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https://doi.org/10.1182/blood.2022015443

TO THE EDITOR:

A model of painful vaso-occlusive crisis in mice with sickle cell disease

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Sickle cell disease (SCD) is the most common hemoglobinopathy and is caused by a point mutation in the β-globin chain that is susceptible to polymerization, giving red blood cells (RBCs) a rigid sickle shape and causing vaso-occlusion.[1] Several factors enhance vaso-occlusion,[2,3] which lead to a vaso-occlusive crisis (VOC) that is accompanied by severe acute pain often requiring hospitalization and opioid treatment.[4] Because pain itself maintains VOC,[2,3] effective pain management is of particular importance for the relief of VOC. To better understand the mechanisms underlying acute pain during VOC, a model is needed that mirrors the clinical features of VOC. Considering that low ambient temperature can be a trigger for painful VOC,[3] we developed a model of acute painful VOC caused by exposure to cold in mice with SCD. We investigated whether exposure to cold causes acute pain in mice with SCD and whether this reflects the clinical features of VOC.

Male and female transgenic Berkeley mice[5] (5-9 months old) that express human sickle hemoglobin S (HbSS) or normal human hemoglobin A (HbAA) were used. Mechanical hyperalgesia was defined as a decrease in paw withdrawal threshold using calibrated von Frey monofilaments according to the up-down method.[6] Heat hyperalgesia was defined as a decrease in paw withdrawal latency in response to a radiant heat stimulus.[7,8] Deep tissue hyperalgesia was defined as a decrease in grip force measured by a grip force meter.[7,9] Baseline (BL) measurements were taken over 3 days before each experiment. Paw withdrawal threshold and paw withdrawal latency were measured for both hind paws and averaged. Different modalities of hyperalgesia were studied in different groups of mice. HbSS mice that exhibited BL mechanical threshold and withdrawal latency less than 2 standard deviations (SDs) and grip force less than 1 SD of the average value for HbAA mice were considered nonhyperalgesic HbSS (HbSS[nh]). Consistent with our previous report,[10] the number of HbSS(nh) mice was ~15% of all HbSS mice, and this decreased with age. HbSS(nh) mice were exposed to cold to evoke acute hyperalgesia, an experimental analogue of pain in patients.

For exposure to cold, mice were placed in a plastic cage without bedding in a room with a controlled ambient temperature of 10°C (50°F) for 1 hour and then returned to room temperature (22°C). Hyperalgesia and measures of blood oxygen saturation (SpO_2) were determined before, immediately

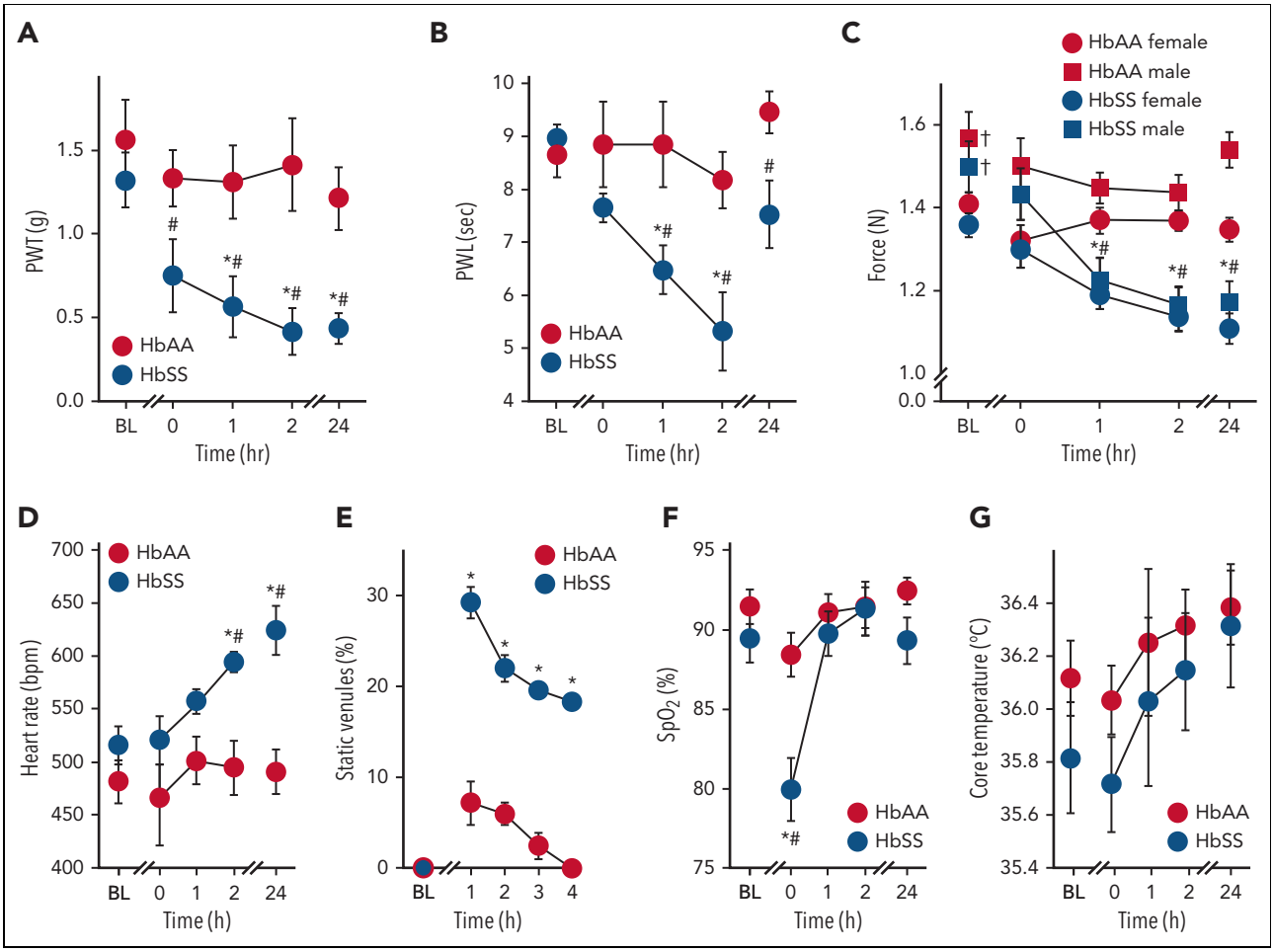

Figure 1. Exposure to cold induced painful VOC in nonhyperalgesic HbSS mice. (A) Mechanical hyperalgesia appeared in HbSS(nh), but not in HbAA mice, 1 hour after exposure to cold and persisted for at least 24 hours. *Different from BL at $P = .002$ ($F[3,40] = 3916$) and #different from HbAA mice at $P = .002$ ($F[1,40] = 18.1$), 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 5 to 7 mice/group. (B) Unlike HbAA mice, HbSS(nh) mice exhibited heat hyperalgesia, which began 1 hour after exposure to cold and disappeared by 24 hours. *Different from BL at $P = .004$ ($F[4,44] = 4.57$) and #different from HbAA mice at $P = .002$ ($F[1,44] = 15.5$, 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 6 to 7 mice/group). (C) Exposure to cold caused prolonged deep tissue hyperalgesia in both sexes of HbSS(nh) mice. Because means of grip force were lower in female mice compared with male mice in both groups (†different from female of the same genotype at $P < .05$, Kruskal–Wallis ANOVA on ranks test with Dunn's method), the data for each sex were analyzed separately. *Different from BL in HbSS(nh) mice of both sexes at $P < .001$ ($F[4,132] = 15.2$) and #different from HbAA mice in both sexes at $P < .001$ ($F[3,132] = 18.2$), 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 6 to 12 mice/group. (D) Cold-induced hyperalgesia was accompanied by an increase in heart rate in HbSS mice ($F[1,44] = 10.5$, $P = .008$, 2-way repeated-measures ANOVA). HbAA mice had no changes in heart rate ($P = 1.0$). *Different from BL at $P < .01$ and #different from HbAA mice at $P < .01$, Bonferroni *t* test, n = 6 to 7 mice/group. (E) Exposure to cold produced microvascular stasis that was greater in HbSS(nh) mice. *Different from HbAA mice at $P < .001$ ($F[1,24] = 240.4$), 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 4 mice/group. (F) Exposure to cold caused transient hypoxia in HbSS(nh), but not HbAA, mice. Hypoxia (hypoxemia) was defined as a decrease in SpO_2 in blood. *Different from BL at $P < .001$ ($F[4,44] = 9.39$) and #different from HbAA mice at $P = .016$ ($F[1,44] = 8.00$), 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 6 to 7 mice/group. (G) Exposure to cold did not produce hypothermia in HbSS or HbAA mice at any time ($F[4,44] = 0.177$, $P = .949$, 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 6 to 7 mice/group).

after, and 1, 2, and 24 hours after exposure to cold. Blood SpO_2 was determined using a pulse oximeter (Kent Scientific, Torrington, CT) applied to the hind paw with simultaneous measurement of heart rate and core temperature in anesthetized mice (1.8%-2% isoflurane). A decrease in SpO_2 in blood was an indication of hypoxia (hypoxemia). Stasis measurements were performed in 20 to 25 flowing venules through implanted dorsal skin fold chambers.[11] The same venules were reexamined for stasis (no flow) every hour for 4 hours after exposure to cold. Percent of static venules was calculated as static venules/total venules × 100. For all experiments, the experimenter was blind to the genotype and treatment.

Blood collection and isolation of plasma and blood cells[10] were performed at 2 hours after exposure to cold. The levels of cyclooxygenase-2 (COX-2) and diacylglycerol lipase β (DAGLβ) proteins in blood cell lysates were determined by western blotting (45 μg of protein per sample), defined as the density of the immunoreactivity of the protein of interest/total protein within each sample (Revert 700 Total Protein Stain, LI-COR) and expressed as a percent of the corresponding average amount in HbAA mice. Specificity of the DAGLβ and COX-2 antibodies was confirmed.[10] The level of endocannabinoid 2-arachidonoylglycerol (2-AG) was analyzed in plasma by liquid chromatography with tandem mass spectrometer and quantified against deuterated internal standard.[10]

Data are presented as mean ± standard error of the mean and were analyzed by 1- or 2-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's post hoc test. $P < .05$ was considered significant.

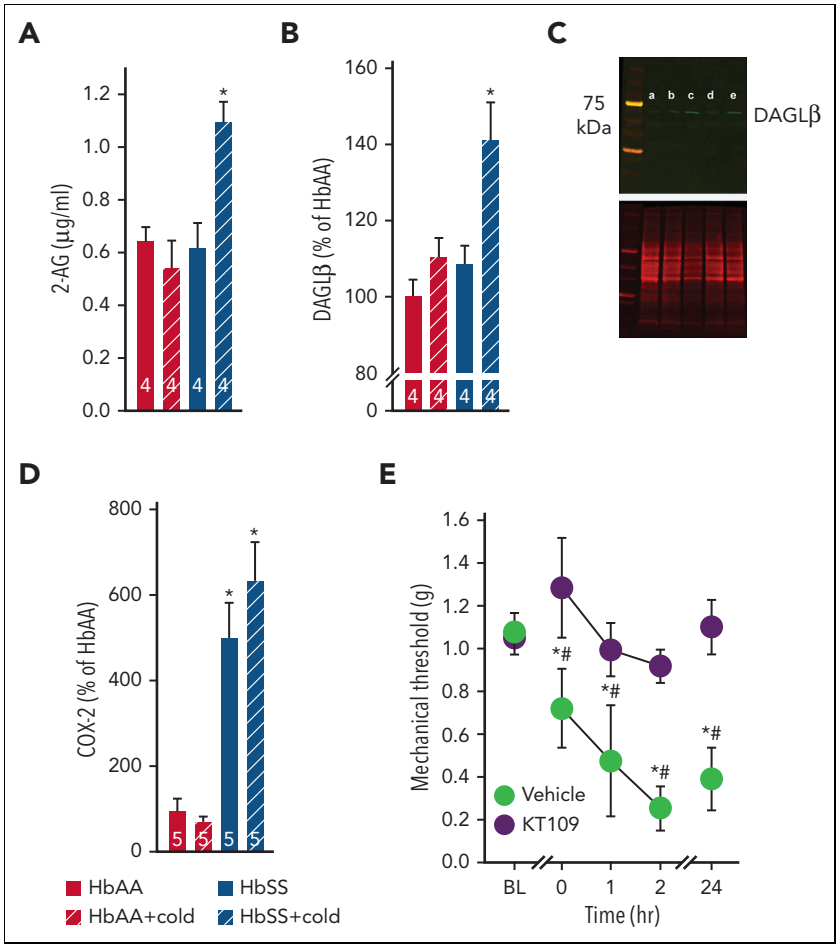

Figure 2. An increase in the amount of DAGLβ in blood cells and the accumulation of 2-AG in plasma contributes to cold-evoked hyperalgesia in HbSS(nh) mice. Data for biochemical studies were collected 2 hours after exposure to cold. (A) In contrast to HbAA mice, the level of 2-AG increased in plasma of HbSS(nh) mice exposed to cold. Numbers inside bars indicate group size. *Different from other groups ($F[3,12] = 8.564$, $P = .003$, 1-way ANOVA with Bonferroni *t* test. (B) The accumulation of 2-AG in the plasma of HbSS(nh) mice was associated with an increased level of DAGLβ in blood cells. The relative level of DAGLβ protein was defined as the amount of immunoreactivity in the HbSS(nh) sample/average amount of immunoreactivity in the HbAA sample × 100%. *Different from other groups ($F[3,12] = 7.401$, $P = .005$, 1-way ANOVA with Bonferroni *t* test). (C) Representative images of immunoreactive bands corresponding to DAGLβ isolated from blood cells [top, HbSS(nh) mice (a,d) and HbSS+cold mice (b,c,e)] and the total protein stain for loading control (bottom). DAGLβ was detected with rabbit anti-DAGLβ (1:500, Abcam). The secondary antibody was IRDye 800CW goat anti-rabbit (1:15 000; LI-COR). (D) The basal level of COX-2 protein in blood cells was higher in HbSS(nh) compared with HbAA mice. *Different from HbAA and HbAA+cold groups ($F[3,16] = 20.530$, $P < .001$, 1-way ANOVA with Bonferroni *t* test). Exposure to cold did not cause additional changes in HbSS(nh) mice. There was no difference in the level of COX-2 between naïve HbAA mice and HbAA mice exposed to cold. 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. (E) Unlike vehicle, systemic administration of KT109, an inhibitor of DAGLβ, prevented acute mechanical hyperalgesia in HbSS mice. KT109 (30 µg) or vehicle (dimethyl sulfoxide: Tween 80:Saline [30:1:69]) was administered by intraperitoneal injection 1 hour before exposure to cold. *Different from BL at $P = .002$ ($F[4,36] = 5.187$) and #different from vehicle at $P = .007$ ($F[1,36] = 11\,852$), 2-way repeated-measures ANOVA with Bonferroni *t* test, n = 5 to 6 mice/group.

Because pain associated with VOC can be severe, we first determined if exposure to cold produced acute mechanical, heat, and deep tissue hyperalgesia. Unlike in HbAA mice, mechanical, heat, and deep tissue hyperalgesia developed in nonhyperalgesic HbSS mice (Figure 1A-C). Mechanical and deep tissue hyperalgesia persisted for 24 hours. In some mice, hyperalgesia did not disappear during the entire test period, which may indicate a possible transition from acute to chronic hyperalgesia. Because there were no sex differences in mechanical and heat hyperalgesia at any time, data for male and female mice were combined. BL measures of grip force were lower in female mice compared with male mice in both groups. Therefore, data for each sex were analyzed separately. Exposure to cold caused deep tissue hyperalgesia in both sexes. Hyperalgesia was accompanied by an increase in heart rate in HbSS mice, but not in HbAA mice (Figure 1D), suggesting the presence of pain-related stress.[2,3,12,13]

Exposure to cold produced vascular stasis in both HbSS and HbAA mice; however, the magnitude and duration of stasis were greater in HbSS mice (Figure 1E). Because changes in peripheral blood flow are mediated by the autonomic nervous system, it is likely that autonomic dysregulation, also observed in patients with SCD,[3,13] contributes to the increased and prolonged stasis in HbSS mice. We have shown in earlier studies that stasis caused by hypoxia-reoxygenation in sickle mice is characterized by vasoconstriction, endothelial cell activation, inflammation, and leukocyte adhesion to the vessel wall.[11,14] These factors contribute to the increase in the transit time of RBCs through capillary beds, prolonging RBC exposure to hypoxia, measured as a decrease in SpO_2 (Figure 1F). A high incidence of hypoxia has been reported among patients with SCD, which increases significantly during VOC.[15] Additionally, no changes in core temperature were observed after exposure to cold in both groups of mice (Figure 1G).

Recently, we showed that an increased level of the endocannabinoid 2-AG in plasma due to an increase in its synthesizing enzyme DAGLβ in blood cells contributes to chronic hyperalgesia in HbSS mice.[10] Likewise, acute hyperalgesia after exposure to cold was accompanied by an increase in these indicators in HbSS mice (Figure 2A-C). An increase in DAGLβ protein may be associated with an overall increase in the number of immune cells[16,17] expressing DAGLβ.[18,19] 2-AG is oxidized by COX-2 to form pronociceptive derivatives, and high levels of COX-2 were observed in the blood cells of HbSS mice regardless of hyperalgesia status[10] and exposure to cold (Figure 2D). We postulate that DAGLβ-mediated accumulation of 2-AG in the presence of high COX-2 level potentiates the formation of pronociceptive metabolites that contribute

to acute hyperalgesia.[10,20] Administration of KT109, which blocks DAGLβ activity,[18,21] 1 hour before exposure to cold blocked the development of mechanical hyperalgesia (Figure 2E).

We have developed a model of cold-induced VOC similar to that in patients with SCD to investigate the mechanisms underlying the induction of VOC and associated pain to develop effective approaches for prevention. Because elevated levels of 2-AG contribute to both acute and chronic hyperalgesia, controlling the level of this endocannabinoid may be a promising target for pain relief in SCD.

Acknowledgments

Liquid chromatography mass spectrometry/mass spectrometry analysis was performed in the University of North Dakota Mass Spectrometry Core Facility supported by the University of North Dakota School of Medicine & Health Sciences Dean's Office.

This work was supported by National Institutes of Health (NIH), National Cancer Institute Grants CA241627 (D.A.S.) and CA263777 (S.G.K.); NIH, National Heart, Lung, and Blood Institute Grant HL147562 (G.M.V.; J.D.B.); NIH, National Institute of Biomedical Imaging and Bioengineering Grant EB029354; the Susan Samueli Scholar Award (K.G.); and NIH, National Heart, Lung, and Blood Institute Grant HL147562 to 03S Supplement to promote diversity (K.G.).

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authorship

Contribution: I.I.K. designed the concept and performed the biochemical and behavioral experiments, analyzed and interpreted data, and wrote the manuscript; J.J. performed behavioral experiments and read the manuscript; V.M.R. performed behavioral experiments and read the manuscript; S.G.K. performed measures of SpO_2, core temperature, and heart rate, and edited the manuscript; M.Y.G. performed the mass spectrometric assay and associated data analysis; S.A.G. performed mass spectrometric assay; S.K. bred and phenotyped sickle and control mice and performed quality control; K.G. designed the concept and experimental design, production, and validation of mouse models and editing; J.D.B. performed stasis measurements, contributed to interpretation of data, and read the manuscript; G.M.V. performed stasis measurements, contributed to interpretation of data, and read the manuscript; V.S.S. contributed to the design of experiments, interpretation of data, and writing of the manuscript; and D.A.S. contributed to the design of experiments, interpretation of data, and writing of the manuscript.

Conflict-of-interest disclosure: K.G. received honoraria from Tautona Group, Novartis, and CSL Behring; and research grants from Cyclerion, 1910 Genetics, Novartis, Grifols, UCI Foundation, and SCIRE Foundation. The remaining authors declare no competing financial interests.

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Footnotes

Submitted 6 June 2022; accepted 27 July 2022; prepublished online on *Blood* First Edition 12 August 2022.

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

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