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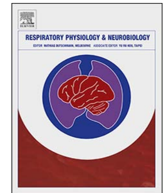

Cyclooxygenase enzyme activity does not impair respiratory motor plasticity after one night of intermittent hypoxia

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ABSTRACT

Although inflammation is prevalent in many clinical disorders challenging breathing, we are only beginning to understand the impact of inflammation on neural mechanisms of respiratory control. We recently demonstrated one form of respiratory motor plasticity is extremely sensitive to even mild inflammation induced by a single night (8 h) of intermittent hypoxia (IH-1), mimicking aspects of obstructive sleep apnea. Specifically, phrenic long-term facilitation (pLTF) following moderate acute intermittent hypoxia (AIH) is abolished by IH-1, but restored by high doses of the non-steroidal anti-inflammatory drug, ketoprofen. Since a major target of ketoprofen is cyclooxygenase (COX) enzymes, we tested the involvement of COX in IH-1 suppression of pLTF using the selective COX inhibitor NS-398. Systemic COX inhibition (3 mg/kg, i.p., 3 h before AIH) had no effect on pLTF in normoxia treated rats (76 ± 40% change from baseline, n = 6), and did not restore pLTF in IH-1 treated rats (–9 ± 7% baseline, n = 6). Similarly, spinal COX inhibition (27 mM, 12 µl, i.t.) had no effect on pLTF in normoxic rats (76 ± 34% baseline, n = 7), and did not significantly restore pLTF after IH-1 (37 ± 18% baseline, n = 7). COX-2 protein is expressed in identified phrenic motor neurons of both normoxia and IH-1 exposed rats, but immunolabeling was minimal in surrounding microglia; IH-1 had no discernable effect on COX-2 immunoreactivity. We conclude that the inflammatory impairment of pLTF by IH-1 is independent of COX enzyme activity or upregulated COX-2 expression.

1. Introduction

Although the precise physiological role(s) of respiratory plasticity are still subject to debate (Gonzalez-Rothi et al., 2015; Fuller and Mitchell, 2017; Mateika and Komnenov, 2017), the ability to express respiratory plasticity can be harnessed to recover lost breathing capacity due to disease or injury (Gonzalez-Rothi et al., 2015). Systemic and neural inflammation accompany clinical disorders compromising breathing (Huxtable et al., 2011). Even mild systemic or neuroinflammation undermines an important form of respiratory motor plasticity, phrenic long-term facilitation (pLTF) following moderate acute intermittent hypoxia (AIH; Vinit et al., 2011; Huxtable et al., 2011; Huxtable et al., 1985). For example, mild systemic inflammation elicited by lipopolysaccharide (LPS) (Huxtable et al., 2011; Vinit et al., 2011) or 1 night of intermittent hypoxia (IH-1) (Huxtable et al., 2015) abolishes moderate AIH-induced pLTF. pLTF sensitivity to mild inflammation has significant implications for exploiting respiratory plasticity for therapeutic advantage. Thus, greater understanding of the mechanisms whereby inflammation undermines plasticity are of considerable importance.

Although detailed mechanism(s) whereby inflammation inhibits pLTF are not yet fully understood, plasticity can be restored by high doses of non-steroidal anti-inflammatory drugs (NSAIDs), such as ketoprofen (12.5 mg/kg) (Huxtable et al., 1985; Huxtable et al., 2015). At typical therapeutic doses, most anti-inflammatory actions of NSAIDs are via cyclooxygenase (COX)-1 and/or COX-2 enzyme inhibition (Cashman, 1996; Cabre et al., 1998; Diaz-Reval et al., 2004; Lleo et al., 2007; Kokki, 2010), suggesting that high- ketoprofen doses restore pLTF after systemic inflammation via COX inhibition. We recently demonstrated spinal administration of p38 MAP kinase inhibitors restores

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pLTF after IH-1 (Huxtable et al., 2015). However, since many p38 MAP kinase inhibitors also inhibit COX activity (Borsch-Haubold et al., 1998) and the specific roles of cyclooxygenases in pLTF inhibition have not been tested, we investigated the impact of selective COX inhibition on pLTF after IH-1.

COX-1 and COX-2 are both expressed in CNS neurons, astrocytes and microglia (Choi et al., 2009). Specifically, COX-2 plays an important pro-inflammatory role, triggering inflammation in response to infection or injury (Bazan, 2001). Further, increased COX-2 activity induced by β-amyloid proteins inhibits other forms of synaptic plasticity such as hippocampal long-term potentiation (LTP); conversely, COX-2 inhibitors restore it (Kotilinek et al., 2008). Since hippocampal LTP shares some mechanistic similarities with AIH-induced pLTF (Mahamed and Mitchell, 2007; Mateika and Sandhu, 2011), we tested the involvement of COX in pLTF impairment after IH-1-induced inflammation, and examined COX-2 expression in phrenic motoneurons, the site of pLTF (Baker-Herman and Mitchell, 2002; Devinney et al., 2015; Dale et al., 2017). Unlike hippocampal LTP, we report here that COX enzymatic activity is not necessary in the mechanism whereby systemic inflammation caused by IH-1 undermines pLTF.

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin–Madison, and conformed to policies in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Experiments were performed on 3–4 month old Harlan male Sprague Dawley rats (colony 217 and 211a) housed under standard conditions, with food and water *ad libitum* and a 12-h light/dark cycle.

2.1. Experimental groups

Rats were placed in custom-designed cylindrical, Plexiglas exposure chambers for 8 h with ClearH_2O® hydrogel (Portland, ME) to provide nutrition and hydration during normoxia or intermittent hypoxia exposures. Gas flow through the chambers was controlled by a customized program (National Instruments, LabVIEW™ 2009, Service Pack 1, version 9.0.1, Austin, TX), and a mass flow controller (Teledyne, Hastings Instruments, Hampton, VA), and O_2 levels were continuously monitored by a Gemini Respiratory Gas Analyzer (CWE, Inc., Ardmore, PA). Over an 8-h period during a standard "dark phase", rats experienced either intermittent hypoxia (2 min of 10.5% O_2, 2 min intervals of 21% O_2) or normoxia (21% O_2). Upon completion of exposures, rats were returned to their home cages until the next day, and then studied in neurophysiology experiments or perfused for immunohistochemistry.

Prior to electrophysiology experiments, rats were assigned to one of three groups to investigate whether pLTF could be restored by systemic application of the COX inhibitor NS-398 (Sigma Chemical Company, St. Louis, MO): 1) Normoxia (Nx) + NS-398 (n = 6); 2) IH–1 + NS-398 (n = 6); and 3) NS-398 Time Controls (TC) (3 Normoxia, 2 IH-1; n = 5). In a second study to investigate whether pLTF could be restored by spinal COX inhibition, rats were divided into six groups: 1) Nx + NS–398 + AIH (n = 6); 2) IH–1 + NS-398 (n = 8); 3) NS-398 Time controls (3 Normoxia, 3 IH-1; n = 8); 4) Nx + veh + AIH (n = 5); 5) IH–1 + veh + AIH (n = 4); and 6) Veh TC (n = 4).

2.2. Electrophysiological experiments

The protocol used in electrophysiological experiments has been described in detail previously (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002; Huxtable et al., 1985; Huxtable et al., 2015). In brief, rats were anesthetized with isoflurane, tracheotomized and pump ventilated (Small Animal Ventilator 683, Harvard Apparatus, Inc., Holliston, MA, USA). After surgery was completed, rats were slowly converted to urethane anesthesia (1.8 g/kg, i.v., Sigma-Aldrich). During a one-hour stabilization period after conversion to urethane, pancuronium bromide (1 mg, i.v.) was given to paralyze the rats. Anesthetic level was assessed by monitoring blood pressure and phrenic nerve responses to toe pinch. Approximately thirty minutes into the stabilization period, an i.v. infusion (1.5–2 ml/hour) began of a solution consisting of Hetastarch (0.3%) and sodium bicarbonate (0.84%) in Lactated Ringers. Infusion rate was adjusted to maintain blood volume, pressure and acid-base balance throughout experiments.

2.2.1. Surgical preparation

Rats were vagotomized and a catheter inserted into the right femoral artery to measure blood pressure and take arterial blood samples. A rectal temperature probe was used to monitor and regulate body temperature. From a dorsal approach, the left phrenic nerve was isolated, cut distally, desheathed and placed on a bipolar silver recording electrode submerged in mineral oil. Nerve activity was amplified (gain X10 K), bandpass filtered (300 Hz to 20 kHz) (A-M Systems, Carlsberg, WA, USA) and integrated (absolute value, Powerlabs 830, AD Instruments, Colorado Springs, CO, USA, time constant 50 ms). The signal was digitized, recorded, and analyzed using Powerlabs 830 (version 7.2.2, AD Instruments).

To examine the effect of spinal COX inhibition, C2 laminectomy was performed and a primed silicone catheter inserted 2 mm caudally so that the catheter tip rested on the C4 dorsal surface, as described previously (Baker-Herman and Mitchell, 2002; MacFarlane and Mitchell, 2009; Huxtable et al., 2015).

2.2.2. Protocol

Baseline nerve activity was established with FIO_2 ∼0.56 (PaO_2 > 300 mmHg) and CO_2 added to the inspired gas (balance nitrogen). The CO_2 apneic threshold was determined by slowly lowering inspired CO2 until phrenic nerve activity ceased. Inspired CO_2 was slowly increased until phrenic nerve activity resumed (recruitment threshold). End-tidal CO_2 was set ∼2 mmHg above the recruitment threshold to establish baseline nerve activity. End-tidal CO_2 was monitored and maintained throughout an experiment using a flow-through capnograph with sufficient response time to measure end-tidal CO2 in rats (Respironics, Andover, MA, USA).

Once phrenic nerve activity was stable, an arterial blood sample was taken to establish baseline conditions; these conditions were maintained for the duration of the experiment. Blood samples (62.5 μl in heparinized plastic catheter) were analyzed for PO_2, PCO_2, pH and base excess using a blood gas analyzer (ABL 800, Radiometer, Copenhagen, Denmark). After establishing baseline conditions, a moderate AIH protocol began consisting of 3 hypoxic episodes (5 min duration, 9–10.5% O2), separated by 5 min of control oxygen levels. Blood samples were taken during the first hypoxic episode, and 15, 30, and 60 min post-AIH. Data were included in analysis only if they complied with the following criteria: 1) PaO_2 during baseline and post-AIH was > 180 mmHg; 2) PaO_2 during hypoxic episodes was between 35–45 mmHg; 3) $PaCO_2$ remained within 1.5 mmHg of baseline throughout the post-AIH period. Maximum hypoxia + hypercapnia was assessed at the end of each experiment. Rats not responding to maximal stimulation were removed from analysis. Phrenic nerve amplitude was evaluated immediately before each blood sample and during minute two of hypoxia. Upon completion of experiments, rats were euthanized with a urethane overdose.

2.3. Data analysis

Peak amplitude of integrated phrenic nerve activity was averaged for 30 bursts at each time point of interest. Changes in phrenic nerve burst amplitude were normalized to baseline values (percent change from baseline).

Statistical comparisons for hypoxic responses were made from data at minute two during the first hypoxic episode using a *t*-test (systemic

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