Carotid body inflammation and cardiorespiratory alterations in intermittent hypoxia

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Running head: Inflammation and intermittent hypoxia

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ABSTRACT

Chronic intermittent hypoxia (CIH), a main feature of obstructive sleep apnoea (OSA), increases hypoxic ventilatory responses and elicits hypertension, partially attributed to an enhance carotid body (CB) responsiveness to hypoxia. Since inflammation has been involved in the CIH-induced hypertension and chemosensory potentiation, we tested if ibuprofen may block CB chemosensory and cardiorespiratory alterations induced by CIH in a rat model of OSA.

We studied effects of ibuprofen (40 mg kg⁻¹ day) on the immunohistochemical IL-1β and TNF-α levels in the CB, the number of c-fos positive-neurons in the nucleus tractus solitarii (NTS), CB chemosensory and ventilatory responses to hypoxia, and arterial blood pressure in male rats exposed 21 days to 5% O₂; 12 episodes h⁻¹; 8 h day⁻¹ or sham condition.

CIH increased CB TNF-α and IL-1β and c-fos positive-neurons in the NTS, enhanced carotid chemosensory and ventilatory hypoxic responses, and produced hypertension. Ibuprofen prevented CB cytokines overexpression, the CIH-induced increases in c-fos positive-neurons in the NTS, the enhanced hypoxic ventilatory responses and the hypertension, but failed to impede the CB chemosensory potentiation.

Results suggest that pro-inflammatory cytokines may contribute to the CIH-induced cardiorespiratory alterations, acting at several levels of the hypoxic chemoreflex and cardiovascular control pathways.

Keywords: Obstructive sleep apnoea, hypoxia, inflammation, hypertension.
INTRODUCTION

The obstructive sleep apnoea (OSA) syndrome, a rising worldwide health disease, is characterized by chronic intermittent hypoxia (CIH), which is considered the main risk factor for developing hypertension and other cardiovascular diseases [1,2,3]. It has been proposed that oxidative stress, inflammation and sympathetic activation are involved in the OSA-induced hypertension [3,4,5,6]. A growing body of evidence suggests that CIH enhances the carotid body (CB) chemosensory responses to hypoxia contributing to the OSA-induced hypertension [6,7,8,9]. Indeed, OSA patients and animals exposed to CIH show potentiated ventilatory, sympathetic and cardiovascular responses to acute hypoxia [6,7,8,9,10]. Furthermore, recordings of carotid chemosensory discharges \textit{in situ} and \textit{in vitro} have shown that CIH selectively increases basal chemosensory discharges in normoxia and potentiates the chemosensory responses to acute hypoxia in rats and cats [9,11,12,13].

The repetitive episodes of hypoxia–reoxygenation during CIH exposure elicits oxidative stress due to the accumulation of reactive oxygen species (ROS), which are involved in the potentiation of the hypoxic CB chemosensory responses [9,11,13,14] and in the pathological consequences observed in animals exposed to CIH and OSA patients [3,4,5,8,9,13]. Recently, we found that ascorbic acid supplementation, which impedes the systemic and local CB oxidative stress in the rat exposed to CIH for 21 days, prevented the enhanced CB chemosensory and ventilatory responses to hypoxia, as well as the hypertension [13]. These observations support a main contribution for oxidative stress in the generation of the CB chemosensory potentiation and the cardiorespiratory alterations induced by CIH. Nevertheless, a direct effect of ROS in the
CB oxygen process is still matter of debate. Indeed, the application of H$_2$O$_2$ does not produce CB chemosensory excitation [15,16]. Moreover, modifications of ROS production in the CB do not modify the catecholaminergic secretory response to hypoxia, indicating a lack of a causal link between ROS levels and chemoreceptor activity [17]. Thus, it is likely that other molecules downstream of the ROS signals, may mediate the enhancing effects of ROS on CB chemoreception under intermittent hypoxia. Among other molecules upregulated in the CB by CIH, such as ET-1 and iNOS [14,18,19], pro-inflammatory cytokines has been proposed as mediators of the CB chemosensory potentiation induced by CIH [9,14]. Indeed, recently we found that CIH for 21 days increases the expression of the tumour necrosis factor alpha (TNF-α) and the interleukin 1 beta (IL-1β) in the rat CB [9], molecules which are considered excitatory modulators of the CB oxygen chemoreception [20,21].

The progression of the hypertension in OSA patients and animals exposed to CIH is also associated to increased levels of pro-inflammatory cytokines [1,3,5]. An increased ROS production induced by hypoxia-reoxygenation evokes the synthesis and secretion of pro-inflammatory cytokines [22]. Thus, we hypothesized that a treatment with an anti-inflammatory drug may prevent both the CB chemosensory potentiation and the cardiorespiratory alterations in rats exposed to CIH. Accordingly, we studied the effects of the non-steroidal anti-inflammatory drug ibuprofen on the increased immunorreactive levels of TNF-α and IL-1β in the rat CB, the potentiation of the CB chemosensory and ventilatory responses to hypoxia and the hypertension induced by CIH in conscious rats. We also tested the effects of antioxidant treatment with ascorbic acid on the CIH-induced increase expression of TNF-α and IL-1β in the CB to find out if the up-regulation
of these cytokines was downstream of the ROS signalling pathways. Since the nucleus of the tractus solitarii (NTS) plays a major role in the integration of baro- and chemosensory signals [23], and by the fact that CIH increases the number of c-fos positive-neurons in the rat NTS, indicating changes in the neuronal activity [24, 25], we also addressed the effects of ibuprofen on the number c-fos positive-neurons in the NTS of rats exposed to CIH.

**Methods**

**Animals and exposure to intermittent hypoxia**

Experiments were performed on 40 adult male Sprague-Dawley rats, weighting initially 200g. Rats were fed with standard chow diet *ad libitum*, and kept on a 12-hour light/dark schedule (8:00am - 8:00 pm). Animals were randomly assigned to CIH or to Sham conditions. Researchers unaware of the identity of the treatment performed the physiological recordings and immunohistochemical studies. The experimental procedures were approved by the Bio-Ethical Committee of the Biological Sciences Faculty, P. Universidad Católica de Chile, and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Unrestrained, freely moving rats housed in individual chambers were exposed to hypoxic cycles of 5 % inspired O₂ for 20 s, followed by room air for 280 s, applied 12 times h⁻¹; 8 h day⁻¹ or sham condition for 21 days [13]. The O₂ level in the chambers was continuously monitored with an oxygen analyzer (Ohmeda 5120, BOC Healthcare, USA) and the CO₂ was maintained low by continuous air extraction. In the Sham condition, the
hypoxic exposure was replaced by means of flushing equal flow of compress air into the chambers. The room temperature was kept at 23-25°C.

**Chronic subcutaneous ibuprofen treatment**
Two days before the beginning of CIH or Sham exposures, animals were anesthetised with 3% isofluorane in O₂, and osmotic minipumps (2ML4, Alzet Scientific Products, USA) were implanted subcutaneously on the back. The pumps were filled with 400 mg ibuprofen (IB) in 2 ml NaCl 0.9%, to achieve a delivering concentration ~ 40 mg kg⁻¹ day, at a rate of 2.5 μl h⁻¹. Control animals were implanted with pumps containing NaCl 0.9%.

**Ascorbic acid treatment**
In separate experiments, ascorbic acid (1.25 g l⁻¹) was administered through the drinking tap water from the first day of the CIH exposure as previously described [13]. The water solution was freshly prepared every day, and preserved in dark containers to avoid oxidation.

**Recording of physiological responses**
Acute experiments were performed in the morning of the day after the 21-day of CIH exposure. Rats were anesthetized with sodium pentobarbitone (40 mg kg⁻¹ i.p.), followed by additional doses when necessary to maintain a level of surgical anaesthesia (stage 3 plane 2). Rats were placed in supine position and the body temperature monitored by a rectal probe was maintained at 38.0±0.5 °C with a heating pad. The trachea was cannulated for airflow recording, and connected to a pneumotachograph to obtain tidal
volume ($V_T$), respiratory frequency ($F_R$), and minute volume ($V'_I$). One femoral artery was cannulated with a polyethylene tube, filled with 50 IU ml$^{-1}$ of heparin solution for measuring arterial blood pressure with a transducer (Statham P23, USA). Heart rate ($H_R$) was measured from the ECG recordings. Physiological variables were acquired with an analogue-digital system (PowerLAB 8SP, ADInstruments, Australia) and analyzed with the Chart 7.2-Pro software. To assess the effects of CIH on the reactivity of the peripheral hypoxic chemoreflex, we measured ventilatory responses elicited by several isocapnic levels of PO$_2$ (5 to 670 mmHg), maintained until the response was in a steady state (~10-20 s).

**Recording of carotid body chemosensory discharge**

At the end of the ventilatory physiological recordings, one carotid sinus nerve was dissected and placed on a pair of platinum electrodes, and covered with warm mineral oil. The neural signal was pre-amplified (Grass P511, USA), filtered (30 Hz–1 kHz) and fed to an electronic spike-amplitude discriminator, allowing the selection of action potentials of given amplitude above the noise to be counted with a frequency meter to measure the frequency of carotid chemosensory discharge ($f_x$), expressed in Hz. Carotid sinus barosensory fibres were eliminated by crushing the common carotid artery wall between the carotid sinus and the carotid body. The other carotid sinus nerve was cut to prevent vascular and ventilatory reflexes evoked by the activation of the CB. The chemosensory discharge was measured at several isocapnic levels of PO$_2$ (~5 to 670 mmHg).
Immunohistochemistry for cytokines in the CB

Quantitative immunohistochemistry was used to address the relative levels of TNF-α and IL-1β in the CB as previously described [13]. Anesthetized rats were perfused intracardially with phosphate saline buffer (PBS) at pH 7.4 for 10 min followed by buffered 4% paraformaldehyde (PFA, Sigma, USA). The carotid bifurcations with the CBs were dissected and post-fixed in the same fixative solutions for 12 h at 4°C. Samples were then dehydrated in ethanol, included in paraffin, cut in 5 µm sections and mounted on silanized slides. After deparaffinization, samples were submitted to microwave based antigen retrieval protocol (700W for 6 min in citrate buffer 1M pH 6.0). Samples were incubated with 0.3% H₂O₂ to inhibit endogenous peroxidase and then in normal horse serum blocking solution (Vectastain Elite ABC Kit, Vector Lab, USA). Slides were incubated with specific antibodies overnight at 4°C in humidity chambers for detection of TNF-α (sc-1350, 1:20, goat anti- TNF-α, Santa Cruz Biotech., USA) and IL-1β (sc-7884, 1:100, rabbit anti- IL-1β, Santa Cruz Biotech., USA). After rinse slides in cold PBS, samples were incubated with secondary antibodies conjugated to biotin followed by a ready-to-use stabilized ABC reagent (Vectastain Elite ABC Kit, Vector Lab, USA), and revealed at 37°C in a dark chamber with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, USA). To avoid false positives during DAB chromogen quantification, special attention was kept to prevent DAB signal saturation. Samples were counterstained with Harris Haematoxylin and mounted with Entellan® (Merck, USA). Photomicrographs of the CB tissue were taken at 100x with a CCD-camera coupled to an Olympus CX 31 microscope (Olympus Corp, Japan), digitized and analyzed using a colour deconvolution algorithm with the ImageJ software (NIH, USA).
The positive immunoreactive intensity, averaged from four CB fields (9,200 µm² each) was expressed as optical integrated intensity.

**Immunohistochemistry for c-fos expression in the NTS**

Anaesthetized rats were perfused through the left ventricle with 4% PFA in PBS (pH 7.4), and the brains were post-fixed in the same fixative for 2 h and transferred to 30% sucrose with 0.02% sodium azide in PBS until they sank. The brainstems were cut frozen under dry ice in the coronal plane, at 50-µm thickness, using a sliding microtome. Three alternate series of sections from each brainstem were obtained. One series was stained with cresyl violet, and the other two were used for immunohistochemistry. Free-floating coronal sections of the rat brainstem including the caudal portion of the NTS were incubated in 0.3% H₂O₂ in PBS for 30 min, rinsed in PBS and transferred to the blocking solution (0.4% Triton-X100, 0.02% sodium azide, 3% normal goat serum in PBS) for 1 h. Sections were incubated overnight at room temperature with the Fos polyclonal antibody (1:20,000; Ab-5, rabbit polyclonal, Oncogene, USA). Sections were rinsed in PBS for 1 h before being incubated in the secondary antibody solution (1:1,000, Biotin-SP-conjugated AffiniPure goat anti-rabbit IgG, Jackson Immuno-Research, USA). After rinsing for 40 min, sections were incubated for 1 h in Vectastain ABC Elite kit (Vector Laboratories, USA), rinsed and incubated in a 0.05% DAB solution containing 0.003% H₂O₂, and 0.05% nickel chloride to obtain a dark blue reaction product. Photomicrographs of the coronal sections were taken at 4x with a CCD-camera coupled to an Olympus CX 31 microscope (Olympus Corp, Japan), digitized and analyzed using the ImageJ software (NIH, USA). The activation of the caudal NTS
region induced by CIH was assessed by counting c-fos-immunoreactive neurons bilaterally in 5 coronal sections from each rat.

**Statistical Data Analysis**

Data was expressed as means ± SEM. Differences between 3 or more groups were assessed with one or two-way ANOVA tests, followed by Newman-Keuls or Bonferroni posthoc comparisons.

**RESULTS**

The effect of CIH on $f_R$, $V_T$, $V'_I$, systolic ($P_s$) and diastolic arterial pressure ($P_D$) and $H_R$ measured at the beginning of the recordings, while rats breathed spontaneously room air, are summarized in Table 1. Baseline $f_R$, $V_T$, $V'_I$ and $H_R$ did not differ between any groups ($p \geq 0.05$, one way ANOVA). Exposure of rats to CIH for 21 days increased the mean arterial blood pressure (MABP) due to a significant increase in both $P_s$ and $P_D$ as compared to Sham rats (fig. 1 and Table 1, $p \leq 0.001$ Newman-Keuls test after one way ANOVA). Ibuprofen treatment for 21 days prevented the CIH-induced hypertension (fig. 1). We found that sham rats treated with ibuprofen showed a decrease in arterial blood pressure (Table 1, fig.1). Nevertheless, the decrease in arterial blood pressure was not statistically different as related to the sham rats ($p \geq 0.05$, Newman-Keuls test after One-way ANOVA).

Rats exposed to CIH showed higher reflex ventilatory responses to acute hypoxia as compared with Sham, CIH-IB, and Sham-IB rats (fig. 2, $p \leq 0.001$, Newman-Keuls test after two-way ANOVA). Thus, treatment with Ibuprofen prevented the potentiation of reflex ventilatory responses to acute hypoxia induced by CIH. On the contrary, the CIH-
induced potentiation of the CB chemosensory response to acute hypoxia was not prevented by ibuprofen (fig. 3). The two-way ANOVA analysis showed that the overall CB chemosensory curve for PO₂ was not different between the CIH and CIH-IB rats (p≥0.05). Ibuprofen treatment did not modify the carotid chemosensory response in the Sham rats.

We found significant increased levels of TNF-α immunoreactivity (TNF-α-ir) and IL-1β immunoreactivity (IL-1β-ir) in the CB from rats exposed to CIH for 21 days, which was prevent by ibuprofen treatment (fig. 4a, b). As is shown in fig. 4c-d, ibuprofen reduced the increased TNF-α-ir and IL-1β-ir by a 70-40% respectively, as compared with the increased optical integrated intensity measured in CIH-treated rats. We did not find differences between the TNF-α -ir and IL-1β -ir levels in the CBs from sham rats treated with or without ibuprofen (p≥0.05, See supplemental fig.S1). The increased levels of TNF-α-ir and IL-1β-ir in the rat CB exposed to CIH depended on the oxidative stress since ascorbic acid treatment, during the hypoxia protocol for 21 days, reduced the enhanced levels of TNF-α-ir and IL-1β-ir in the CB from rats exposed to CIH (fig. 5). We found that CIH increased the number of c-fos positive neurons in the caudal portion of the rat NTS, while ibuprofen treatment attenuated the number of positive neurons (fig. 6). Ibuprofen itself did not change the expression of c-fos. Indeed, we did not find changes in the number of c-fos positive neurons in the NTS from sham rats treated with or without ibuprofen (p≥0.05, see supplemental fig. S2).
Discussion

The main findings of this study showed that ibuprofen, which prevented the CIH-increased TNF-α and IL-1β in the CB and the number of c-fos positive-neurons in the caudal NTS, failed to impede the potentiation of the carotid chemosensory responses to acute hypoxia, but effectively prevent the potentiation of the chemoreflex ventilatory responses to hypoxia as well as the hypertension. Thus, the CIH-induced potentiation of the CB chemosensory responses does not depend on the increased TNF-α and IL-1β levels in the CB, although the increased level of these pro-inflammatory cytokines play an essential role in the generation of the cardiorespiratory alterations induced by CIH, probably acting at different levels of the hypoxic ventilatory reflex arc and cardiovascular control pathways. In addition, our results showed that ascorbic acid, which prevents the CIH-induced potentiation of the chemosensory responses to hypoxia and the local oxidative stress in the rat CB [13], blocked the increased TNF-α and IL-1β in the rat CB, indicating that the increased cytokines levels in the CB are secondary to the oxidative stress. The inhibitory effect of ibuprofen on cytokine accumulation induced by CIH in the CB is consistent with its known anti-inflammatory effect. Although, ibuprofen is considered a non-selective inhibitor of cyclooxygenases 1 and 2, it is known that ibuprofen inhibits the nuclear translocation of the transcription factor, NF-κB, which mediates the TNFα and IL-1β production [26].

Cytokines and CB chemosensory potentiation

The enhanced production of ROS induced by hypoxic-reoxygenation cycles evokes the expression of genes and the synthesis of pro-inflammatory cytokines, mediated by the activation of transcription factors such as NF-κB and HIF-1α [23]. In response to
oxidative stress, HIF-1α induces the expression of several proteins including ET-1 that transiently increased in the rat CB exposed to CIH [13], but oxidative stress also enhances the expression of pro-inflammatory cytokines such as IL-1β and TNF-α in the CB, suggesting that chemoreceptor cells can synthesize and release cytokines. Inflammatory processes have been involved in the enhanced reactivity of the CB chemosensory response to hypoxia in rats exposed to sustained hypoxia [27]. Indeed, Lam et al. [20] found that sustained hypobaric hypoxia recruits macrophages to the rat CB, increases the mRNA expression of IL-1β and TNF-α, IL-1R1 and TNF-R1 receptors. Liu et al. [21] found that the concurrent administration to rats exposed to sustained hypoxia of ibuprofen and dexamethasone reduced the potentiated CB chemosensory response to hypoxia, blocked the immune cell invasion and reduced the cytokine RNA expression. Intermittent hypoxia also produces a progressive increase of the TNF-α and IL-1β in the rat CB, but the CIH-induced increases of TNF-α and IL-1β-ir was not associated with CB tissue invasion of immune cell or increased plasma levels, suggesting that CIH augmented the local production of cytokines in the CB [14]. Present results showed that the CB chemosensory potentiation to hypoxia induced by CIH was not prevented by ibuprofen, while the increased levels of IL-1β and TNF-α in the CB were abolished by the anti-inflammatory treatment. Thus, our results suggest that the mechanisms underlying the hypoxic CB chemosensory potentiation induced by sustained and intermittent hypoxia are different.

**Cytokines and cardiorespiratory alterations induced by intermittent hypoxia**

The progression of the hypertension in OSA patients and animals exposed to CIH is associated to increased levels of pro-inflammatory cytokines [1,2,5]. Our results strongly
suggest that pro-inflammatory cytokines contribute to the CIH-induced cardiorespiratory alterations acting at different levels of the hypoxic chemoreflex and cardiovascular control pathways. Popa et al. [28] reported that ibuprofen treatment blocked the increased ventilatory response to hypoxia and the increased IL-1 and IL-6 protein levels in the brainstem of rats exposed to chronic hypoxia, supporting the proposal that ibuprofen blocks inflammatory processes in the CNS, which contributed to the ventilatory acclimatization to sustained hypoxia. Present results showed a clear dissociation of the effects of ibuprofen on the CB potentiated chemosensory responses to hypoxia and to the reflex ventilatory response to hypoxia as well as on the hypertension induced by CIH. Thus, actions of cytokines on the arterial blood pressure and ventilatory reflex responses to hypoxia in rats exposed to CIH may occur in multiple sites, including the NTS and the CNS. Nevertheless, we cannot rule out if the persistent CB chemosensory potentiation in rats exposed to CIH and treated with ibuprofen would be detrimental to the cardiorespiratory function under long-term exposure to intermittent hypoxia. Future studies addressing the contribution of the CB chemosensory potentiation to cardiovascular and ventilatory alterations induced by long-term exposures to CIH are needed.

The available evidence suggests that the cardiorespiratory alterations induced by CIH are originated from the enhanced CB chemosensory responsiveness to hypoxia [6,7,8,9] signals that are transmitted to the NTS, where the respiratory gas and blood pressure sensory signals are primarily integrated. Kline [23] found evidence that CIH increased the postsynaptic neuron activity in the rat NTS, elicited by an augmented afferent sensory input and enhanced spontaneous synaptic discharge. The idea that CIH
increases the neural activity in the NTS is also supported by previous studies showing that c-fos immunoreactivity, a marker for neural activation, increased in the rat NTS following CIH [24, 25]. Moreover, it has been proposed that inflammation in the NTS contributes to the neurogenic hypertension [29,30]. Waki et al. [30] found that abnormal gene expression of pro-inflammatory molecules such as the junctional adhesion molecule 1 are highly expressed in the NTS of spontaneously hypertensive rats and elicits leukocyte accumulation within the vasculature in the NTS. Accordingly, they proposed that cytokines and chemokines might contribute to elevate the arterial pressure by increasing the neuronal activity in the NTS of spontaneous hypertensive rats [30]. Our results showing that ibuprofen reduced the c-fos immunoreactivity in neurons of the caudal NTS support a novel role for inflammation in the hypertension induced by CIH.

Clinical Perspectives

The OSA syndrome is recognized as an independent risk factor for cardiovascular diseases [1, 2]. Indeed, ~50% of OSA patients develop diurnal hypertension attributed to oxidative stress and inflammation [1,2,3,4,5]. The gold standard therapy for patients with severe OSA is the application of continuous positive airway pressure (CPAP) during sleep, which reduces the production of ROS and inflammatory molecules and reverses the hypertension [1,2 for review]. However, there are no specific treatments, based on antioxidant or anti-inflammatory drugs, for OSA patients presenting low adherence to CPAP as well as patients with mild or moderate OSA [1,2]. Thus, a potential therapeutic use of antioxidants and/or anti-inflammatory drugs in OSA deserves further attention.
Present result showing that ibuprofen treatment prevented the cardiorespiratory alterations induced by CIH, suggests that anti-inflammatory drugs may potentially be used to ameliorate the hypertension associated with OSA. However, the prolonged use of anti-inflammatory drugs, including ibuprofen may have some risk. Indeed, non-steroidal anti-inflammatory drugs may increase the risk of cardiovascular thrombotic events, myocardial infarction and stroke, and the risk of gastrointestinal bleeding and ulceration [31]. In addition, prolonged use of ibuprofen may lead to onset of a new hypertension or worsening a pre-existing hypertension [31]. Our results showed that the CIH-induced over-expression of IL-1β and TNF-α in the CB is mediated by ROS and prevented by ascorbic acid, indicating that the increased cytokines levels are secondary to the oxidative stress. Studies performed in rats exposed to CIH have shown that antioxidant treatment prevents the hypertension [13,32,33]. Indeed, recently we reported that CIH increased plasma lipid peroxidation and 3-nitrotyrosine formation in the rat CB, enhanced the CB chemosensory and ventilatory responses to hypoxia, and produced hypertension [13]. Ascorbic acid treatment prevented the systemic and local CB oxidative stress, the potentiated chemosensory and ventilatory responses to hypoxia as well as the hypertension [13]. Thus, our results support a plausible therapeutic use of antioxidants and anti-inflammatory drugs in OSA patients. Antioxidants seem to be a better choice than anti-inflammatory drugs for the treatment of the OSA-induced hypertension, but anti-inflammatory drugs such as ibuprofen used with precaution and in low doses may also be beneficial. Based on the current available information, a long-term combined treatment of antioxidants and anti-inflammatory drugs needs further studies in animal models before to be tested in clinical trials.
In summary, present results suggest that the mechanisms underlying the upregulation of pro-inflammatory cytokines in the CB induced by CIH are linked to oxidative stress, as well as the enhanced CB chemosensory responsiveness to hypoxia. However, the CIH-induced potentiation of CB chemosensory responses to acute hypoxia does not depend on the increased TNF-α and IL-1β levels. Nevertheless, pro-inflammatory cytokines may contribute to the potentiation of the hypoxic ventilatory chemoreflex response to hypoxia and the progression of the hypertension induced by CIH. This suggests that multiple mechanisms may be involved in the cardiorespiratory alterations induced by CIH.
Support statement

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References


33. Kumar GK, Rai V, Sharma SD, Ramakrishnan D, Peng YJ, Souvannakitti D Prabhakar NR. Chronic intermittent hypoxia induces hypoxia-evoked
Table 1. Basal ventilatory and cardiovascular variables measure at normoxia.

<table>
<thead>
<tr>
<th>Variable</th>
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<th>CIH</th>
<th>Sham+IB</th>
<th>CIH+IB</th>
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Sham: control animals; CIH: rats exposed to chronic intermittent hypoxia; Sham+IB: control rats that received ibuprofen; CIH+IB: rats treated with ibuprofen during the exposure to chronic intermittent hypoxia; VT: tidal volume; fR: respiratory frequency; V'T: minute ventilatory volume; PS: systolic blood pressure; PD: diastolic blood pressure; HR: heart rate. *p≤0.05, ***p≤0.001 Newman-Keuls test after One-way ANOVA.
FIGURE LEGEND

Figure 1. Ibuprofen treatment for 21 days prevented the CIH-induced hypertension in rats. MABP, mean arterial blood pressure measured in 6 Sham rats ( ), 8 CIH-treated rats ( ), 6 Sham+IB rats ( ) and 10 CIH+IB ( ). *** p≤0.001 compared to sham. Newman-Keuls after one-way ANOVA.

Figure 2. Ibuprofen prevented the potentiation of the hypoxic ventilatory response induced by CIH exposure. Ventilatory minute (V') respiration was measured in responses to several levels of inspired PO2 in 6 Sham rats ( ), 8 CIH-treated rats ( ), 6 Sham+IB rats ( ) and 10 CIH+IB ( ). *** p≤0.001 compared to sham, Bonferroni test after 2-way ANOVA.
Figure 3. Ibuprofen failed to prevent the enhanced CB chemosensory responses to hypoxia in rats exposed to CIH. Summary of the carotid chemosensory responses induced by several levels of inspired $P_{O_2}$ in 4 Sham rats (□), 5 CIH-treated rats (■), 6 Sham+IB rats (♦) and 10 CIH+IB (▼). $f_{CSN}$, frequency of chemosensory discharges in Hz. ** $p \leq 0.01$, * $p \leq 0.05$ CIH compared to sham; # $p \leq 0.05$ CIH+IB compared to Sham, Bonferroni test after 2-way ANOVA.

Figure 4. Effects of ibuprofen treatment on the CIH-induced increase expression of TNF-$\alpha$ and IL-1$\beta$ in the rat CB. Upper panels, micrographs showing positive immunoreactivity for TNF-$\alpha$ (a) and IL-1$\beta$ (b) in CBs from a Sham rat, CIH exposed rat, and CIH rat treated with ibuprofen (CIH+IB). Insets, negative controls devoid of positive staining. Scale bars 20 µm. Lower panels, quantitative analysis of the positive TNF-$\alpha$ -ir (c) and IL-1$\beta$ -ir (d) measured in the CBs from 6 Sham rats, 6 CIH rats and 5 CIH+IB rats. *** $p \leq 0.001$ compared to Sham.
Figure 5. Increase CB expression of TNF-α and IL-1β induced by CIH is related oxidative stress. Upper panels, micrographs showing TNF-α –ir (a) and IL-1β –ir (b) in one CB from a Sham rat, CIH exposed rat, and CIH rat treated with ascorbic acid (CIH+AA). Note that ascorbic acid treatment abolished the CIH-induced increases in TNF-α and IL-1β. Insets, negative controls devoid of positive staining. Scale bars 20 µm. Lower panels, summary of the effects of ibuprofen on TNF-α -ir (c) and IL-1β -ir (d) in rat.
CBs. Sham rats (n=4), CIH rats (n=6) and CIH+AA rats (n=5). *** p≤0.001 compared to sham.

Figure 6. Effects of ibuprofen treatment on the CIH-induced increases in the number of c-fos positive neurons in the caudal portion of the rat NTS. Representative micrographs showing positive immunoreactivity for c-fos in the NTS from a Sham rat, CIH exposed rat, and CIH rat treated with ibuprofen (CIH+IB) (a). Low magnification images scale bar 600 µm; High magnification images scale bar 200 µm. Note that ibuprofen prevents the
increase in c-fos–ir in the rat NTS following CIH. b) Summary of the effects of ibuprofen (b). Sham rats (n=5), CIH rats (n=5) and CIH+IB rats (n=5). ** p≤0.01 compared to sham.