Archival Report

Biological Psychiatry

Microglial Acid Sensing Regulates Carbon Dioxide-Evoked Fear

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ABSTRACT

BACKGROUND: Carbon dioxide (CO_2) inhalation, a biological challenge and pathologic marker in panic disorder, evokes intense fear and panic attacks in susceptible individuals. The molecular identity and anatomic location of CO_2-sensing systems that translate CO_2-evoked fear remain unclear. We investigated contributions of microglial acid sensor T cell death–associated gene-8 (TDAG8) and microglial proinflammatory responses in CO_2-evoked behavioral and physiological responses.

METHODS: CO_2-evoked freezing, autonomic, and respiratory responses were assessed in TDAG8-deficient ($^{-/-}$) and wild-type ($^{+/+}$) mice. Involvement of TDAG8-dependent microglial activation and proinflammatory cytokine interleukin (IL)-1β with CO_2-evoked responses was investigated using microglial blocker, minocycline, and IL-1β antagonist IL-1RA. CO_2-chemosensitive firing responses using single-cell patch clamping were measured in TDAG8$^{-/-}$ and TDAG8$^{+/+}$ mice to gain functional insights.

RESULTS: TDAG8 expression was localized in microglia enriched within the sensory circumventricular organs. TDAG8$^{-/-}$ mice displayed attenuated CO_2-evoked freezing and sympathetic responses. TDAG8 deficiency was associated with reduced microglial activation and proinflammatory cytokine IL-1β within the subfornical organ. Central infusion of microglial activation blocker minocycline and IL-1β antagonist IL-1RA attenuated CO_2-evoked freezing. Finally, CO_2-evoked neuronal firing in patch-clamped subfornical organ neurons was dependent on acid sensor TDAG8 and IL-1β.

CONCLUSIONS: Our data identify TDAG8-dependent microglial acid sensing as a unique chemosensor for detecting and translating hypercapnia to fear-associated behavioral and physiological responses, providing a novel mechanism for homeostatic threat detection of relevance to psychiatric conditions such as panic disorder.

Keywords: Acid sensing, Carbon dioxide, Fear, Microglia, Panic, TDAG8

http://dx.doi.org/10.1016/j.biopsych.2016.04.022

Fear encompasses threat-associated behavioral and physiological responses crucial to survival. Most of our current biological understanding of fear genesis comes from studies in which animals are exposed to exteroceptive aversive stimuli such as pain or predator exposure (1,2). Fear responses also can be evoked by stimuli producing an internal threat to homeostasis and imminent danger to survival. A widely studied interoceptive stimulus, carbon dioxide (CO_2) inhalation, produces intense fear, autonomic, and respiratory responses that can evoke panic attacks. In humans, CO_2 sensitivity lies on a continuum (3), with panic disorder (PD) patients being highly sensitive to low CO_2 doses, whereas healthy volunteers experience panic-like symptoms only at higher concentrations (4,5). In the extracellular space, CO_2 combines with water to produce protons, leading to systemic acidosis (6,7), which is responsible for the panicogenic effects of CO_2. Prior studies indicate that acid-sensing ion channels in the amygdala contribute to CO_2-evoked fear responses (8). However, recent studies in patients with amygdala damage resulting from Urbach-Wiethe disease indicate that the amygdala is not required for the expression of fear and panic to CO_2 inhalation (9), suggesting that distinct chemosensory systems may exist for homeostatic threats such as CO_2 that may not engage traditional fear mechanisms.

Microglia, innate immune cells of the central nervous system (CNS) (10), are recruited in physiological responses to homeostatic fluctuations (11). Microglia transform rapidly from a resting to a proinflammatory activated state on sensing subtle imbalance in ionic homeostasis (12,13), in accordance with their role in maintenance of the CNS microenvironment. Extracellular acidification induces rapid alteration in microglial morphologic features and actin integrity (14,15), suggesting their potential engagement in the effects of acidotic stimuli such as CO_2. This study addresses possible mechanisms responsible for generation of panic-relevant fear responses, focusing on the T cell death–associated gene-8 (TDAG8), an acid-sensing G-protein coupled receptor (16,17). TDAG8 is expressed in microglial cells resident in sensory

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ISSN: 0006-3223

circumventricular organs (CVOs). Sensory CVOs such as the subfornical organ (SFO) are integrative sites lacking a blood-brain barrier and have access to systemic and CNS compartments for maintenance of homeostasis (18). Importantly, the SFO has been identified as a site where interoceptive stimuli can be sensed and relayed to panic-generating CNS areas (19). Previous work associates the SFO with panic-like responses to intravenous lactate (20,21). The SFO therefore may be a primary locus for detecting interoceptive challenges relevant to panic. Given the expression of microglial acid-sensor TDAG8 in a panic-regulatory area, we investigated potential recruitment of the receptor in CO_2-evoked behavior and physiological features. We hypothesized that acid-sensor TDAG8-mediated microglial activation within the SFO contributes to the behavioral and physiological sequelae of CO_2 inhalation. Our data suggest that acid-sensor TDAG8 acts in the SFO to promote CO_2-evoked behavioral (freezing) and physiological (cardiovascular) responses via a mechanism involving microglial activation and the proinflammatory cytokine interleukin (IL)-1β.

METHODS AND MATERIALS

Animals

$TDAG8^{-/-}$ mice, a generous gift from Dr. Owen Witte, University of California at Los Angeles, were generated on a BALB/c background (22). All experiments reported here were performed on 8- to- 16-week-old homozygous male mice carrying wild-type ($TDAG8^{+/+}$) or knockout ($TDAG8^{-/-}$) allele. All behavioral experiments were performed between 8 AM and 1 PM during the 12-hour light cycle. Study protocols were approved by the Institutional Animal Care and Use Committees of University of Cincinnati and Wright State University, in vivariums accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Carbon Dioxide Behavior Studies

Experimental layout is shown in Figure 2A (details in the Supplement). Mice were habituated to the CO_2 chamber for 10 minutes 1 day before the CO_2 challenge (day 0). On day 1, mice were exposed to air and 5% and 10% CO_2 concentrations for 10 minutes, during which behavior was videotaped. This CO_2 concentration range is translationally relevant to challenge studies in humans (5). The following day (day 2), animals were returned to the chamber for 5 minutes in the absence of CO_2. Freezing, defined as the complete lack of movement except for respiration, was scored using the FreezeScan software (CleverSys, Inc., Reston, VA) by a trained observer blinded to genotype and treatment. After an initial dose-response study, all subsequent experiments were conducted using 5% CO_2.

Radiotelemetry Surgery and Recording

Mice were implanted with telemetric devices (PA-C20; Data Sciences International, New Brighton, MN) to measure blood pressure (BP) and heart rate. To assess responses to CO_2 inhalation, the data were analyzed as response during CO_2 inhalation minus average response 10 minutes before CO_2 inhalation. This is consistent with clinical studies in which cardiovascular effects during CO_2 inhalation were analyzed over baseline pre-exposure measurements (23). For measurements during day 2 context exposure, delta of mean response was calculated over baseline (average of 2 hours before entry into the room) as described previously (24).

Whole-Body Plethysmography

Ventilatory parameters in unrestrained, nonanesthetized mice were measured using whole-body plethysmography, as described previously (25) with modifications. For setup and data collection details, see the Supplement.

Drug Administration

Minocycline (10 μg/500 nL; Sigma-Aldrich, St. Louis, MO) was administered intracerebroventricularly once daily for 4 days before CO_2-inhalation exposure. For the minocycline–IL-1β experiment, IL-1β (5 ng/500 nL; R&D Systems, Minneapolis, MN) was administered to minocycline-treated mice 20 minutes before CO_2 inhalation. For IL-1β necessity and sufficiency experiments, mouse recombinant IL-1RA [an endogenous receptor antagonist that binds selectively to the IL-1 receptor (IL-1R) and prevents signaling via this receptor (1.8 μg/2 μL; R&D Systems)] or IL-1β itself (5 ng/500 nL) was administered 20 minutes before CO_2 or air inhalation, respectively. Doses and duration of minocycline, IL-1RA, and IL-1β were adapted from previous studies (26–28). For surgery details, see the Supplement.

Immunofluorescence

Coronal brain sections were immunolabeled with primary antibodies against green fluorescent protein (GFP) (1:3000; cat. no. A-11122; Invitrogen, Grand Island, NY), anti-ionized calcium binding adapter molecule (1:1000; cat. no. 234-003; Synaptic Systems, Inc., Gottingen, Germany), anti-HuC/D (1:200; cat. no. A-21271; Invitrogen), anti-glial fibrillary acidic protein (1:1000; cat no. ab4674; Abcam, Cambridge, MA) using standard immunofluorescence procedures (see the Supplement for details).

Morphologic Analysis

Methodology to measure morphologic changes in microglia after CO_2 inhalation was adapted from previous studies (29,30). Flattened images from Z-stacks were examined using ImageJ software (National Institutes of Health, Bethesda, MD) to quantify increased soma perimeter and attenuated microglial branching complexity and process length (deramification) that are parameters for assessing microglial activation. (For details, see the Supplement.)

Measurement of Cytokines

Cytokine concentrations were measured using the Bio-Plex Mouse Cytokine Assays (Bio-Rad, Hercules, CA). For details on tissue collection, see the Supplement.

Slice Electrophysiology

Coronal SFO slices (300 μm) were used for whole-cell patch clamp recordings of CO_2-evoked neuronal firing as