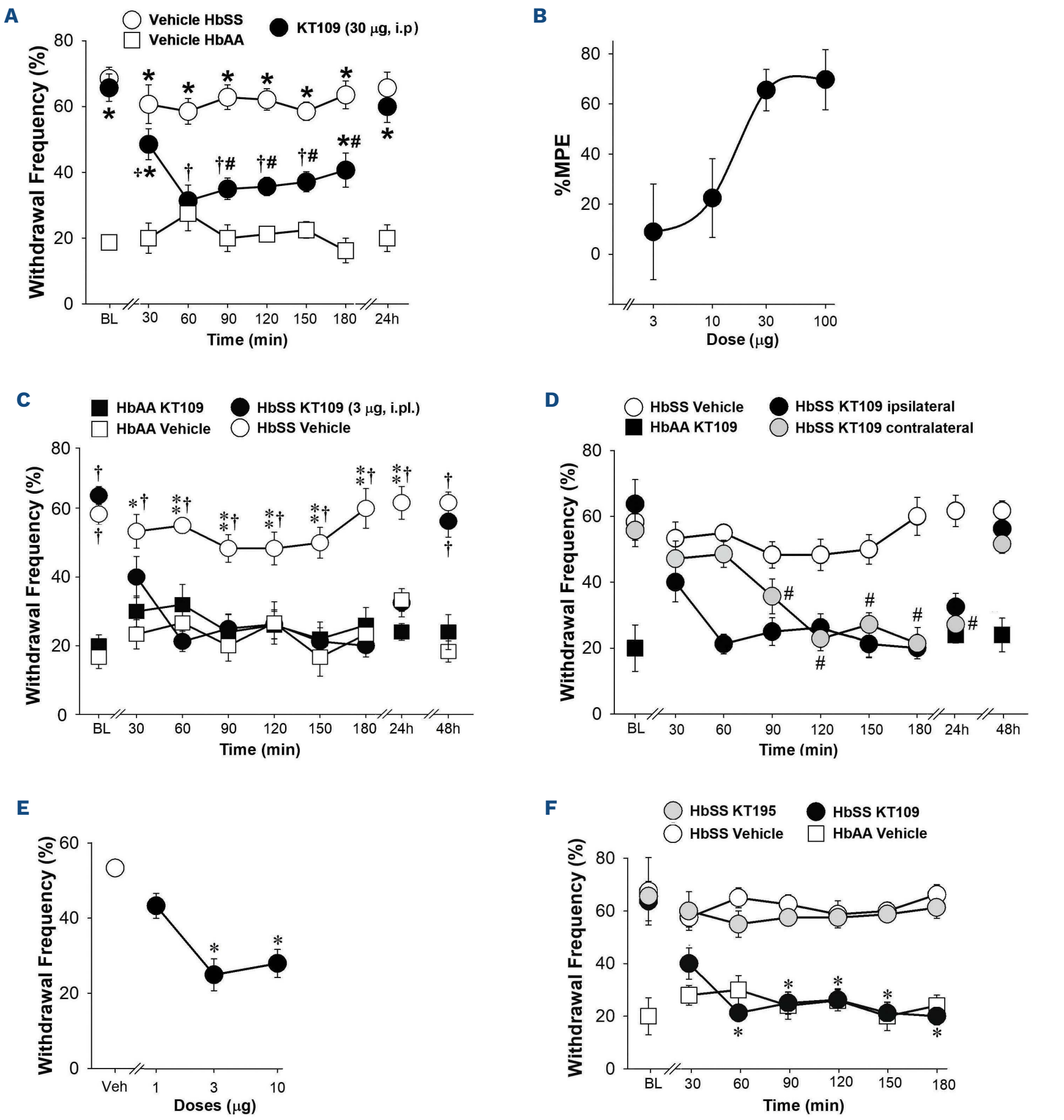

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Figure 5. Systemic and intraplantar administration of KT109 inhibited mechanical hyperalgesia in HbSS mice. (A) HbSS mice exhibited significant mechanical hyperalgesia prior to drug injections (BL, baseline). KT109 (30 μg) was administered by intraperitoneal injection. Vehicle was DMSO:Tween 80:saline (30:1:69 v:v:v). A reduction in hyperalgesia occurred at 60 min and persisted through the 3 h testing period ($F[12,90]=3.72$, $P<0.001$ for treatment, n=4-7 mice/group, 2-way repeated measures analysis of variance [ANOVA]). KT109 had no effect in HbAA mice, and the vehicle was without effect in either strain ($P=1.0$ in HbAA mice, $P=0.57$ in HbSS mice, 2-way repeated measures ANOVA). *Different from vehicle in HbAA mice at $P<0.001$, #Different from vehicle in HbAA mice at $P<0.05$, †different from vehicle in HbSS mice at $P<0.001$; †different from vehicle in HbSS at $P<0.05$ (2-way repeated measures ANOVA with Bonferroni *t* test). (B) A dose-dependent effect was observed for 3-100 μg KT109 ($F[5, 33]=5.341$, $P<0.001$ for treatment, n=4-8 mice/dose, one-way ANOVA). Data for doses were converted to a percent of the maximum possible effect (%MPE). Percent MPE was defined as the average response in the vehicle-treated HbSS mice (V HbSS) minus the post-drug (PD) response in the KT109-treated HbSS mice divided by the average response in the vehicle-treated HbSS mice (V HbSS) minus the average response in vehicle-treated HbAA (V HbAA) mice and multiplied by 100%: %MPE = (V HbSS – PD HbSS)/(V HbSS – V HbAA) x 100%. A dose response analysis confirmed that the dose of 30 μg (intraperitoneal) was the minimally effective dose. The EC_{50} was 13.1 μg (95% confidence interval: 0.61-283 μg) (GraphPad Prism). Doses were plotted on a log scale. (C) Vehicle was DMSO:Tween 80:saline (13:0.5:86.5 v:v:v). HbSS mice injected with vehicle remained different from HbAA mice injected with vehicle throughout the testing period. KT109 (3 μg, intraplantar) blocked mechanical hyperalgesia ipsilateral to the injection through 24 h ($F[3,168]=30.4$, $P<0.001$ for treatment effect, n=5-8 mice/group, 2-way repeated measures ANOVA). *Different from HbSS mice injected with KT109 at $P<0.05$, **different at $P<0.001$, †different from HbAA mice at $P<0.001$ (2-way repeated measures ANOVA with Bonferroni *t* test). (D) Mechanical hyperalgesia was also blocked in the paw contralateral to the injection ($F[1,88]=83.2$, $P<0.001$ for treatment effect, 2-way repeated measures ANOVA), but the effect was not observed until 90 min after intraplantar injection of the drug (#different from vehicle in HbSS mice at $P<0.001$). Limited data from (A) are included for perspective. (E) Testing doses of 1, 3 and 10 μg (intraplantar) confirmed that 3 μg was the minimum effective dose to reduce mechanical hyperalgesia ipsilateral to the injection in HbSS mice. *Different from vehicle at $P<0.001$, one-way ANOVA with Bonferroni *t* test; n=5-8 mice/dose. Doses were plotted on a log scale. (F) The analog KT195 did not reduce mechanical sensitivity ipsilateral to the injection in HbSS mice when administered at the effective dose of KT109 (3 μg, intraplantar). ($F[12,100]=4.2$, $P<0.001$ for treatment effect, n=6-8 mice/group, 2-way repeated measures ANOVA). *Different from KT195 and vehicle at $P<0.001$, two-way ANOVA repeated measures with Bonferroni *t* test. BL: baseline; i.p.: intraperitoneal; i.pl.: intraplantar.

KT109 reduced the level of 2-AG and its downstream products in HbSS mice

To assess the role of DAGLβ and the effect of KT109 on the production of 2-AG, PGE_2 and PGE_2-G, these lipids were measured in plasma after intraperitoneal administration of 30 μg of KT109, the smallest dose that reduced mechanical and heat hyperalgesia. Blood was collected at 60 min after injection, a time that coincided with the maximum systemic anti-hyperalgesic effect. PGE_2 was measured because of its pro-nociceptive activity and because hydrolysis of 2-AG by monoacylglycerol lipase produces arachidonic acid, a precursor for PGE_2 (Table 1). The endocannabinoid AEA was also measured because of its importance in endogenous analgesia. Consistent with their roles in contributing to hyperalgesia, the levels of PGE_2 and PGE_2-G were elevated in the plasma of HbSS mice compared to the levels in HbAA mice following intraperitoneal administration of vehicle (note the difference in units: pmol for PGE_2 and fmol for PGE_2-G). The level of AEA was lower in the samples of plasma from HbSS mice. KT109 reduced the level of 2-AG in HbSS mice to a level that was also lower than that in HbAA mice. The levels of PGE_2-G and PGE_2 in HbSS mice treated with KT109 were reduced to the levels measured in HbAA mice; however, KT109 had no effect on the level of AEA in plasma of HbSS mice. These effects of KT109 are consistent with its role in blocking the production of 2-AG. The recovery of hyperalgesia 24 h after administration of KT109 in HbSS mice was associated with an increase in 2-AG in plasma to the level before administration (153.3 ± 19.3 pmol/mL, $P=0.57$).

Discussion

These data demonstrate for the first time the exceptional contribution of DAGLβ in blood and the associated accumulation of 2-AG, its synthetic product, to hyperalgesia in mice with SCD. A high level of DAGLβ in blood cells distinguished HbSS mice with hyperalgesia from HbAA and non-hyperalgesic HbSS mice. Moreover, the simultaneous increase in both DAGLβ and COX-2 in blood cells ensures the accumulation of 2-AG and the formation of its pro-nociceptive derivatives that are sufficient for hyperalgesia. Two strategies were used to increase 2-AG: injection (intravenous) of exogenous 2-AG and injection of JZL184 to inhibit hydrolysis of endogenous 2-AG. Whereas administration of 2-AG did not promote hyperalgesia in HbAA mice with low levels of DAGLβ and COX-2 in blood cells, the same dose of 2-AG caused hyperalgesia in non-hyperalgesic HbSS mice with a low level of DAGLβ but a high level of COX-2. Although these two enzymes may be regulated independently, functionally they act in concert to achieve maximum hyperalgesia. The hyperalgesic effect of an increase in endogenous 2-AG in response to the administration of JZL184 in non-hyperalgesic HbAS mice, which may represent SCD trait (*Online Supplement*), emphasizes the importance of the proposed mechanism.

Several factors may contribute to the accumulation of 2-AG in HbSS mice. Since SCD in patients and mice is associated with an increase in the number of immune cells,[11,39-41] most of which express DAGLβ,[29,42,43] an increase in the level of DAGLβ protein in hyperalgesic HbSS mice may be associ-

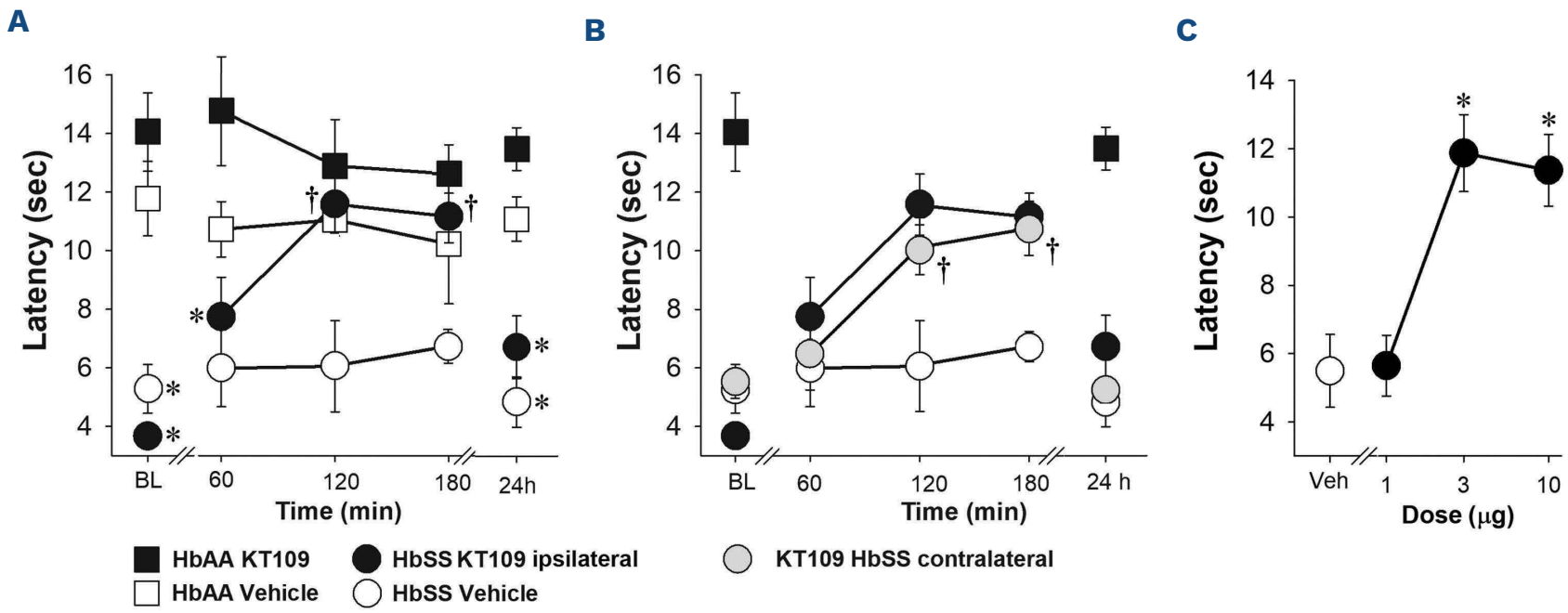

Figure 6. Intraplantar administration of KT109 reduced heat hyperalgesia in HbSS mice. (A) HbSS mice had a shorter latency to withdraw from the heat stimulus prior to drug injection. *Different from the same treatment group in HbAA mice at *P*<0.001 (F[3,60]=20.7, n=4-6 mice/group, 2-way repeated measures analysis of variance [ANOVA] with Bonferroni *t* test). HbSS mice injected with KT109 maintained this difference from HbAA mice injected with KT109 60 min after drug administration in the paw ipsilateral to the injection, but heat hyperalgesia in HbSS mice was blocked at 2 and 3 h after drug administration (†different from HbSS mice treated with vehicle at *P*<0.05). (B) Heat hyperalgesia was also blocked in the contralateral hind paw following intraplantar injection of KT109 into the opposite hind paw in a parallel time course (†different from HbSS mice treated with vehicle at *P*<0.05). Limited data from (A) are included for perspective. (C) Testing doses of 1, 3 and 10 μg (intraplantar) confirmed that 3 μg was the minimum effective dose to reduce thermal hyperalgesia ipsilateral to the injection in HbSS mice. *Different from vehicle at *P*<0.05, one-way ANOVA with Bonferroni *t* test; n=6-8 mice/dose. Doses are plotted on a log scale.

ated with an overall increase in immune cells, although an increase in the activity of the enzyme cannot be excluded as well. Post-translational modifications, including phosphorylation[44] and cysteine palmitoylation[45,46] may contribute to an increase in DAGLβ activity. In addition, increased production of 2-AG may reflect increased availability of substrate. Intracellular mobilization of Ca^{2+} through Gq/11 protein-dependent activation of phospholipase Cβ promotes the hydrolysis of phosphatidylinositol and the formation of diacylglycerol, the precursor of 2-AG.[28,47] Enriched levels of DAGLβ, 2-AG and downstream arachidonic acid and PGE_2 in white blood cells are associated with hyperalgesia in mouse models of inflammation.[29,30,42,43,48,49]

KT109 and its analog KT195 were initially screened for selective binding to serine hydrolases using activity-based protein profiling.[29] In this assay KT109 bound to DAGLβ and ABHD6 exhibited partial binding to isoforms of phospholipase-A_2 (PLA_2), but did not bind to DAGLα or COX-2. KT195 bound to ABHD6 and PLA_2 isoforms[29,30] but not to DAGLα or DAGLβ. In sickle mice, KT109 (30 μg/mouse = 1.3 mg/kg) produced a dramatic decrease in 2-AG and its downstream metabolite, PGE_2-G, with no effect on AEA. The decrease in PGE_2 observed in HbSS mice treated with KT109 may be attributed directly to inhibition of PLA_2, as suggested in the activity-based protein profiling assay, and indirectly to a decreased contribution of the hydrolysis of 2-AG to the pool of arachidonic acid, its precursor. The present data are consistent with a report on lipopolysaccharide-stimulated murine macrophages in which treatment with KT109 (5 mg/kg), but not KT195, reduced 2-AG.[29] Similarly, treatment with KT109 reduced arachidonic acid, PGE_2 and PGD_2 in macrophages. However, there were no changes in 2-AG or arachidonic acid in brain tissue, in which DAGLα contributes primarily to the generation of 2-AG.

Lipids were measured in plasma 60 min after systemic injection of KT109; the time of maximum anti-hyperalgesia. The effective anti-hyperalgesic doses we determined in HbSS mice following systemic (~1.3 mg/kg, intraperitoneal)

Table 1. Effect of KT109 on 2-AG, AEA, PGE_2 and PGE_2-G in plasma.

Lipid	HbAA/ vehicle (N=4)	HbSS/ vehicle (N=5)	HbSS/ KT109 (N=5)
2-AG (pmol)	90±2.9	179±4.0[a]	73±5.7[b]
AEA (pmol)	0.67±0.08	0.24±0.03[a]	0.25±0.04[b]
PGE_2 (pmol)	0.61±0.12	3.28±0.34[a]	1.18±0.16[b]
PGE_2-G (fmol)	0.17±0.08	6.48±0.74[a]	2.69±0.75[b]

Samples of blood were collected 60 min after injection of KT109 (30 μg, intraperitoneal) or vehicle (DMSO:Tween 80:saline, 28:1:71%). The quantification of lipids was based on the area ratio of analytical internal standard/tested lipid. Lipid values are normalized to volume of plasma (mL). All data are expressed as the mean ± standard error of mean. The sample size is indicated in parentheses. One-way analysis of variance was run across treatment groups within the same lipid. For simplicity, representation of differences between treatment groups is restricted to two levels of significance: HbSS/vehicle different from HbAA/vehicle at [a]*P*<0.005; HbSS/vehicle different from HbSS/KT109 at [b]*P*<0.005.

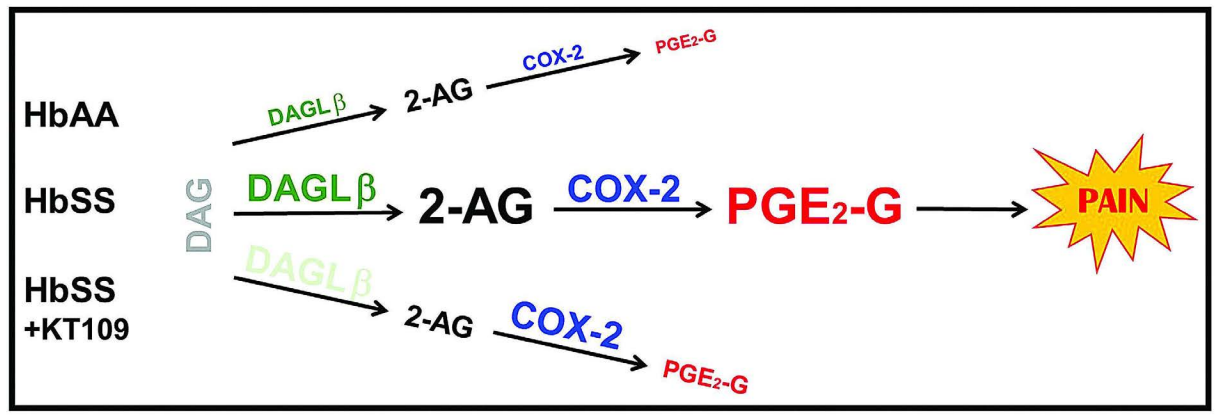

Figure 7. Biochemical pathways involved in the modulation of pain by KT109 in sickle cell disease. In contrast to HbAA mice, hyperalgesic HbSS mice demonstrated an increase in diacylglycerol lipase β (DAGLβ) in blood cells and 2-arachidonoylglycerol (2-AG) in plasma. High levels of cyclooxygenase-2 (COX-2) oxidize 2-AG to generate the pro-nociceptive lipid mediator prostaglandin E2-glyceryl ester (PGE_2-G), which causes pain by sensitizing nociceptors. By inhibiting the enzyme activity of DAGLβ, KT109 reduces the accumulation of 2-AG, a target for COX-2, and thus blocks hyperalgesia in HbSS mice. DAG: diacylglycerol.

administration are consistent with effective doses reported in murine models of acute lipopolysaccharide-induced inflammation, chemotherapy-induced neuropathy and nerve injury.[29,48] It is likely that the anti-hyperalgesia observed *in vivo* was specific to inhibition of DAGLβ and not ABHD6 because the effect was not mimicked by KT195 or KT182 which are each selective for ABHD6.[29,31] Moreover, the effect is independent of cannabinoid receptors as the anti-nociceptive effect of KT109 in acute lipopolysaccharide-induced inflammation was maintained in $CB1^{-/-}$ and $CB2^{-/-}$ mice.[48] Evidence that KT195 bound potently to PLA2 isoforms in an activity-based protein profiling assay[30] but had no effect on hyperalgesia *in vivo* supports the conclusion that the effect of KT109 on hyperalgesia in HbSS mice was specific to inhibition of DAGLβ and downstream production of PGE_2-G and less likely due to a decrease in PGE_2. Moreover, the decreased production of PGE_2-G following administration of KT109 mitigates the seeming paradox of why a *decrease* in 2-AG is anti-hyperalgesic when an *increase* in 2-AG, produced by inhibition of 2-AG hydrolysis, is anti-hyperalgesic in multiple models of peripheral inflammation.[21-23,25]

Our behavioral data following intraplantar administration of KT109 are consistent with the contribution of DAGLβ in blood cells to hyperalgesia in SCD. Intraplantar administration of KT109 decreased hyperalgesia in the contralateral paw with a longer latency than in the paw ipsilateral to the injection. The apparent systemic effect of intraplantar KT109 (0.13 mg/kg) exhibited greater potency than intraperitoneal administration of a 10-fold higher dose, suggesting better absorption of the drug in addition to a local action. Although we cannot exclude an effect mediated by the central nervous system, the localization of DAGLβ to immune cells,[29] the absence of binding of KT109 to DAGLα within the central nervous system, and the longer latency for the contralateral effect of KT109 suggest that circulating immune cells contribute to hyperalgesia in HbSS mice.

Evidence that disruption of DAGLβ in macrophages does not result in a general accumulation of triacylglycerides, and that DAGLβ is specific for polyunsaturated fatty acids only,[30] supports the therapeutic safety of selective DAGLβ inhibitors. Moreover, the increase in COX-2 in blood cells and increase in the pro-nociceptive lipid PGE_2-G in plasma in SCD indicates important therapeutic effects of DAGLβ inhibitors for the treatment of pain in SCD. A schematic representation of the biochemical pathway inhibited by KT109 is summarized in Figure 7.

Disclosures

KG has received grants from the UCI Foundation, SCIRE Foundation, Novartis, Grifols, Cyclerion and 1910 Genetics, and honoraria from Novartis, Tautona Group, and CSL Behring; none of these has any conflict with the work presented in this manuscript. None of the other authors have any competing financial interests.

Contributions

IAK designed and performed the biochemical and behavioral experiments, analyzed and interpreted data, and contributed to writing the manuscript. JG performed behavioral experiments and read the manuscript. MJ performed the siRNA knockdown experiments and contributed to the western blot studies. SGK performed behavioral experiments and edited the manuscript. AEK performed the COX-2 immunoprecipitation studies. MYG performed the mass spectrometric assay and associated data analysis. SAG performed the mass spectrometric assay. SK bred and phenotyped sickle and control mice and performed quality control. KG designed the use of sickle mice, produced all mice and edited the manuscript. VSS contributed to the design of experiments, interpretation of data, and writing the manuscript. DAS contributed to the design of experiments, interpretation of data, and writing the manuscript.

Acknowledgments

The authors would like to thank P. Villalta in the Analytical

Biochemistry Core facility of the University of Minnesota Masonic Cancer Center for direction in the measurement of lipids. Mass spectrometry analysis was performed in the UND MS Core Facility supported by the UND SMHS Dean's Office.

Funding

This study was supported by National Institutes of Health grants HL135895 to DAS, CA236777 to SGK, HL147562 to KG, and a Diversity Supplement 3RO1 HL147562-03S to SGK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Data-sharing statement

The published methods and results of this study will be deposited with PubMed Central in accord with the policies of the National Institutes of Health.

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