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Review

Ibuprofen blocks time-dependent increases in hypoxic ventilation in rats☆

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ABSTRACT

Recently, inflammatory processes have been shown to increase O_2-sensitivity of the carotid body during chronic sustained hypoxia [Liu, X., He, L., Stensaas, L., Dinger, B., Fidone, S., 2009. Adaptation to chronic hypoxia involves immune cell invasion and increased expression of inflammatory cytokines in rat carotid body. Am. J. Physiol. Lung Cell Mol. Physiol. 296, L158–L166]. We hypothesized that blocking inflammation with ibuprofen would reduce ventilatory acclimatization to hypoxia by blocking such increases in carotid body O_2 sensitivity. We tested this in conscious rats treated with ibuprofen (4 mg/kg IP daily) or saline during acclimatization to hypoxia ($P_{I_{O_2}} = 70$ Torr for 7 days). Ibuprofen blocked the increase in hypoxic ventilation observed in chronically hypoxic rats treated with saline; ibuprofen had no effects on ventilation in normoxic control rats. Ibuprofen blocked increases in inflammatory cytokines (IL-1β, IL-6) in the brainstem with chronic hypoxia. The data supports our hypothesis and further analysis indicates that ibuprofen also blocks inflammatory processes in the central nervous system contributing to ventilatory acclimatization to hypoxia. Possible mechanisms linking inflammatory and hypoxic signaling are reviewed.

1. Introduction

Ventilatory acclimatization to hypoxia is a time-dependent increase in ventilation during chronic sustained hypoxia (Powell et al., 1998). Several laboratories have demonstrated that this involves increased O_2-sensitivity of carotid body chemoreceptors resulting in increased afferent input to ventilatory chemoreflexes for a given arterial P_{O_2} after chronic hypoxia (reviewed by Powell, 2007). Additionally, we have shown time-dependent changes in the central nervous system (CNS) processing of carotid body chemoreceptor input resulting in a greater respiratory motor output for a given afferent input (Dwinell and Powell, 1999). Plasticity in the carotid body-ventilatory chemoreflex with chronic hypoxia involves changes in neurotransmitters and ion channels in the carotid bodies and CNS (reviewed by Powell, 2007), and changes in gene expression controlled by Hypoxia Inducible Factor 1, HIF-1 (Kline et al., 2002; Powell and Fu, 2008).

Recently, inflammatory processes have been shown to be important for the increased O_2-sensitivity of carotid bodies with chronic sustained hypoxia (Liu et al., 2009). Using an *in vitro* rat carotid body preparation, these investigators showed that chronic sustained hypoxia increases the frequency of action potentials recorded in the carotid sinus nerve when P_{O_2} is lowered in a solution superfusing the carotid body. They also found increased mRNA expression for inflammatory cytokines in carotid bodies from chronically hypoxic rats. The increased cytokine expression, as well as the increased carotid body neural response to acute hypoxia, was blocked by ibuprofen and dexamethasone treatment during the chronic hypoxia.

To test the physiological significance of these inflammatory signals for plasticity in chronically hypoxic carotid bodies, we studied the effects of a nonsteroidal anti-inflammatory drug, ibuprofen, on ventilatory acclimatization to hypoxia in conscious rats. Also, we measured the effects of chronic hypoxia and ibuprofen on cytokine expression in the CNS. Ibuprofen decreased ventilatory acclimatization to hypoxia and cytokine gene expression in the CNS, providing evidence that inflammatory signaling in the CNS contributes to ventilatory acclimatization to hypoxia.

2. Methods

2.1. Experimental animals

Adult, male rats (Sprague–Dawley, Charles River) were housed in standard rat cages in a vivarium, with a 12:12-h light–dark cycle and fed a standard rat diet *ad libitum*. All experiments were approved by the University of California, San Diego, Animal Care

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and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals." At the end of an experiment, rats were euthanized with an overdose of sodium pentobarbital (Sleep Away, 150 mg/kg IV) and death was confirmed by open chest exsanguination via cardiac ventricular incision.

2.2. Experimental groups

Animals were housed in individual cages in either normoxia (N) or chronic hypoxia (CH). Rats acclimatizing to CH were placed in a hypobaric chamber for 7 days at 0.5 atm ($P_{I_{O_2}} = 70$ Torr); N rats were housed in the same room outside the chamber. CH rats were significantly lighter than N rats at the time of experiments (268 ± 8 g vs. 349 ± 7 g). The chamber was opened once daily for ~20 min for regular cage maintenance and drug injection or for removing animals for experimentation. Four experimental groups were studied: (1) NS = chronic normoxia with saline injections, (2) NI = normoxia with ibuprofen injections, (3) HS = CH injected with saline, and (4) HI = CH injected with ibuprofen.

2.3. Experimental drugs

Ibuprofen solution (2 mg/mL) was prepared from commercially available syrup (20 mg/mL, McNeil Children's Motrin Berry-Flavored, Lot SHM046) diluted with sterile normal saline (0.9% NaCl). Ibuprofen injections (4 mg/kg IP, ≈0.6 mL) and saline injections of a similar volume (0.6 mL) were made daily.

2.4. Surgical procedures

After 5 days in designated experimental conditions, we catheterized animals to sample arterial blood gases. Anesthesia was induced with 5% isofluorane in 100% O_2 and isofluorane was decreased to 2.5–3% for maintenance. A customized catheter (polyethylene PE-50 drawn out under heat to an end diameter approximately equivalent to PE-10 tubing) was inserted in the femoral artery through a skin incision. The catheter was advanced into the abdominal aorta, secured to the artery with suture (5.0 silk) tied around tubing glued to the outside of the catheter, filled with heparin (10,000 Units/mL) and heat-sealed at distal end. The distal end of the catheter was led subcutaneously along the back to emerge from a metal button (Instech) sutured to underlying muscle at the base of the neck. A spring sheath secured to the button protected the distal end of the catheter.

Finally, a telemetric thermometer (G-2 Emitter, Respironics) was implanted in the abdominal cavity through a midline incision. Muscle and skin were sutured together separately and antibiotic ointment (Fura-Zone, Squire) was placed over every incision site.

2.5. Physiological measurements

After 1 week of acclimatization, a whole body plethysmograph (7L, plexiglass) was used to measure ventilatory responses to hypoxia and hypercapnia measurements as previously described (Reid and Powell, 2005). Briefly, flow (3 L/min) was maintained through the chamber during ventilatory measurements and pressure changes due to warming and humidification of inhaled gases were measured (MP45 with 2 cm H_2O diaphragm, Validyne) and used to calculate tidal volume by the method of Drorbaugh and Fenn (1955). Arterial blood was sampled through polyethylene tubing (PE 50) leading out the top of the chamber and attached to the arterial catheter with 25 gauge hypodermic tubing. O_2 and CO_2 concentrations in the chamber were changed with a mass flow controller (Sable Systems MFC-4) and monitored with a mass spectrometer (Marquette, 1100). Ventilatory data was digitized (Labview, National Instruments, v2.5.0) and analyzed using a custom Matlab-based program for V_T, f_R and their product, $\dot{V}_I$.

Ventilatory measurements were performed with baseline O_2 levels equal to the environment in which the animals were housed the previous week (NS and NI = 21%; HS and HI = 10%). The animals acclimated to the box for 30 min and were then given a 5–10 min challenge (10% O_2 for NS and NI; 21 or 30% O_2 for HS and HI) to test responsiveness. Then the protocol began to measure ventilation in normoxia (21% O_2), hypoxia (10% O_2) and hypercapnia (7% CO_2, 30% O_2) with a return to baseline conditions (e.g. 21% O_2 for NS and NI) for 15 min between conditions. Different gas levels were maintained for 15 min and ventilation was measured in a stable sample with at least 20 breaths between 10 and 15 min after changing gas concentrations. Arterial blood was sampled (0.2 mL) during ventilatory measurements for P_{O_2}, P_{CO_2}, pH and hematocrit analysis (GEM Premier 3000, Instrumentation Laboratory). Blood gas values were corrected to body temperature as measured with the telemeter.

In a separate cohort of rats ($n = 3$–5 per experimental group), we measured metabolic rates in normoxia and hypoxia (10% O_2). The protocol was similar to that for the ventilatory measurements described above. O_2 and CO_2 concentrations were measured in the plethysmograph under steady state conditions and then gas flow into the chamber was blocked and the chamber was sealed for 3 min. O_2 and CO_2 concentrations were measured at the end of this period, then flow through the chamber was restored and $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were calculated using mass balance.

2.6. Cytokin gene expression measurements

In a third cohort of rats exposed to the same experimental conditions ($n = 5$ per group) we measured expression of mRNA for IL-1β, IL-6, and TNFα in biopsies of the nucleus tractus solitarii (NTS) using quantitative PCR (qPCR). Rats were euthanized and their brainstems were removed and immediately frozen in liquid nitrogen. Samples were allowed to thaw just enough to make a transverse section between the *calamus scriptorius* and 2 mm rostral. This section was refrozen in liquid nitrogen and then allowed to thaw just enough to allow the dorsal region, containing the nucleus tractus solitarii (NTS), to be cut away from the remainder of the section. The NTS biopsies were placed in a 1.5 mL Eppendorf tube and weighed. We extracted mRNA in 150 μL buffer in the Eppendorf tubes (RNeasy Minikit, Qiagen, and rapid homogenization), adjusted the final volume to 600 μL with buffer and performed an added step of on-column DNase digestion (Qiagen). Concentration of mRNA was determined with a micro-volume UV–vis spectrophotometer (Nanodrop, 2000). A cDNA synthesizing kit (Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR) was used to reverse transcribe the mRNA and prepare the samples for qPCR using Power SYBR Green (Applied Biosystems). qPCR reactions were performed in duplicate on the same day with reaction mix (SYBR green, primers, RNase free H_2O, and cDNA) for each reaction made together to reduce pipetting errors. Primer sequences were as follows (FW, forward; RW, reverse):

Rat IL-1β:	FW CACCTCTCAAGCAGAGCACAG
	RW GGGTTCCATGGTGAAGTCAAC
Rat IL-6:	FW TCCTACCCCAACTTCCAATGCTC
	RW TTGGATGGTCTTGGTCCTTAGCC
Rat TNFα:	FW AAATGGGCTCCCTCTCATCAGTTC
	RW TCTGCTTGGTGGTTTGCTACGAC

Housekeeping genes used to normalize the above cytokines were Rat β-2 microglobulin (B2M) and ribosomal protein L13a (RPL13A), which are unchanged by hypoxia in the central nervous system (Tang et al., 2010). Primer sequences were as follows (FW, forward; RW, reverse):