Human intermittent hypoxia-induced respiratory plasticity is not caused by inflammation

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ABSTRACT Ventilatory instability, reflected by enhanced acute hypoxic (AHVR) and hypercapnic (AHCVR) ventilatory responses is a fundamental component of obstructive sleep apnoea (OSA) pathogenesis. Intermittent hypoxia-induced inflammation is postulated to promote AHVR enhancement in OSA, although the role of inflammation in intermittent hypoxia-induced respiratory changes in humans has not been examined. Thus, this study assessed the role of inflammation in intermittent hypoxia-induced respiratory plasticity in healthy humans.

In a double-blind, placebo-controlled, randomised crossover study design, 12 males were exposed to 6 h of intermittent hypoxia on three occasions. Prior to intermittent hypoxia exposures, participants ingested (for 4 days) either placebo or the nonsteroidal anti-inflammatory drugs indomethacin (nonselective cyclooxygenase (COX) inhibitor) and celecoxib (selective COX-2 inhibitor). Pre- and post-intermittent hypoxia resting ventilation, AHVR, AHCVR and serum concentration of the pro-inflammatory cytokine tumour necrosis factor (TNF)-α were assessed.

Pre-intermittent hypoxia resting ventilation, AHVR, AHCVR and TNF-α concentrations were similar across all three conditions (p$\geqslant$0.093). Intermittent hypoxia increased resting ventilation and the AHVR similarly across all conditions (p=0.827), while the AHCVR was increased (p=0.003) and TNF-α was decreased (p=0.006) with only selective COX-2 inhibition.

These findings indicate that inflammation does not contribute to human intermittent hypoxia-induced respiratory plasticity. Moreover, selective COX-2 inhibition augmented the AHCVR following intermittent hypoxia exposure, suggesting that selective COX-2 inhibition could exacerbate OSA severity by increasing ventilatory instability.

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Introduction

Obstructive sleep apnoea (OSA) is a chronic medical disorder associated with increased cardiovascular and cerebrovascular morbidity and mortality [1, 2]. OSA is characterised by repetitive apnoeas during sleep due to collapse of the pharynx resulting in exposure to intermittent hypoxia (IH) and hypercapnia. Ventilatory instability, reflected in part by enhanced acute ventilatory responses to hypoxia (AHVR) [3] and hypercapnia (AHCVR) [4], is positively correlated with OSA severity [5] and contributes to OSA pathogenesis [6].

IH exposure is thought to contribute to increased ventilatory instability in OSA as both acute (minutes to hours) and chronic (days to years) exposure increase the AHVR [7–11] and the AHCVR [11–14], although these are not consistent findings [8, 15]. The progressive increase in the AHVR and AHCVR following IH may be considered as forms of respiratory plasticity (defined as a continuing alteration in the neural system controlling breathing based on prior experience [16]). In relation to the increased ventilatory response to hypoxia following brief IH exposure, this form of respiratory plasticity has been labelled "progressive augmentation" [17]. The molecular pathways underlying IH enhanced ventilatory chemosensitivity are poorly understood, but involve increased oxidative stress [3, 8, 18–24] and inflammation [18, 25]. In rats, prevention of IH-induced systemic and carotid body oxidative stress abolishes the enhanced carotid body chemosensory response to hypoxia [20] and the AHVR [26]. Furthermore, using an experimental human model of IH, we previously reported that the increase in AHVR with IH was correlated with IH-induced oxidative stress [8]. Whether oxidative stress enhancement of carotid body chemoreception enhances the AHVR directly is still under debate, as direct application of reactive oxygen species (ROS) to the carotid body does not produce excitation and modification of carotid body ROS production does not modify catecholaminergic secretory responses to hypoxia [18, 22, 27]. Moreover, inhibition of pro-inflammatory cytokines downstream from oxidative stress with the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen (a nonselective cyclooxygenase (COX) inhibitor) abrogates the IH-induced AHVR enhancement [18]. This abolition of AHVR enhancement was in conjunction with minimised carotid body tumour necrosis factor (TNF)-α and interleukin (IL)-1β levels, and the number of c-fos positive neurons in the caudal nucleus tractus solitarii (NTS).

As OSA is associated with elevated markers of inflammation [28], these findings suggest that NSAIDs may provide a therapeutic pathway for decreasing OSA severity *via* decreasing ventilatory instability [18, 25], but the role of inflammation in IH-induced augmentation of ventilatory chemosensitivity to hypoxia and hypercapnia in humans has not been examined. Thus, using our experimental human model of IH shown to increase both oxidative stress and the AHVR [8, 29], this study assessed the role of inflammation in IH-induced respiratory plasticity in healthy humans using two widely prescribed NSAIDs: indomethacin (nonselective COX inhibitor) and celecoxib (selective COX-2 inhibitor). It was hypothesised that inflammation is involved in IH-induced respiratory changes in humans and that NSAID use would prevent these respiratory changes.

Methods

Approvals

This study was approved by the Conjoint Health Research Ethics Board at the University of Calgary (Calgary, AB, Canada) and performed according to the Declaration of Helsinki. After initial contact, volunteers were familiarised to the experimental set-up, instrumentation and provided with an informed consent form.

Participants

15 healthy males volunteered for this double-blind (*i.e.* to experimenter and participant), placebo-controlled, randomised crossover study between September 2010 and October 2011. Recruitment was ended after achieving our desired sample size (online supplementary material). Two volunteers declined to participate and 13 provided written informed consent (online supplement and fig. S1). Subsequently, one participant did

not complete the study due to an adverse reaction to the first allocated medication [30]. Thus, 12 participants completed the experimental protocol and their characteristics have been published previously [30]. Briefly, participant characteristics were as follows (mean±SEM): age 25.8±1.5 years, body mass index 24.9±0.7 kg·m^{-2}, normotensive (mean arterial blood pressure 83.0±0.7 mmHg) and no sleep-disordered breathing (respiratory disturbance index (RDI) 1.8±0.1 events·h^{-1} and mean arterial oxyhaemoglobin saturation (S_{aO_2}) 94.8±0.1%). Additional details are presented in the online supplementary material.

Experimental protocol

Participants were exposed to experimental IH in the Laboratory of Human Cerebrovascular Physiology (University of Calgary) on three occasions administered in a double-blind, placebo-controlled, randomised crossover fashion. For 4 days preceding each IH exposure, participants ingested either placebo (*p.o.* three times daily at 08:00 h, 14:00 h and 20:00 h), indomethacin (50 mg *p.o.* three times daily at 08:00 h, 14:00 h and 20:00 h) or celecoxib (200 mg *p.o.* twice daiily at 08:00 h and 20:00 h). To maintain the double-blinded nature of the study, a placebo pill was included in the celecoxib condition as the second pill. On experimental days (day 5), the dosage regimen was maintained through to the end of physiological testing. Therefore, only the final medication dose (scheduled for 20:00 h) was omitted in all conditions. After each IH exposure, there was a minimum 4-day washout period [30]. Additional details regarding medication dosages are provided in the online supplementary material.

On experimental days, participants arrived at ∼08:00 h and provided capillary and venous blood samples shortly thereafter. Next, using the technique of dynamic end-tidal forcing [31] participants underwent an acute test to assess ventilatory responses to isocapnic hypoxia and hypercapnic hyperoxia. Next, participants were exposed to 6 h of IH. Post-IH, capillary and venous blood samples were collected and ventilatory responses to isocapnic hypoxia and hypercapnic hyperoxia were reassessed. A 6-h IH exposure was chosen because our previous findings using a 4-day IH protocol revealed the greatest increase in the AHVR and oxidative stress occurred after the first 6-h IH exposure [8].

Intermittent isocapnic hypoxia

Utilising a custom-built, normobaric hypoxic room, IH consisted of cycling participants' end-tidal oxygen tension (P_{ETO_2}) between 88 Torr (S_{aO_2}≈96–97%) and 45 Torr (S_{aO_2}≈80–85%) every 60 s for 6 h, thus replicating an RDI of 30 events·h^{-1} as observed in patients with moderate-to-severe OSA. End-tidal carbon dioxide tension (P_{ETCO_2}) was maintained at normal levels throughout IH exposures, as described previously [31]. Respired gases were continuously sampled from a nasal cannula at 150 mL·min^{-1} and analysed for CO_2 and O_2 fractions by a dual gas analyser (Normocap Oxy; Datex-Ohmeda, Louisville, CO, USA), permitting breath-by-breath determination of P_{ETO_2} and P_{ETCO_2} throughout IH exposures. Additionally, S_{aO_2} was monitored with an earlobe pulse oximeter (3900P; Datex-Ohmeda, Madison, WI, USA). Participants were not permitted to sleep during the IH exposures. Additional details are provided in the online supplementary material.

Pre- and post-IH assessment of resting ventilation, AHVR and AHCVR

The primary study outcome was the effect of the anti-inflammatory medications on the AHVR augmentation following IH. The pre-IH test started with 10 min of air breathing to determine resting P_{ETCO_2} and P_{ETO_2}. Next, P_{ETCO_2} was increased to +1 Torr above resting levels and P_{ETO_2} was maintained at euoxic (88 Torr) for 5 min (isocapnic euoxia baseline). Subsequently, P_{ETCO_2} was maintained at +1 Torr while P_{ETO_2} was cycled between 45 and 88 Torr every 90 s until six hypoxic bouts were completed. After the sixth hypoxic bout, P_{ETCO_2} was maintained at +1 Torr while P_{ETO_2} was increased to 300 Torr for 5 min (isocapnic hyperoxia rest), after which P_{ETCO_2} was increased to +9 Torr above resting levels for an additional 5 min (hypercapnic hyperoxia), followed by a return to isocapnic euoxia for a 5-min recovery. Figure 1 shows a schematic of this acute test; technical details and data analyses are provided in the online supplementary material.

Inflammatory markers

Serum TNF-α and IL-1β concentrations were measured *via* ELISA (Ready-SET-Go!; eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Both TNF-α and IL-1β kits had a sensitivity of 4 pg·mL^{-1} and range of 4–500 pg·mL^{-1}.

Statistical analyses

All dependent variables were assessed for normality using the Shapiro–Wilk test. Subsequently, a three- (drug: placebo, indomethacin or celecoxib) by-two (IH: pre- and post-) factor repeated-measures ANOVA was used to compare dependent variables. TNF-α data were not normally distributed and were analysed using an aligned rank transform and repeated measures ANOVA [32]. If main effects were significant, *post hoc* comparisons were performed incorporating a Bonferonni correction for multiple comparisons. α was
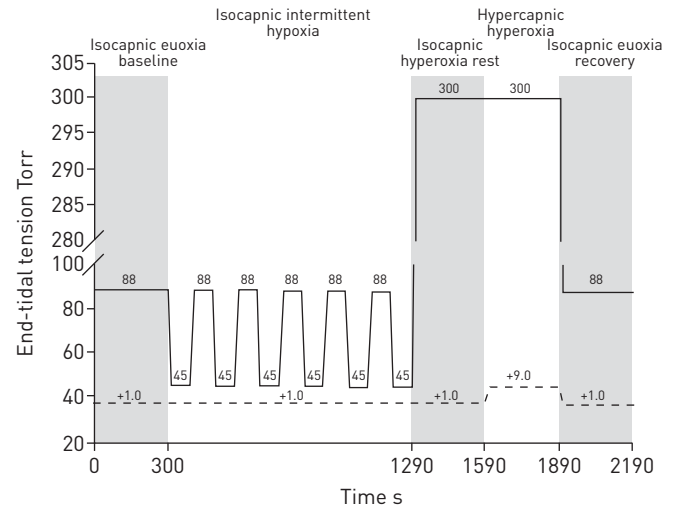
FIGURE 1 Schematic of the acute test undergone by participants before and after exposure to 6 h of isocapnic intermittent hypoxia. Solid line: end-tidal oxygen tension; dashed line: end-tidal carbon dioxide tension.

set *a priori* at 0.05 and statistical analyses were performed using SPSS (version 21; IBM Corporation, Armonk, NY, USA). Results are presented as mean±SEM. Post-IH blood sampling was unsuccessful for one participant. Thus, that participant was removed from the analyses of TNF-α and IL-1β concentrations.

Results
Isocapnic euoxia baseline

There was a significant effect (p=0.020) of ingesting the medications for 4 days on resting, air-breathing P_{ETCO_2} (placebo 37.7±0.6 Torr; nonselective COX inhibition 36.6±0.6 Torr, p=0.026 *versus* placebo; and selective COX-2 inhibition 37.1±0.7 Torr, p=0.298 *versus* placebo and p=0.721 *versus* nonselective COX inhibition), which was, by design, translated into the isocapnic euoxia baseline (table 1).

By design, IH had no effect on P_{ETCO_2} during the isocapnic euoxia baselines (p=0.819). In contrast, P_{ETO_2} during the isocapnic euoxia baseline was similar across all drug conditions (p=0.502) and pre- and post-IH conditions (p=0.949). Likewise, the drug main effect was not significant for minute ventilation (V'_E) (p=0.383), tidal volume (V_T) (p=0.985) and respiratory frequency (f_R) (p=0.428), although IH had a significant effect on V'_E (p=0.003) and f_R (p=0.004), but not V_T (p=0.120). Compared to pre-IH values,

TABLE 1 Respiratory parameters during the isocapnic euoxia baseline prior to undergoing the six cycles of hypoxia and during the isocapnic hyperoxia rest period prior to undergoing the hypercapnic challenge

	Placebo		Nonselective COX inhibition		COX-2 inhibition	
	Pre-IH	**Post-IH**	**Pre-IH**	**Post-IH**	**Pre-IH**	**Post-IH**
Isocapnic euoxia baseline						
P_{ETCO_2} Torr	38.8±0.4	38.9±0.4	37.7±0.5#	37.7±0.5#	38.3±0.6	38.2±0.6
P_{ETO_2} Torr	87.9±0.2	87.8±0.2	87.9±0.2	87.9±0.2	87.6±0.2	88.1±0.1
V'_E L·min^{-1}	11.8±0.4	14.9±0.8***	12.6±0.9	16.4±2.1	12.4±0.7	16.2±1.1**
V_T L	1.0±0.12	0.98±0.09	0.92±0.07	1.03±0.11	0.91±0.07	1.05±0.10
f_R breaths·min^{-1}	13.2±0.9	16.1±0.9**	14.1±0.7	16.0±0.9*	14.1±0.5	16.5 ± 1.2*
Isocapnic hyperoxia rest						
P_{ETCO_2} Torr	38.7±0.5	38.6±0.5	37.3±0.5#	37.4±0.5	38.3±0.6	38.3±0.6
P_{ETO_2} Torr	300.5±0.2	300.9±0.3	300.4±0.3	301.0±0.8	300.5±0.2	300.0±0.1
V'_E L·min^{-1}	18.0±0.9	21.8±2.0*	18.5±2.6	24.7±3.9	16.6±1.0	20.6±2.0*
V_T L	1.13±0.12	1.20±0.12	1.20±0.15	1.31±0.18	1.08±0.09	1.14±0.10
f_R breaths·min^{-1}	17.1±1.0	18.7±0.9	15.7±1.2	18.5±0.9*	15.9±0.8	18.1±0.7**

Data are presented as mean±SEM. COX: cyclooxygenase; IH: intermittent hypoxia; P_{ETCO_2}: end-tidal carbon dioxide tension; P_{ETO_2}: end-tidal oxygen tension; V'_E: pulmonary minute ventilation; V_T: tidal volume; f_R: respiratory frequency. *: p⩽0.05 *versus* pre-IH value; ** : p⩽0.01 *versus* pre-IH value; ***: p⩽0.001 *versus* pre-IH value; #: p⩽0.05 *versus* placebo pre-IH values.

post-IH V'_E was higher within the placebo (p<0.001) and COX-2 inhibition conditions (p=0.003), and showed a trend to be greater within the nonselective COX inhibition condition (p=0.072). f_R was increased in all three drug conditions following IH (p⩽0.049).

Ventilatory responses to hypoxia

Figure 2 shows pre- and post-IH group averages of the overlaid hypoxia cycles during the acute test. For all drug conditions, pre- and post-IH P_{ETCO_2} (p⩾0.569), P_{ETO_2} (p⩾0.158) and V_T (p⩾0.087) were not
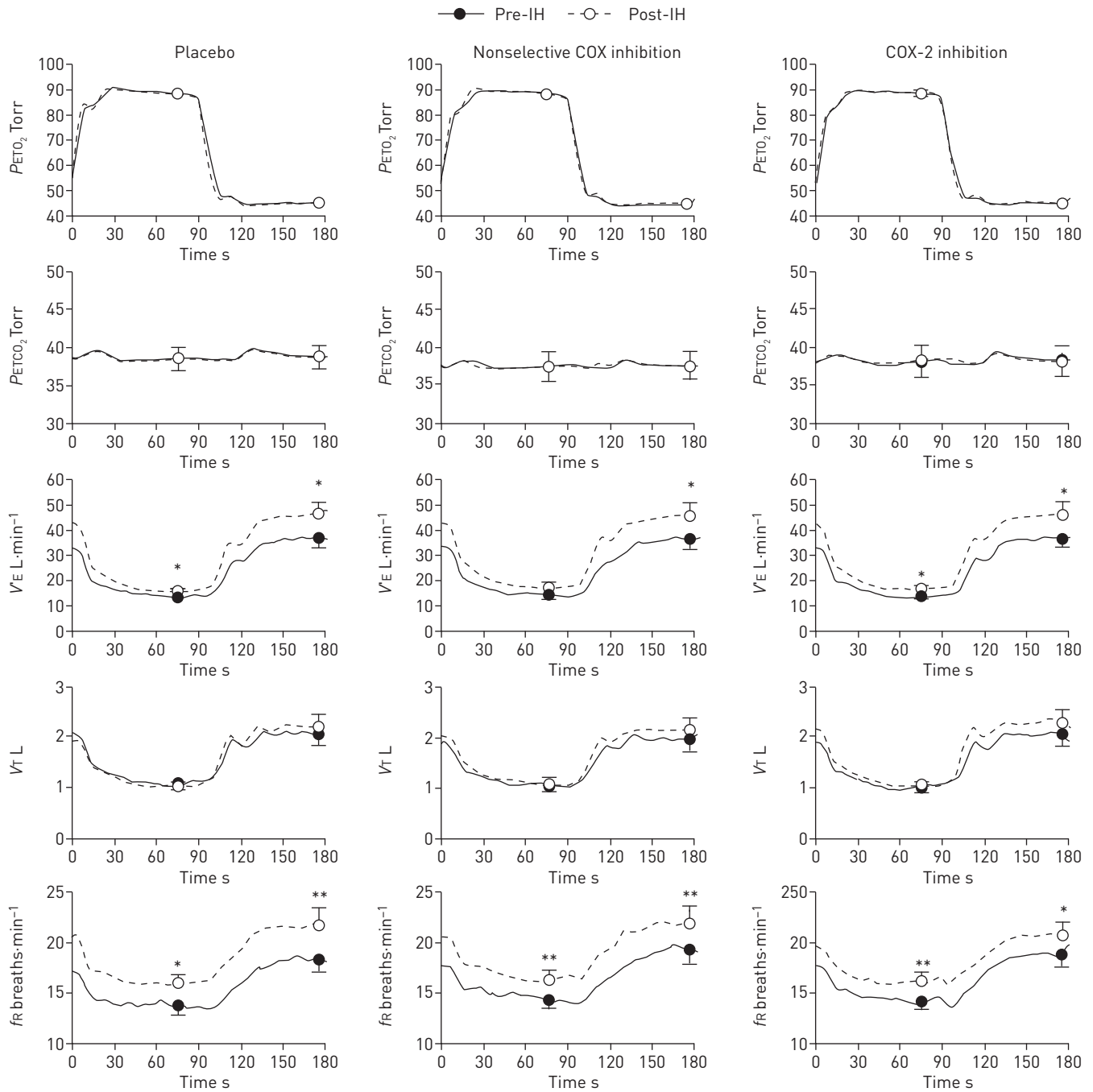

FIGURE 2 Group mean ventilatory responses to isocapnic hypoxia assessed during the acute tests performed before and after the 6-h intermittent hypoxia (IH) exposure for the placebo, nonselective COX inhibition and selective COX-2 inhibition conditions. For each of the six cycles of hypoxia performed during the acute tests, all variables were interpolated at 1 s, overlaid and averaged, thus creating a single group mean 180-s response (0–90 s: isocapnic-euoxia; 91–180 s: isocapnic hypoxia). Pre- and post-IH data are presented as means±SEM for the final 30 s of the two stages of the challenge (isocapnic euoxia: 0–90 s and isocapnic hypoxia: 91–180 s). P_{ETO_2}: end-tidal oxygen tension; P_{ETCO_2}: end-tidal carbon dioxide tension; V'_E: pulmonary minute ventilation; V_T: tidal volume; f_R: respiratory frequency. *: p<0.05 versus pre-IH value; **: p<0.01 versus pre-IH value.

different. In contrast, $V'E$ (p<0.001) and fR (p<0.001) were elevated after IH in all conditions. For $V'E$, there was a significant interaction between the two stages of the hypoxic test (0–90 s: isocapnic euoxia; 91–180 s: isocapnic hypoxia) and IH exposure within all drug conditions (p⩽0.023), with a greater $V'E$ in hypoxia after the IH exposure (p⩽0.023).

Figure 3 shows the pre- and post-IH AHVR for all conditions. Irrespective of IH exposure (drug main effect), the AHVR was similar across all three conditions (p=0.642). The IH main effect was significant (p=0.005), with the post-IH AHVR being greater in all conditions (p⩽0.026). Finally, the drug-by-IH interaction was not significant (p=0.827), indicating the IH-induced AHVR augmentation was similar between the placebo (fig. 3a), nonselective COX inhibition (fig. 3b) and the selective COX-2 inhibition (fig. 3c) conditions.

Isocapnic hyperoxia rest

Pre- and post-IH mean ventilatory data during the final minute of the 5-min isocapnic hyperoxia rest are shown in table 1. By design, the difference in P_{ETCO_2} between the three drug conditions during the isocapnic euoxia baseline was maintained (drug main effect p=0.013), while pre- and post-IH P_{ETCO_2} were the same (IH main effect p=0.751). Furthermore, P_{ETO_2} was similar across all three drug conditions (p=0.275) and pre- and post-IH (p=0.567). Similar to the isocapnic euoxia baseline, the drug main effect was not significant for $V'E$ (p=0.253), V_T (p=0.317) or fR (p=0.212) while the IH main effect was significant for $V'E$ (p=0.012) and fR (p=0.003), but not V_T (p=0.266). Post-IH, $V'E$ was greater within the placebo (p=0.025) and COX-2 inhibition conditions (p=0.031), and showed a trend to be greater within the nonselective COX inhibition condition (p=0.059). fR was increased in the non-selective (p=0.015) and selective COX-2 inhibition (p=0.002) conditions, with a trend for an increase within the placebo condition (p=0.068).

Ventilatory responses to hypercapnia

Pre- and post-IH group averages for the hypercapnic component of the acute test are shown in figure 4. Within all drug conditions, pre- and post-IH P_{ETCO_2} (p⩾0.533), P_{ETO_2} (p⩾0.142) and V_T (p⩾0.181) were not different. In contrast, $V'E$ (p⩽0.050) and fR (p⩽0.034) were increased following IH. In contrast to hypoxia, the interaction between the stages of the hypercapnic hyperoxia test (0–180 s isocapnic hyperoxia; 181–480 s: isocapnic hypoxia) and IH was significant for only the selective COX-2 inhibition condition (p=0.007), with a greater increase in $V'E$ during hypercapnia after IH exposure (p=0.007). This was reflected in a greater AHCVR following IH exposure (fig. 5c), although when comparing the AHCVR across all three conditions the drug main effect was not significant (p=0.093). Moreover, the drug-by-IH interaction was also not significant (p=0.086). When comparing the AHCVR across the three conditions using a 3×2 repeated measures ANOVA, the result that neither the drug main effect, nor the drug-by-IH interaction was significant is probably the consequence of the increase in AHCVR with selective COX-2 inhibition, although significant, being relatively small.

Inflammatory markers

TNF-α concentrations were below the detectable limit (4 pg·mL^{-1}) for one participant. Thus, TNF-α concentrations for 10 participants are shown in figure 6. There was no drug (p=0.394) or IH (p=0.292) effect, but the drug-by-IH interaction was significant (p=0.046), driven by a decrease in TNF-α after IH within the selective COX-2 inhibition condition (p=0.009; fig. 6c). Serum IL-1β concentrations were below the detectable limit (4 pg·mL^{-1}) for eight participants (online supplementary table S2). Thus, no statistical analyses were performed.
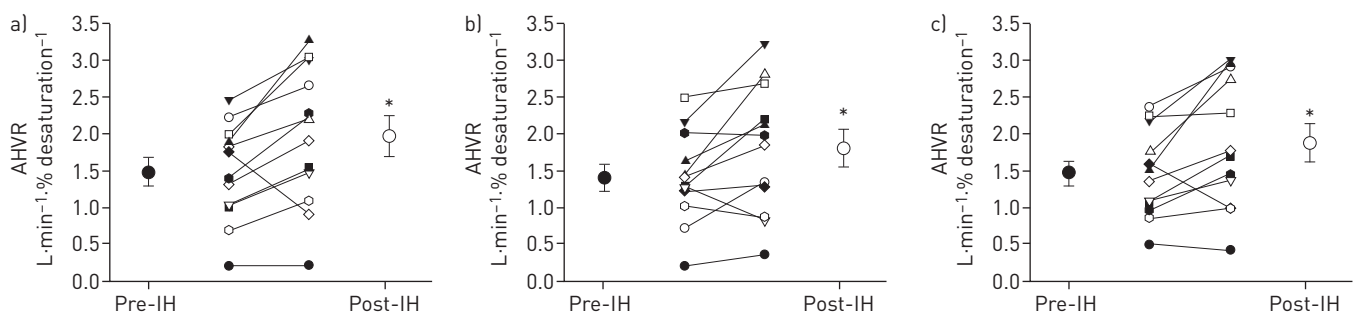

FIGURE 3 Individual and mean±SEM acute hypoxic ventilatory response (AHVR) before (filled symbols) and after (clear symbols) exposure to 6 h of intermittent hypoxia (IH) within the a) placebo, b) nonselective cyclooxygenase (COX) inhibition and c) selective COX-2 inhibition conditions. *: p<0.05 versus pre-IH value.
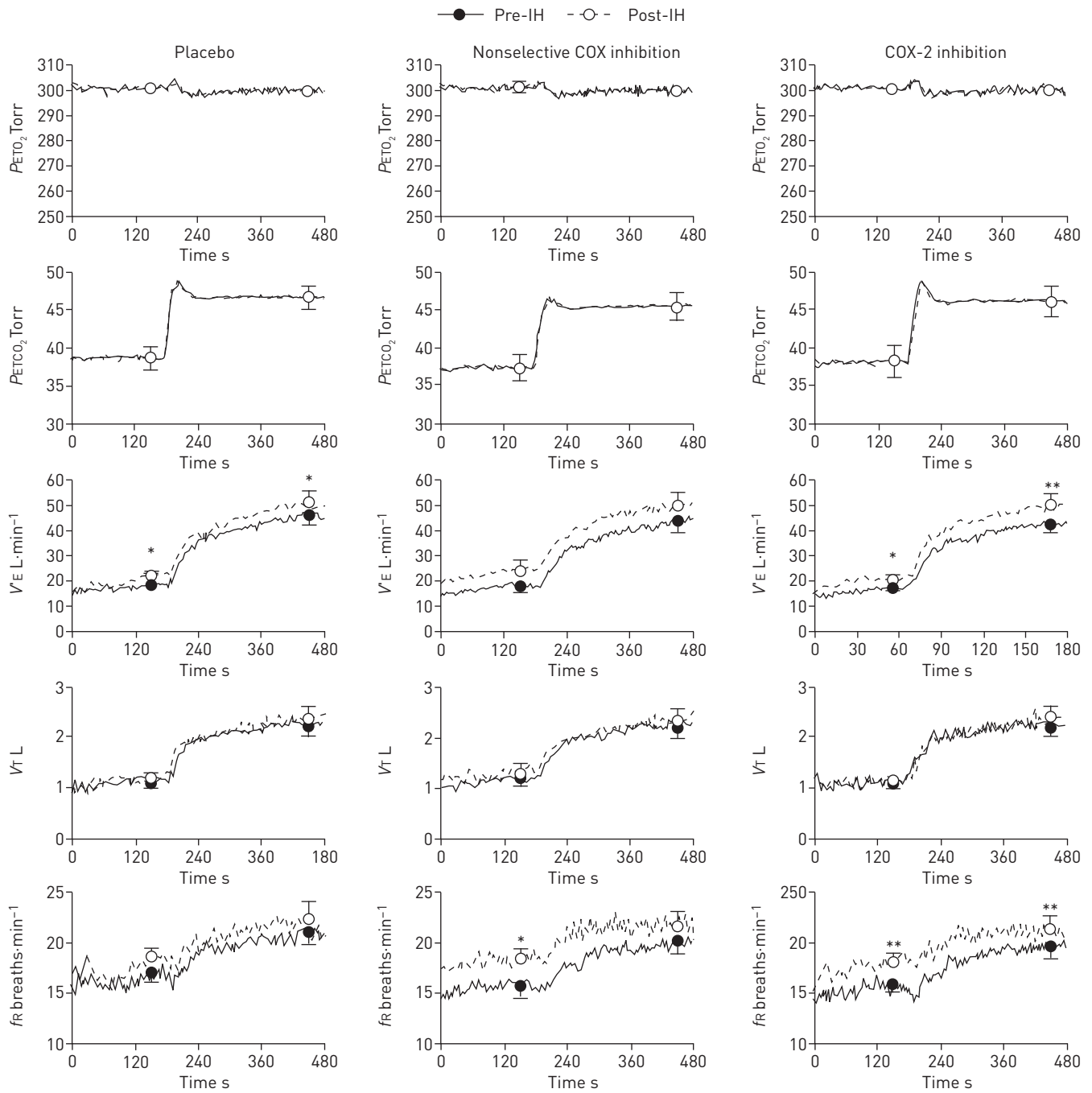
FIGURE 4 Group mean ventilatory responses to hypercapnic hyperoxia assessed during the acute test performed before, and after, the 6-h intermittent hypoxia (IH) exposure for the placebo, nonselective cyclooxygenase (COX) inhibition and selective COX-2 inhibition conditions. For all variables, data from the final 3 min of isocapnic hyperoxia rest and the full 5 min of hypercapnic hyperoxia were interpolated at 1 s to permit calculation of the group mean. Pre- and post-IH data points are the means±SEM of the final 60 s for the two stages of the challenge (isocapnic hyperoxia 0–180 s and isocapnic hypoxia 181–480 s). P_{ETO_2}: end-tidal oxygen tension; P_{ETCO_2}: end-tidal carbon dioxide tension; V'_E: pulmonary minute ventilation; V_T: tidal volume; f_R: respiratory frequency. *: $p<0.05$ versus pre-IH value; **: $p<0.01$ versus pre-IH value.

Results of pre- and post-IH capillary blood samples collected are contained within the online supplement (table S2).

Discussion

This is the first study to assess the mechanistic role of inflammation in IH-induced respiratory plasticity in healthy humans. The major finding is that neither nonselective nor selective COX-2 NSAIDs (indomethacin and celecoxib, respectively) prevented the elevation of isocapnic euoxia resting ventilation or the
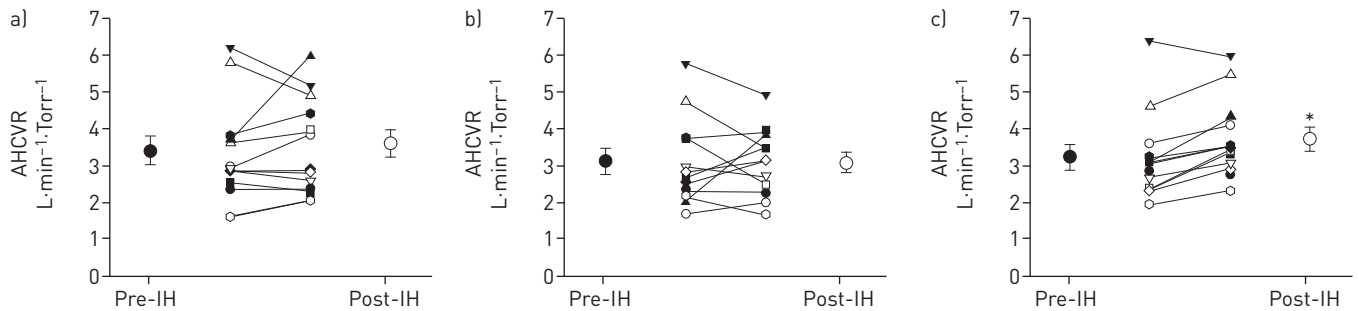
FIGURE 5 Individual and mean±SEM acute hypercanic ventilatory response (AHCVR) before (filled symbols) and after (clear symbols) exposure to 6 h of intermittent hypoxia (IH) within the a) placebo, b) nonselective cyclooxygenase (COX) inhibition and c) selective COX-2 inhibition conditions. *: p<0.05 versus pre-IH value.

augmentation of the AHVR following IH (*i.e.* quantified using an acute test performed before, and after, the 6-h IH exposure). Moreover, selective COX-2 inhibition facilitated a small, but significant, enhanced AHCVR following IH. These findings indicate that IH-induced inflammation does not contribute to respiratory plasticity occurring with acute IH exposure in healthy humans. Additionally, selective COX-2 inhibition may promote ventilatory instability following IH *via* augmenting the AHCVR.

Resting ventilation

Compared to the placebo, pre-IH resting air-breathing P_{ETCO_2} was ~1 Torr lower within the nonselective COX inhibition condition, but similar within the selective COX-2 inhibition condition while P_{ETCO_2} was similar between the nonselective and the selective COX-2 inhibition conditions. The reason for this lower P_{ETCO_2} after 4 days ingestion of the nonselective COX inhibitor indomethacin is unclear. To our knowledge, the impact of chronic COX inhibition on resting ventilation has not been investigated. In contrast, 90 min after a single 100-mg dose of the nonselective COX inhibitor indomethacin, ventilation has been reported to have greater variability (*i.e.* greater magnitude of waxing and waning) [33]. Whether this increased variability is maintained, enhanced or decreased after 4 days' ingestion is unknown, but if maintained or enhanced, this may explain the lower P_{ETCO_2} observed.

Ventilatory long-term facilitation (vLTF), characterised by a sustained increase in resting ventilation for several minutes-to-hours upon completion of a brief IH exposure, is a form of respiratory plasticity [17, 34] and results from elevated respiratory motor output [35]. In the current study, post-IH resting isocapnic euoxic ventilation was higher within the placebo and selective COX-2 inhibition conditions, with strong trends for increased ventilation within the nonselective COX inhibition condition. As post-IH isocapnic euoxia ventilation was assessed ~20 min after participants exited the hypoxic chamber (after blood sampling and acute test instrumentation), the elevated ventilation may represent the induction of vLTF. Interestingly, the greater post-IH ventilation resulted from increased f_R as V_T was not different from pre-IH levels (table 1). This respiratory pattern is different from that observed in anaesthetised rats where LTF of respiratory motor output is typically conveyed as an increase in burst amplitude in respiratory related nerves (*e.g.* phrenic), the neural equivalent of tidal volume [36]. In contrast, studies in unanaesthetised rats more commonly report LTF of respiratory motor output resulting from an increase in breathing frequency [36]. Furthermore, studies reporting vLTF in humans following a brief hypercapnic

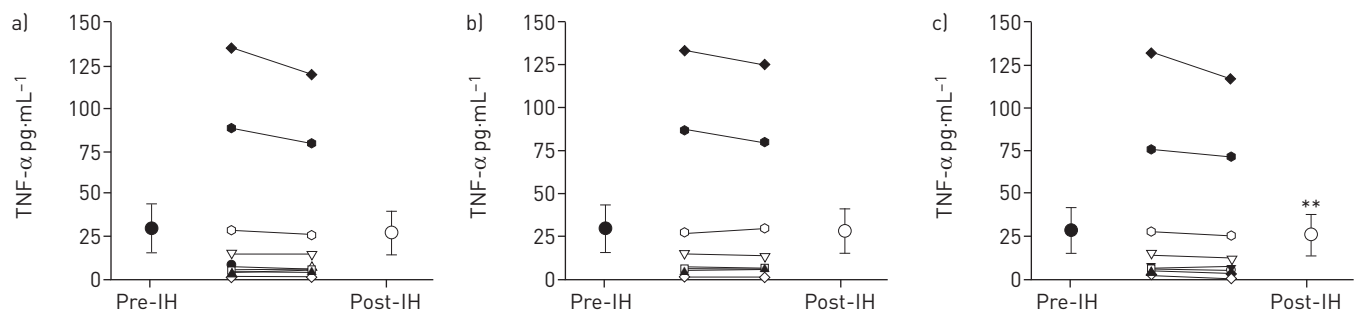

FIGURE 6 Individual and mean±SEM (n=10) serum tumour necrosis factor (TNF)-α concentrations before (filled symbols) and after (clear symbols) exposure to 6 h of intermittent hypoxia (IH) within the a) placebo, b) nonselective cyclooxygenase (COX) inhibition and c) selective COX-2 inhibition conditions. **: p<0.01 versus pre-IH value.

IH protocol (eight 4-min periods of hypoxia (inspiratory oxygen fraction (F_{IO_2}) 8%), separated by 5 min of normoxia (F_{IO_2} 21%)) describe an increase in both tidal volume and breathing frequency [37, 38]. Thus, findings regarding the ventilatory pattern of IH-induced LTF is equivocal, and probably the result of varying IH protocols. Based upon animal studies showing LTF of respiratory motor output following brief IH exposure, it has been proposed that for sleep apnoea patients, exposure to IH across a night of sleep may also lead to vLTF [39, 40]. To our knowledge, the current study is the first to report an increase in resting ventilations in healthy humans after an IH exposure mimicking moderate sleep apnoea and of similar duration to a night of sleep.

Similar to resting isocapnic euoxic ventilation, isocapnic hyperoxic ventilation was also greater during the post-IH acute test, again a result of increased breathing frequency. As hyperoxia quiets carotid body activity [41], the increased ventilation during isocapnic hyperoxia following IH supports *in vitro* evidence that continued respiratory motor output enhancement is an adaptation of central respiratory control centres [42].

Finally, for both resting isocapnic euoxia and isocapnic hyperoxia ventilation, neither anti-inflammatory medication prevented the IH-induced frequency-based vLTF. Thus, inflammatory markers inhibited *via* the nonselective COX inhibitor indomethacin and the selective COX-2 inhibitor celecoxib (*e.g.* nuclear factor (NF)-κB and COX pathways) do not appear to contribute to the observed frequency-based vLTF.

AHVR and AHCVR

The increased AHVR (*via* an increase in breathing frequency) within the placebo condition is similar to previous reports [7–12]. Although the mechanism is incompletely understood, oxidative stress has been proposed to be a primary mediator, as antioxidant treatment before [20] and during [18] IH exposure prevents carotid body sensory long-term facilitation and the enhanced chemosensory response to hypoxia [20] as well as augmentation of the AHVR [3, 18]. In addition, in healthy humans, we have reported that the IH-induced increase in AHVR was positively correlated (r=0.88) with systemic measures of oxidative stress [8]. As directly applying ROS to the carotid body does not result in excitation, nor does altering carotid body ROS production modify catecholaminergic secretory responses to hypoxia [18, 22, 27], there is still debate regarding whether IH-induced AHVR enhancement is the direct result of increased oxidative stress. Recently, IH-induced inflammation (resulting from increased oxidative stress) and production of pro-inflammatory cytokines (*e.g.* TNF-α and IL-1β) has been proposed to contribute to the augmented AHVR following IH [25]. In support of this hypothesis, DEL RIO *et al.* [18] reported that administration of the nonselective COX inhibiting anti-inflammatory ibuprofen to rats exposed to IH prevented AHVR augmentation. Mechanistically, although ibuprofen minimised the increase in carotid body TNF-α and IL-1β, it did not prevent the potentiated carotid body chemosensory response to hypoxia. Rather, ibuprofen decreased the number of c-fos positive neurons in the caudal nucleus tractus solitarii (a measure of nucleus tractus solitarii activation), indicating that IH-induced inflammation may act *via* central, not peripheral, pathways in augmenting the AHVR. In either case, the current findings in healthy humans of an augmented AHVR following IH within both the nonselective and selective COX-2 inhibition conditions are in contrast to these observations.

Aside from species differences, two dissimilarities may explain these opposing results. First, the current study utilised an acute (6 h) IH exposure while DEL RIO *et al.* [18] exposed rats to chronic IH (8 h·day^{-1} for 21 days). Between the two IH paradigms, the profile of the inflammatory response probably differs, with longer duration IH exposures being required to stimulate inflammation [26, 43]. Therefore, IH-induced inflammation may be mechanistically more important in ventilatory changes following chronic IH, whereas IH-induced oxidative stress [8] and/or alterations in gaseous mediators of hypoxia sensing within the carotid bodies (*e.g.* carbon monoxide and hydrogen sulphide) [44], may regulate respiratory changes following acute IH, but the latter still needs to be investigated [45]. Second, different NSAIDs were used in the two studies: nonselective COX inhibitor indomethacin and selective COX-2 inhibitor celecoxib *versus* the nonselective COX inhibitor ibuprofen. Although these medications are all reversible COX inhibitors that also inhibit the upstream pro-inflammatory transcription factor NF-κB, they all have different pharmacological properties (*e.g.* ibuprofen is a proprionic acid derivative, whereas indometacin is a indoleacetic acid derivative), pharmacokinetics (*e.g.* median inhibitory concentration for COX-1 and COX-2) [46] and off-target pharmacodynamics (*e.g.* differential effects on kinases implicated in carotid body oxygen sensing [45, 47]) that potentially contributed to these contrasting results.

As carotid body and respiratory centre concentrations of TNF-α and IL-1β are not easily assessed in humans, pre- and post-IH serum concentrations of the pro-inflammatory cytokines were quantified. As IL-1β was quantifiable in only three participants, interpretation of these data is limited, but these findings do indicate that the role of IL-1β is minimal, if any, in the observed IH-induced respiratory changes. As we observed a significant increase in the AHVR following IH (*i.e.* main outcome of the study), no

additional participants were recruited to further explore the relationship between IL-1β and respiratory changes following IH. Thus, the discussion is limited to TNF-α. The maintenance of serum TNF-α concentration after IH within the placebo condition is in contrast with results reporting an increase in TNF-α in rats after only 3 h of IH exposure, but is similar to a previous human study using a less severe IH exposure [48]. This maintenance of serum TNF-α concentrations following IH exposure during the placebo condition does not necessarily indicate that inflammation is not involved in augmenting the AHVR, as systemic TNF-α may not reflect carotid body concentrations [25] nor central inflammation. On the contrary, as the dosage regimens for the nonselective COX inhibitor and the selective COX-2 inhibitor celecoxib were used to produce systemic anti-inflammatory effects, and both inhibit the pro-inflammatory transcription factor NF-κB in addition to their respective COX isoenzymes, similar to resting ventilation, the similar increase in the AHVR across all conditions suggests inflammatory markers derived *via* NF-κB and the COX pathways are not involved in the acute IH-induced AHVR augmentation. Alternatively, these results could indicate the medication dosages were insufficient, although the dosages were sufficient to decrease urinary prostanoid concentrations (prostacyclin, prostaglandin (PG)E_2, thromboxane A2 and $PGF_{2\alpha}$ in the expected manner [30]). Furthermore, IH did not alter prostanoid concentrations, except for an increase in PGE_2 concentrations within the placebo condition [30]. Therefore, as both nonselective and selective COX-2 inhibition prevented the increase in PGE_2 with IH exposure, but did not abolish the increase in resting isocapnic euoxia and isocapnic hyperoxia ventilation, nor the augmented AHVR, prostanoids do not appear to have a role in the observed respiratory changes.

An increased AHCVR following IH has been reported previously [11–13], but is not a consistent observation, with shorter duration IH studies reporting no change [8, 15]. Thus, the unchanged AHCVR within the placebo and nonselective COX inhibition conditions following the 6-h IH exposure are consistent with previous studies using more acute IH exposures [8, 15]. In contrast, the augmented AHCVR within the selective COX-2 inhibition condition may be related to changes in cerebral blood flow (CBF). Typically, following acute IH, CBF is maintained or slightly elevated [31]. Previously, we reported a significant decrease in CBF during air-breathing rest within the same 12 participants following IH exposure within the selective COX-2 inhibition condition [30]. As CBF is inversely related to ventilation *via* regulating carbon dioxide removal, and thus, the extracellular pH of the central chemoreceptors [49], the observed decrease in post-IH CBF may have contributed to the observed augmented AHCVR.

Clinical implications

Greater ventilatory instability is associated with increasing OSA severity [5] and occurs in humans following IH exposure *via* enhanced ventilatory responses to hypoxia [7–12] and hypercapnia [11–14]. Animal models implicate increased inflammation in promoting the IH-induced AHVR augmentation, suggesting that anti-inflammatory medications may offer a therapeutic pathway to decrease ventilatory instability and, therefore, OSA severity [18, 25]. The current findings in humans do not support this potential therapeutic use, at least for acute IH exposures. Moreover, the use of selective COX-2 inhibition medication may enhance ventilatory instability (*via* an enhanced AHCVR) and OSA severity. Future studies are needed to determine the effect of longer IH exposures on inflammation in healthy humans and the impact of anti-inflammatory medications on IH-induced respiratory plasticity. In addition, the impact of anti-inflammatory medication on the AHVR and AHCVR in untreated OSA needs to be investigated.

The limitations of this study must be acknowledged. First, although the IH model used replicates the oxygen desaturation profile characteristic of moderate-to-severe OSA, it is not a perfect reproduction as it lacks the increased negative intrathoracic pressure, hypercapnia and sleep fragmentation associated with obstructive apnoeas [28]. Second, the study participants were healthy males free of the comorbidities that often accompany OSA. While these limitations may curtail extrapolation of our findings to OSA, the IH model and study population provide the opportunity to evaluate the role of inflammation in IH-induced respiratory plasticity without confounders [8, 30].

Conclusion

This study demonstrates that IH-induced inflammation is not involved in acute IH-induced AHVR enhancement in healthy humans. Additionally, the use of selective COX-2 inhibition in untreated OSA patients or healthy individuals undergoing IH exposure (*e.g.* altitude training) may warrant caution due to the potential enhancement of ventilatory instability *via* enhancing the AHCVR. Finally, while the extent to which these findings extend to OSA or healthy humans exposed to chronic IH still needs exploration, these results merit attention and further investigation.

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