ARTICLE - Red Cell Biology & its Disorders

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

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Methods

Mice

HbSS mice exclusively express human sickle hemoglobin S (HbS) whereas HbAA mice (experimental controls) express normal human hemoglobin A (HbA). Homozygous HbSS or HbAA mice do not express murine alpha- and beta-globins. Hemizygous HbAS mice containe one copy of normal human β-globin and one copy of sickle human β-globin. Hemizygous BERK sickle mice show vascular pathobiology and inflammation and increased mechanical, thermal and deep tissue hyperalgesia, although to a lesser extent than in homozygous HbSS mice.[9,50]

Western blot analysis

The amount of DAGLβ and COX-2 in blood cell lysates (45 μg of protein per sample) were determined from western blot. Samples were sonicated in RIPA buffer, and protein was quantified using a BCA kit (Sigma-Aldrich). DAGLβ and COX-2 were resolved on a 4-20% gradient SDS-PAGE gel and transferred to a PVDF membrane. Immunoreactivity was visualized using an Odyssey Imaging System (LI-COR). The density of each immunoreactive band was determined using ImageJ software (National Institutes of Health). The amount of DAGLβ and COX-2 proteins was 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 average amount of HbAA.

The specificity of the DAGLβ antibody was tested by knocking down DAGLβ in mouse fibrosarcoma cells clone NCTC 2472 with small interfering RNA (siRNA) specific

for the DAGLβ gene. siRNA transfection was performed in accordance with the Polyplus INTERFERin protocol. Commercially available siRNAs were used to silence DAGLβ: siRNA #1 (ID s107015, Catalog #4390771, Ambion) and siRNA #2 (ID s107016, Catalog #4390771, Ambion). A scrambled siRNA sequence (Catalog #AM4611) was used to demonstrate the specificity of DAGLβ transfection. The housekeeping gene GAPDH, (Catalog #AM4624) was selected as a positive control to show that silencing of the target gene did not affect cell viability. Cells were homogenized in RIPA buffer and stored at -80°C until analyzed. Western blot analysis was performed on 45 µg of protein and the amount of loading protein was verified by Revert™ 700 Total Protein Stain.

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 (R&D systems). The antibody-COX-2 protein-nickel resin complex was collected by centrifugation, and the supernatant was applied to the PVDF membrane for determination of immunoreactivity. Incubation of COX-2 antibody with nickel resin alone was used as a negative control.

Behavioral measures of hyperalgesia

Mechanical hyperalgesia. Mice were placed on an elevated mesh platform under glass enclosures and allowed to habituate for 30 minutes prior to testing. Paw withdrawal thresholds were determined using the up-down method[32]. A series of eight von Frey monofilaments (0.07, 0.16, 0.4, 0.6, 1, 1.2, 2, and 4 g) was used. Testing was initiated with a monofilament that delivered 0.6 g. In the absence of a withdrawal response, a stronger monofilament was applied. If a withdrawal occurred, a weaker

stimulus was presented. Six responses, starting with the negative response immediately before the first paw withdrawal, were recorded. The resulting pattern was tabulated and the 50% paw withdrawal threshold (mechanical threshold) was calculated. The interstimulus interval was 5 s. To determine withdrawal response frequency, a von Frey monofilament with a bending force of 3.6 mN was applied to the plantar surface of each hind paw 10 times, with an interval of approximately 5-10 seconds between applications. *Heat hyperalgesia.* Mice were placed on a glass platform, covered with glass containers, and habituated for ~ 30 minutes. Radiant heat was applied to the plantar surface of each hind paw from below and paw withdrawal latency was determined. Mean withdrawal latency was calculated from 3 trials. The intensity of the heat source was adjusted so that mice withdrew their hind paws at ~9 seconds during baseline testing. A cutoff time of 16 seconds was imposed to prevent tissue damage.

Measurement of 2-AG, PGE_2, PGE_2-G and AEA

Lipids were extracted from each sample in the presence of AEA-*d8*, 2-AG-*d5*; PGE_2-G-*d5* and PGE_2-*d4* as internal standards.[17] Levels of 2-AG, AEA, PGE_2-G and PGE_2 were analyzed by LC-NSI-MS/MS on a Quantiva™ triple quadrupole (Thermo Scientific™) interfaced to a Dionex Ultimate™ 3000 Rapid separation LC HPLC system (Thermo Scientific™) using a home-packed Luna C18 column (5 µm, 120 Å, 200 mm x 75 µm ID, Phenomenex) at room temperature.