

Spinal glial activation and oxidative stress are alleviated by treatment with curcumin or coenzyme Q in sickle mice

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Supplementary Appendix for the Letter to the Editor:

Oxidative stress in a mouse model of sickle cell disease is associated glial activation that is alleviated by treatment with curcumin or coenzyme Q

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Methods

Mice

Transgenic female sickle mice (HbSS-BERK) and control mice (HbAA-BERK) were used. HbSS-BERK are homozygous for knockout of both murine alpha and beta globins and carry linked transgenes for human alpha and beta-S globins. Thus, HbSS-BERK mice produce only human alpha and beta S globin chains (human hemoglobin S) and have severe disease that simulates human sickle cell anemia.¹⁻³ Control HbAA-BERK have the same mixed genetic background as HbSS-BERK, but exclusively express normal human alpha and beta globins (hemoglobin A). Mice were bred and phenotyped for sickle and normal human hemoglobin by IEF.² Genotyping for the mouse knockout and human hemoglobin transgenes was done by Transnetyx (Cordova, TN). All experiments were performed following approved protocols from the University of Minnesota's Institutional Animal Care and Use Committee and conform to the statutes of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.⁴

Drug treatment of mice

Mice were treated daily for 4 weeks with Coenzyme Q10 (CoQ10) and curcumin (both from Sigma, St Louis, MO) at 15 mg/kg and 45 mg/kg, respectively, in olive oil by gavage of 0.1 ml/25 g body weight.^{5,6} Sickle and control mice received either vehicle, curcumin, CoQ10, or both CoQ10 and curcumin (cotreatment).

Pain Behavior Analysis

All behavioural tests were performed in a quiet room maintained at a constant temperature of 23-25°C during the proestrous/estrous cycle once a week for a total of four weeks before that day's treatment. Mice were habituated to each test protocol and environment for three consecutive days before collecting baseline measurements as described earlier.² Three measurements were recorded for each test, or as described, for the following behaviors.

Mechanical hyperalgesia. The paw withdrawal frequency (PWF) evoked by evoked by 10 consecutive applications of a 1.0 g (4.08 mN) von Frey (Semmes-Weinstein) monofilament (Stoelting Co., Wood Dale, IL) were used to assess mechanical sensitivity. Mice were placed on a wire-mesh under a glass container (10x6.5x6.5 cm) and were allowed to acclimate for 60 min. The von Frey filament was applied to the plantar surface of each hind paw, avoiding the toes, heel and pads, for 1-2 seconds with a force sufficient to bend the filament. An inter-stimulus interval of at least 5 seconds was observed. Only vigorous withdrawal responses were counted.

Thermal hyperalgesia. To test for heat sensitivity, mice were placed on the glass floor of Hargreave's apparatus and a radiant heat stimulus was applied to the plantar surface of the hind paw from below with a projector lamp bulb (CXL/CXR, 8 V, 50 W). Paw withdrawal latency (PWL) to the nearest 0.1 second was automatically recorded when the mouse withdrew its paw from the stimulus. A 20 second stimulus cutoff was used to prevent damage to the paw. To test for cold sensitivity, mice were placed on an aluminum plate set in an ice bath (3°C) and the latency to initial lifting of either forepaw from cold plate (PWL) and the number of times mice lifted or rubbed the forepaws together (PWF) over a period of 2 minutes were determined.

Laser Scanning Confocal Microscopy (LSCM)

Sample preparation. Spinal cords were fixed in Zamboni's solution (0.03% w/v picric acid and 2% w/v paraformaldehyde) and transferred into 20% sucrose with 0.05% sodium azide after 24-48 hr at 4°C (Kohli 2010). Fixed tissues were embedded in OCT and cryosections (15 µm) were stained with rabbit anti-Iba1, a microglial cell marker (Wako, Richmond VA), goat anti-GFAP, an astrocyte marker (Abcam, Cambridge, MA), or guinea pig anti-Substance P (SP; Abcam). Cy2- or Cy3-conjugated species-specific donkey secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used to detect immunoreactive proteins and samples were mounted with Vectashield (Vector Labs, Burlingame, CA).

Quantitative analysis. Spinal cord sections from L4 and L5 were imaged using LSCM (Olympus FluoView 1000 BX2, Olympus Corporation, Center Valley, PA). SP immunoreactivity in the superficial dorsal horn was quantitated in optical sections of 1.68- μ (30 steps) captured with a 20X objective (UPLAPO Olympus BX61). GFAP-immunoreactivity was quantitated in optical sections 0.48- μ (38 steps) captured with a 40X objective (UPLFLN Laser 40XO Olympus BX61). For each dorsal horn, a threshold intensity corresponding to the average intensity of labeled regions within the superficial dorsal horn was measured using Image J (NIH). Data was collected from three sections, averaged and expressed as percentage of ir pixels as described by Malmberg.⁷ Iba-1 immunoreactivity was quantitated by counting number of microglial cells exhibiting a clear somata and discernible processes per single field of view (FOV) from optical sections of 0.48- μ (38 steps) captured at 40X with a 2.6X electronic zoom following the protocol by Hains et al.⁸ Activated microglia were classified based on the following criteria: somata exhibiting hypertrophy and retracted processes. Three representative non-adjacent FOV from the dorsal horn were selected. For uniformity the FOV were collected from the superficial dorsal horn, superficial lateral dorsal horn and in proximity to the central canal. Each segment and the total number of glial cells in the three FOV were calculated for each group. The average of the three sections/mouse for 8 mice was averaged for each group.

Reactive oxygen species

Lumbar spinal cords sections (30 μ m) were incubated with the superoxide indicator dihydroethidium (DHE; Life Technology, Grand Island, NY) at 5 μ M and imaged using LSCM (Olympus FluoView 1000 BX2). The percentage of fluorescence pixels divided by the total area was quantitated.

Statistical analysis

All data were analyzed using Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA). A one-way ANOVA with Bonferroni's multiple comparison correction and t-test was used to compare treatments. A two-way ANOVA with Bonferroni's multiple comparison correction was used to compare the pain behaviour responses between treatments. A p-value of < 0.05 was considered significant. All data are presented as mean \pm SEM.

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Supplementary Table S1: Definitions of pain related terminology

Analgnesia	absence of pain in response to stimulation which would normally be painful
Gliosia	a nonspecific reactive change of glial cells (astrocytes, microglia, and oligodendrocytes) in response to damage to the central nervous system
Hyperalgesia	increased sensitivity to pain or enhanced intensity of pain sensation and a raised threshold to painful stimuli
Mechanical hyperalgesia	increased sensitivity in response to touch or pressure
Neurogenic inflammation	inflammation arising from the local release from neurons of inflammatory mediators such as Substance P, Calcitonin Gene-Related Peptide (CGRP), neurokinin A (NKA), and endothelin-3 (ET-3).
Nociception	processing of harmful stimuli by the nervous system to sense potential harm or pain; and a feeling of distress, suffering, or agony, caused by stimulation of specialized nerve endings
Nociceptor sensitization	a reduction in threshold and an increase in responsiveness of specialized nerves
Thermal hyperalgesia	increased sensitivity in response to heat or cold