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OXIDATIVE STRESS AUGMENTS CHEMOREFLEX SENSITIVITY IN RATS EXPOSED TO CHRONIC INTERMITTENT HYPOXIA

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Abstract

Chronic exposure to intermittent hypoxia (CIH) elicits plasticity of the carotid sinus and phrenic nerves via reactive oxygen species (ROS). To determine whether CIH-induced alterations in ventilation, metabolism, and heart rate are also dependent on ROS, we measured responses to acute hypoxia in conscious rats after 14 and 21 d of either CIH or normoxia (NORM), with or without concomitant administration of allopurinol (xanthine oxidase inhibitor), combined allopurinol plus losartan (angiotensin II type 1 receptor antagonist), or apocynin (NADPH oxidase inhibitor). Carotid body nitrotyrosine production was measured by immunohistochemistry. CIH produced an increase in the ventilatory response to acute hypoxia that was virtually eliminated by all three pharmacologic interventions. CIH caused a robust increase in carotid body nitrotyrosine production that was greatly attenuated by allopurinol plus losartan and by apocynin but unaffected by allopurinol. CIH caused a decrease in metabolic rate and a reduction in hypoxic bradycardia. Both of these effects were prevented by allopurinol, allopurinol plus losartan, and apocynin.

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Keywords

chemoreceptor; intermittent hypoxia; antioxidant; reactive oxygen species

1. Introduction

In anesthetized animals and *ex vivo* preparations, exposure to chronic intermittent hypoxia (CIH) has been shown to enhance sensory long-term facilitation of the carotid chemoreceptor (Del Rio et al., 2010; Peng et al., 2001; Peng et al., 2003; Peng et al., 2006b; Rey et al., 2004), augment long-term facilitation of phrenic discharge (Peng and Prabhakar, 2003; Peng et al., 2006b), and sensitize the ventilatory response to acute hypoxia (Del Rio et al., 2010; Peng and Prabhakar, 2003; Rey et al., 2004). Oxidative stress generated by cyclical hypoxia and reoxygenation is thought to play a causative role in these forms of respiratory plasticity affecting the afferent and efferent arms of the carotid chemoreflex (Del Rio et al., 2010; Peng et al., 2009; Peng and Prabhakar, 2003). CIH-induced augmentations in chemoreflex control of ventilation have also been demonstrated in intact, unanesthetized animals (Julien et al., 2008; Morgan et al., 2016; Peng et al., 2006b; Reeves et al., 2003).

In the present study, we sought to advance understanding of the role of oxidative stress in CIH-induced chemosensitization in the following ways: a) we used a repeated measures experimental design to assess cardiorespiratory changes caused by 21 d of CIH in intact, unanesthetized rats; b) we measured ventilatory responses to acute, graded hypoxia, taking into account inter-individual differences in SpO_2 and metabolic rate responses, c) we used systemic administration of losartan to antagonize angiotensin II type 1 receptors (AT_1R) and allopurinol and apocynin to inhibit xanthine oxidase and NADPH oxidase, two intracellular sources of superoxide, and d) we measured nitrotyrosine production in carotid bodies harvested from normoxic controls and CIH-exposed rats concomitantly treated with allopurinol, allopurinol plus losartan, apocynin, or vehicle.

2. Material and Methods

2.1. Animals

Fifty-one adult male Sprague-Dawley rats (body weight, 320–483 g) served as subjects. *Ad libitum* access was provided to drinking water and standard chow (Harlan Teklad #8604). Data from 10 normoxic control rats included herein were published in a previous report (Morgan et al., 2016). Experiments were carried out in accordance with recommendations set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication #8023, revised 1978). The protocol was approved by the University of Wisconsin-Madison School of Medicine and Public Health's Institutional Animal Care and Use Committee.

2.2. Barometric plethysmography

Tidal volume (VT) and respiratory frequency (f_B) were measured by the method of Drorbaugh and Fenn (Drorbaugh and Fenn, 1955) using a custom-designed system comprised of a 2-L plethysmograph connected to a 2-L reference compartment (Olson,

1994). The plethysmograph pressure signal was sampled at 100 Hz. Each day prior to placement of animals in the plethysmograph, a pressure calibration signal was obtained via repeated rapid injections and withdrawals of a known volume of air (0.2 ml) using a motor-driven pump with a frequency of 150 strokes/min, a frequency designed to mimic the maximal breathing frequency elicited by our hypoxic ventilatory response tests (see below). Peak-to-peak pressure deviations were used to calculate VT using the Drorbaugh and Fenn formula (Drorbaugh and Fenn, 1955); therefore, our measurements represent a mean of inspiratory and expiratory VT. A noise detection algorithm that utilized a moving average of the pressure waveforms was used to identify periods of sniffing and/or pressure-changing noise due to movement, grooming, and other activity, and labelled these as artifacts (see below). Minute ventilation ($\dot{V}_E$) was calculated as the product of VT and f_B . Rat body temperature was measured telemetrically using a sensor surgically implanted in the abdominal cavity (#VM-FH Mini-Mitter; Starr Life Sciences, Oakmont, PA) and verified by measurement of rectal temperature. Temperature within the plethysmograph was controlled at 25.4° C and humidity was maintained at 60–70%. A prefiltered proportional-integral-differential-type algorithm regulated the mass flow controllers that balanced impedance to flow into and out of the plethysmograph, so that flow through the system was maintained at 2 L/min. A single set of O₂ and CO₂ analyzers (#FCX-MV, Fujikura Ltd., Tokyo, Japan and #LB-2; Beckman Instruments, Fullerton CA) sampled the inspired and expired air in sequential fashion to determine oxygen consumption and carbon dioxide production ($\dot{V}O_2$ and $\dot{V}CO_2$, respectively). Inspired air was sampled prior to humidification and entry into the plethysmograph and expired gas was dried by a dessicant prior to analysis. A three-point gas calibration was performed each day prior to testing. The calibration gas concentrations bracketed the range of values expected for inspired and expired oxygen and carbon dioxide fractions during the hypoxic response tests (0.22 O₂ and 0.02 CO₂, 0.10 O₂ and 0.01 CO₂, 0.00 O₂ and 0.00 CO₂). This calibration routine provided us with daily assessments of linearity and drift of the two sensors. In the sets of calibrations represented in this study, drifts of both sensors averaged < 0.031%. A computer with custom-written software controlled airflow through the plethysmograph and saved all ventilatory and metabolic measures in 15-s segments. Pressure traces that contained > 40% artifact were excluded from further analysis. Arterial oxygen saturation (SpO₂) was measured by pulse oximetry via neck collar (MouseOx®; Starr Life Sciences Corp., Oakmont, PA). The rats were familiarized with the collar during several abbreviated graded hypoxia sessions in the plethysmograph prior to the baseline, pre-exposure measurements of the hypoxic ventilatory response. Heart rate was obtained from the oximeter signal. PowerLab 16/35 and LabChart Pro (ADInstruments, Colorado Springs, CO) were used to acquire SpO₂ and heart rate data. The animals were easily able to reposition themselves in the plethysmograph; therefore, they were confined, but not restrained, during hypoxic ventilatory response testing.

2.3. Hypoxic ventilatory response testing

At least 2 wk were allowed for recovery from telemeter implantation surgery. Acute exposure to graded hypoxia was achieved by supplementation of the air within the plethysmograph with nitrogen. Data were collected during the final 5 min of a 15-min baseline period of ambient air breathing (inspired oxygen fraction [FIO₂], 0.21). This initial normoxic observation period was extended in 5-min increments if the animal showed

behavioral signs of restlessness such as excessive movement or if respiratory frequency was unstable. Data were also collected during the final 5 min of 15-min periods in which FIO_2 was maintained at 0.15, 0.12, and 0.09. This length of exposure was chosen because 15 min was adequate time for the expired fractions of O_2 and CO_2 to reach new steady states after a step-down in FIO_2 . To ensure stability of hypoxic ventilatory responses prior to CIH and NORM (normoxia) exposures, all rats underwent repeated baseline measurements (2–4 tests conducted on separate days) until the ventilatory response slopes varied by no more than 15%. These slopes were then averaged to determine the baseline, pre-exposure values that were subsequently used for comparisons with slopes obtained after 7 d of drug treatment in normoxia, and 14 and 21 d of subsequent exposure to CIH or NORM. All testing occurred between 0800 and 1600 hours, and in CIH-exposed rats, plethysmograph testing commenced within 30 min of last intermittent hypoxia cycle (see below).

2.4. CIH and NORM exposures

Rats, in their home cages, were placed into a Plexiglas chamber and exposed to intermittent hypoxia for 10 h/d (between 600 and 1600 hours) for 21 d. Airflow through the intermittent hypoxia chamber was controlled at 2.5 L/min per rat, a rate sufficient to maintain CO_2 tension within the chamber at $<0.5\%$. Oxygen concentration in the chamber was monitored using a heated zirconium sensor (Fujikura America, Pittsburgh, PA). A microprocessor-controlled timer was used to operate solenoid valves that controlled the flow of oxygen and nitrogen into the chamber. The system was set to provide hypoxic exposures at 4-min intervals. During the first min of each cycle, nitrogen was flushed into the chamber at a rate sufficient to achieve an FIO_2 of 0.10 within 20–30 s. This level of FIO_2 was maintained for an additional 60 s. Oxygen was then introduced at a rate sufficient to achieve an FIO_2 of 0.21 within 10–15 s and to maintain this oxygen level for the duration of the 4-min cycle. Thus, our CIH paradigm produced a saturation profile that closely mimics moderately severe obstructive sleep apnea in humans (15 events/h, nadir SpO_2 , 75%). Control rats (NORM) were housed under normoxic conditions adjacent to the hypoxia chamber for 21 d. There, they were exposed to light, noise, and temperature stimuli similar to those experienced by the CIH rats.

2.5. Drug treatments

Rats were randomly assigned to receive one of three drug treatments starting 7 d prior to CIH exposure and continuing throughout the 21-d exposure: allopurinol (65 mg/kg/d)(n=9), allopurinol (65 mg/kg/d) plus losartan (25 mg/kg/d)(n=9), or apocynin (40 mg/kg/d)(n=8). These doses of allopurinol and losartan were shown in previous studies to reduce urinary uric acid excretion and block the arterial pressure rise caused by angiotensin II infusion (Dopp et al., 2011; Marcus et al., 2012). Because of the limited solubility of allopurinol in water, the daily doses of allopurinol and the allopurinol plus losartan combination were prepared as 1% w/v methylcellulose suspensions and given by oral gavage. A control group of CIH-exposed rats received an identical volume of methylcellulose vehicle each day by gavage (n=9). Apocynin was given via the drinking water. The actual average dose of apocynin, calculated using water consumption measurements, was 47.3 mg/kg/d. The effects of daily drug treatments alone (prior to CIH exposure) on ventilatory responses to acute hypoxia were assessed after one wk of treatment. Data from drug-treated, CIH-exposed rats

were compared with those from rats exposed to normoxia without drug treatment (n=10) that were previously published (Morgan et al., 2016). To determine whether longer-term treatments would affect ventilatory responses to acute hypoxia, a separate group of normoxic control rats (n=6) received allopurinol plus losartan for 28 d.

2.6. Carotid body nitrotyrosine formation

We measured nitrotyrosine, a marker of the free radical oxidant, peroxynitrite, as an indicator of oxidative stress in carotid bodies (Del Rio et al., 2010, 2011a; Moya et al., 2016). At the end of 21 d of CIH or NORM exposures, the rats were deeply anesthetized with isoflurane and transcardially perfused with PBS to clear the red cells, followed by perfusion with 4% paraformaldehyde in PBS (300–500 ml, 25 ml/min). The carotid bifurcations, including the carotid bodies and superior cervical ganglia, were excised and placed in the same fixative overnight at 4° C. The tissues were then transferred to 30% sucrose solution and stored at 4° C. until embedded in paraffin prior to sectioning. Carotid bifurcations were cut into 5- μ m sections and mounted on silanized slides. Deparaffinized samples were incubated with H₂O₂ to inhibit endogenous peroxidase and then in blocking serum solution (Vector Laboratories Inc., Burlingame, CA). The slices were incubated with an anti-3-NT polyclonal antibody (1:20 in PBS/bovine serum albumin 1%; Molecular Probes A-21285) overnight at 4° C. Slices were incubated with a universal biotinylated secondary antibody followed by a ready-to-use ABC reagent (Vectastain Elite ABC Kit; Vector Laboratories Inc.), and revealed with 3,3-diaminobenzidine tetrachloride (Sigma). Samples were counterstained with Harris' haematoxylin and mounted. Photomicrographs were taken at 100X with a CCD camera coupled to an Olympus CX 31 microscope (Olympus Corporation, Tokyo, Japan), digitized and analyzed with the Image J software (National Institutes of Health, Bethesda, MD). A color deconvolution algorithm was used to quantify 3-NT immunoreactivity as previously described (Del Rio et al., 2010, 2011a; Del Rio et al., 2011b). The immunoreactive intensity, averaged from eight fields for each sampled carotid body, was expressed as optical integrated intensity. This analysis was performed on carotid bodies harvested from 5 rats in each treatment and exposure group with the exception of the NORM-exposed, untreated control rats that were part of a previous study (Morgan et al., 2016).

2.7. Plasma catecholamines

We measured these indirect indicators of sympathetic nervous system activity because stimulation of the carotid chemoreceptor reflexly elicits sympathoexcitation and because sympathetic overactivity is thought to play an important role in causing hypertension in humans with sleep disordered breathing (Carlson et al., 1993). In addition, circulating catecholamines may exert an important influence on breathing pattern (Matsumoto et al., 1981; Zanella et al., 2014). At the end of 21 d of exposure to CIH or NORM, blood for catecholamine determinations was obtained by cardiac puncture in anesthetized rats prior to transcardiac perfusion, placed in ice-cold heparinized tubes, and centrifuged (4° C; 10,000 rpm) for 10 min. Plasma was extracted and stored at –80° C. until analyzed. Norepinephrine and epinephrine determinations were made using HPLC with electrochemical detection in the CRU Core Analytical laboratory at the University of Iowa as previously described (Hoffman et al., 2002). Catecholamine determinations were performed after exposure for 21

d to CIH or NORM, with or without drug treatment, in the same rats that underwent ventilatory measurements.

2.8. Data analysis

Prior to randomization into treatment groups and CIH or NORM exposure, cardiorespiratory variables measured during 3 levels of acute hypoxia in all 51 rats were compared with the normoxic values using one-way, repeated measures ANOVA. To quantify hypoxic chemoreflex sensitivity, the ventilatory equivalent for $\dot{V}CO_2$ and the mean inspiratory flow rate (VT:inspiratory time (T_i) ratio), an index of respiratory “drive”, both normalized for $\dot{V}CO_2$ were expressed relative to SpO_2 . Because of the curvilinear nature of these responses, we initially attempted to characterize them using a curve-fitting approach. We tried logistic fits and second- and third-order polynomials, but were not able to find non-linear models that characterized the responses in all animals. Therefore, linear regression analysis was used to derive the slopes of the stimulus-response relationships. No between-group differences in slopes of these relationships or in normoxic baselines for ventilatory variables were detected via one-way ANOVA prior to the initiation of CIH or NORM ($p=0.077-0.192$; Table 1); therefore, relationships between ventilatory variables and SpO_2 after initiation of drug treatments and at 14 and 21 d of CIH or NORM exposure were expressed as a percentages of the pre-exposure values. The percent change in the slope of these response variables, along with absolute values of $\dot{V}O_2$ and $\dot{V}CO_2$ and body mass and change in heart rate, were evaluated using repeated measures, general linear models (Minitab, State College PA) that included the individual rat as a random variable nested in the exposure type, the exposure type, drug treatment group, and time as fixed variables, and a term to evaluate the interaction between time and exposure type. Between group differences in plasma catecholamines and carotid body nitrotyrosine formation were compared using one-way ANOVA. When the omnibus statistical tests revealed statistically significant results, Dunnett post hoc tests were used to make comparisons with normoxic controls. Differences with p values <0.05 were considered statistically significant. In the text, tables and figures, data are shown as means $\pm$ SEM.

3. Results

3.1. Rat weights

Body mass increased over time in all treatment groups ($p<0.001$). The rate of weight gain was relatively reduced in the CIH- and NORM-exposed rats who received allopurinol plus losartan as compared with the other treatment/exposure groups ($p<0.001$) (Table 2).

3.2. Cardiorespiratory and metabolic responses to acute reductions in FIO_2 prior to CIH or NORM exposure or drug treatment

SpO_2 fell progressively from approximately 95% during air breathing to an average of 66% at the lowest level of FIO_2 . As we have reported previously (Morgan et al., 2014), there was considerable inter-subject variation in SpO_2 at each level of hypoxic inspire (range, 57–80% at FIO_2 of 0.09). Acute exposure to graded, steady-state hypoxia caused progressive increases in $\dot{V}_E$, VT, and f_B ($p<0.001$ for each) and concomitant progressive decreases in metabolic rate ($p<0.001$ for both $\dot{V}O_2$ and $\dot{V}CO_2$) (Figure 1). Acute graded hypoxia elicited

a biphasic heart rate response ($p < 0.001$). Mild hypoxia (FIO_2 , 0.15) caused a modest increase in heart rate, whereas moderate and severe hypoxia caused substantial, progressive, decreases in heart rate relative to the normoxic values. In addition, ventilatory equivalents for both $\dot{V}O_2$ and $\dot{V}CO_2$ and mean inspiratory flow rate normalized for $\dot{V}CO_2$ ($VT/T_i/\dot{V}CO_2$), two indicators of chemoreflex response, were substantially increased during acute exposure to graded hypoxia ($p < 0.001$ for both; data not shown). These cardiorespiratory responses to acute hypoxia are consistent with those reported in previous publications (Morgan et al., 2014; Morgan et al., 2016).

3.3. Effects of drug treatments on responses to acute hypoxia prior to CIH or NORM exposures

Plethysmography was repeated after 7 d of drug treatment prior to the commencement of CIH or NORM exposures. None of the drug treatments *per se* affected ventilatory or metabolic responses to acute hypoxia ($p = 0.207$ – 0.341) (Figure 2).

3.4. Effects of CIH and drug treatments on ventilatory responses to acute hypoxia

Figure 3 shows changes over time in slopes of the relationships between ventilatory variables and SpO_2 in the six exposure/drug treatment groups. After 14 and 21 d of exposure, statistically significant differences from untreated normoxic control rats were observed for $\dot{V}_E$ and f_B (both $p < 0.001$) in the CIH-exposed, vehicle treated rats. In contrast, the $\dot{V}_E$ and f_B slopes were unchanged in rats receiving drugs treatments. No statistically significant differences were observed in VT slope ($p = 0.240$).

3.5. Effects of CIH and drug treatments on hypoxic chemosensitivity

Changes in the slopes of the $\dot{V}_E/\dot{V}CO_2/SpO_2$ and $VT/T_i/\dot{V}CO_2/SpO_2$ relationships in the six exposure/drug treatment groups are shown in Figure 4. Both indices of hypoxic chemosensitivity were augmented, relative to untreated normoxic control rats, in CIH-exposed, vehicle-treated rats ($p = 0.011$ and 0.012 , respectively). In contrast, neither variable was altered by CIH-exposure when drug treatments were administered.

3.6. Effect of CIH and drug treatments on metabolic rate

Statistically significant between-group differences were observed in $\dot{V}CO_2$ and $\dot{V}O_2$ measured in normoxia ($p = 0.017$ and 0.005) (Figure 5). Both indicators of metabolic rate were decreased in normoxia following CIH exposure in vehicle-treated rats, whereas they remained stable after CIH exposure in rats treated with allopurinol, allopurinol plus losartan, and apocynin. $\dot{V}CO_2$ and $\dot{V}O_2$ in normoxia were unchanged over time in NORM-exposed rats.

3.7. Effect of CIH and drug treatments on carotid body oxidative stress

We observed statistically significant between-group differences in nitrotyrosine formation in the carotid body ($p < 0.001$) (Figures 6 and 7). Nitrotyrosine production was increased in CIH-exposed, vehicle-treated rats relative to normoxic controls treated with allopurinol plus losartan. This effect of CIH was also observed in rats that were treated with allopurinol,

whereas it was greatly attenuated in rats treated with allopurinol plus losartan and virtually abolished in rats treated with apocynin.

3.8. Effect of CIH on heart rate responses to moderate acute hypoxia

Figure 8 shows changes in heart rate from the normoxic baseline to 0.12 FIO₂ prior to, and after 14 and 21 d of, CIH or NORM exposure in the six exposure/treatment groups. In the two groups of NORM-exposed rats, acute hypoxia produced the expected decrease in heart rate. In contrast, CIH exposure greatly attenuated (in most cases reversed) hypoxic bradycardia in all treatment groups ($p=0.001$). Heart rate responses to mild and severe acute hypoxia (FIO₂, 0.15 and 0.09, respectively) were unaffected by CIH exposure and drug treatments (data not shown).

3.9. Plasma catecholamines were not elevated after exposure to CIH

We observed no between-group differences in plasma norepinephrine concentrations ($p=0.564$). A small, not statistically significant ($p=0.072$), between-group difference was observed in plasma epinephrine concentrations (Figure 9).

4. Discussion

Previously we demonstrated, in the conscious, intact Sprague-Dawley rat, a CIH-induced increase in chemoreflex sensitivity that was manifest as augmentation of the ventilatory response to acute hypoxia (Morgan et al., 2016). The present data confirm this finding and show, via pharmacological interventions, an important role for oxidative stress in producing the effect of CIH on hypoxic chemosensitivity. Evidence for this conclusion includes: 1) virtual abolition of CIH-induced chemoreflex sensitization by concomitant treatment with apocynin, a drug that inhibits NADPH oxidase, an important intracellular source of superoxide; 2) attenuation of CIH-induced chemoreflex sensitization by allopurinol, an inhibitor of xanthine oxidase, another superoxide-generating enzyme; and 3) nearly complete elimination of CIH-induced chemoreflex sensitization by the combined treatment of allopurinol and losartan, which was aimed at reducing oxidative stress by concomitantly inhibiting xanthine oxidase and blocking the effects of angiotensin II on superoxide formation (Gonzalez-Vicente et al., 2016).

4.1. CIH-induced oxidative stress alters cardiorespiratory function

CIH exposure in rodents and in humans with obstructive sleep apnea is well-known to produce oxidative stress (Jelic et al., 2008; Lavie, 2009; Lim et al., 2014; Philippi et al., 2010). Multiple studies using blockers of ROS formation and ROS scavengers have demonstrated a crucial role for ROS in CIH-induced alterations in vascular function, blood pressure, carotid body sensory activity, and chemoreflex control of ventilation (Del Rio et al., 2010; Dopp et al., 2011; Lim et al., 2014; Marcus et al., 2010; Marcus et al., 2012; Peng et al., 2013; Peng et al., 2009; Peng and Prabhakar, 2003). The present study demonstrates, in the intact unanesthetized rat, that oxidative stress plays a crucial role in the ventilatory and metabolic adaptations elicited by CIH exposure.

The mechanisms by which CIH causes oxidative stress are incompletely understood; however, reoxygenation after a period of hypoxia causes an almost instantaneous burst of superoxide ion release (Hashimoto et al., 1994; Lim et al., 2014; Littauer and de Groot, 1992; Ryma et al., 1991). The pattern of intermittent hypoxia that accompanies obstructive sleep apnea, *i.e.* a relatively slow period of oxygen desaturation followed by rapid reoxygenation, which is modeled in the present study, seems to be an especially potent generator of ROS (Lim et al., 2014). ROS increase intracellular calcium levels, leading to increased synthesis of hypoxia-inducible factor (HIF)-1 α , which codes for pro-oxidant enzymes, and degradation of HIF-2 α , which has antioxidant effects (Nanduri et al., 2015; Nanduri et al., 2013). The resultant imbalance in pro-oxidant and anti-oxidant systems, then, creates oxidative stress. Thus, CIH produces ROS, which beget more ROS in a feed-forward manner (Semenza and Prabhakar, 2015). This process occurs not only in carotid body but also in other CNS regions [*e.g.* nucleus of the solitary tract (NTS), paraventricular nucleus of the hypothalamus (PVN), rostral ventrolateral medulla (RVLM)] that participate in chemoreflex control of ventilation and sympathetic outflow (Mifflin et al., 2015; Semenza and Prabhakar, 2015); nevertheless, previous investigators have demonstrated that an intact carotid sinus nerve is required for the ventilatory sensitizing and pro-hypertensive effects of CIH (Fletcher et al., 1992; Iturriaga et al., 2015).

4.2. Site(s) and actions of CIH-stimulated ROS production

In the present study, CIH-induced increases in carotid body nitrotyrosine immunoreactivity were prevented by concomitant treatment with apocynin and with allopurinol plus losartan; however, because these drugs were given systemically, we cannot pinpoint the carotid body as their sole site of action. These drugs likely have important ROS-reducing effects in central components of the extended chemoreflex arc (*e.g.* NTS and PVN) that relay and modulate carotid sinus nerve afferent information.

Experiments in cultured pheochromocytoma cells indicate that xanthine oxidase activation is a mechanism by which CIH activates NADPH oxidase leading to the generation of ROS (Nanduri et al., 2015). Xanthine oxidase activation is also required for CIH-induced upregulation of HIF-1 α and degradation HIF-2 α (Nanduri et al., 2015; Nanduri et al., 2013). Thus, increased production of xanthine oxidase-derived ROS in carotid body might be expected to play an important role in CIH-induced chemoreflex sensitization. The present data provide only partial support for this notion, however, because allopurinol alone did not prevent CIH-induced augmentation of carotid body nitrotyrosine formation, even though it did attenuate the stimulatory effect of CIH on the hypoxic ventilatory response. Taken together these data suggest that xanthine oxidase-derived oxidative stress contributes to CIH-induced alterations in hypoxic chemosensitivity at loci of ventilatory control apart from the carotid body. The present finding that combined treatment with allopurinol and losartan reduced the CIH-induced increase in nitrotyrosine production whereas allopurinol alone did not suggests that angiotensin II contributes importantly to CIH-induced oxidative stress in carotid body.

Thus, the augmented hypoxic ventilatory response caused by CIH is likely to be dependent, at least in part, on angiotensin II signaling. CIH increases the expression of angiotensinogen,

angiotensin converting enzyme, and AT₁R in carotid body (Lam et al., 2014; Marcus et al., 2010). Our laboratory has recently demonstrated that AT₁R blockade with losartan prevents CIH-induced increases in lumbar sympathetic nerve activity, normalizes CIH-induced increases in carotid body superoxide production, and blocks CIH-induced increases in the gp91^{phox} subunit of NADPH oxidase in carotid body (Marcus et al., 2010). It is becoming increasingly evident that angiotensin II-mediated, NADPH-dependent oxidative stress plays an important role in the pathophysiology of hypertension and heart failure (Griendling et al., 1994; Li et al., 2015; Schultz, 2011; Zimmerman et al., 2002). Our previous and present data indicate that ROS generated by activation of the renin angiotensin system are involved in cardiorespiratory adaptations to intermittent hypoxia and therefore may contribute importantly to the cardiovascular sequelae of sleep apnea in humans.

4.3. Role of NADPH oxidase-derived ROS

Previous investigators have demonstrated that CIH-induced sensory plasticity of the carotid body is critically dependent on NADPH oxidase (Nanduri et al., 2015; Nanduri et al., 2013; Peng et al., 2009; Peng et al., 2006a; Yuan et al., 2011). In the present study, apocynin treatment virtually eliminated CIH-induced increases in carotid body nitrotyrosine production and hypoxic chemosensitivity. These apocynin effects were similar in magnitude to those of allopurinol plus losartan, but greater than those of allopurinol alone, perhaps because allopurinol would be expected to provide only partial inhibition of the xanthine oxidase-Ang II-NADPH oxidase ROS-generating pathway.

4.4. ROS abrogate the effects of CIH on metabolic rate

Consistent with earlier findings from our laboratory (Morgan et al., 2016), CIH exposure reduced $\dot{V}CO_2$ and $\dot{V}O_2$ in normoxia in vehicle-treated rats. The work of previous investigators has shown that hypometabolism elicited by acute hypoxia in rats is caused, at least in part, by hypoxic stimulation of the carotid body and subsequent reflex withdrawal of sympathetic outflow to brown fat (Madden and Morrison, 2005). In our previous study, we speculated that CIH-induced suppression of metabolic rate might be caused by CIH-induced sensitization of the carotid chemoreflex. The present data seem to agree with this idea, because allopurinol, allopurinol plus losartan, and apocynin, drug treatments that prevented CIH-induced sensitization of the hypoxic ventilatory response, also prevented the drop in metabolic rate in normoxia. The clinical relevance of our data concerning CIH-induced intensification of the hypometabolic response is unclear: this response is present in human neonates, but not adults (Cross et al., 1958; Mortola, 2004).

4.5. CIH-induced alterations in heart rate response to acute hypoxia

The observed reversal by CIH of hypoxic bradycardia is consistent with previous work from our laboratory (Morgan et al., 2016) and other investigators who attributed this effect to altered neurotransmission in vagal neurons of the nucleus ambiguus and dorsal motor nucleus of the vagus (Dyavanapalli et al., 2014). CIH-induced degeneration of neurons in the nucleus ambiguus and dorsal motor nucleus of the vagus has also been reported (Yan et al., 2008). Our data suggest that the effect of CIH on hypoxic bradycardia is not dependent on increased ROS production because it was not affected by any of our anti-oxidant treatments. The present data further suggest that CIH-induced reversal of hypoxic bradycardia is not

dependent on sensitization of the carotid chemoreflex because it was unaffected by the drug interventions that prevented CIH-induced ventilatory adaptations. This effect of CIH on heart rate, which is consistent with diminished parasympathetic cardiac regulation, may be responsible for the decreased baroreflex sensitivity observed in rodents exposed to CIH (Lai et al., 2006; Lin et al., 2007), healthy humans exposed to CIH (Tamisier et al., 2011), and in individuals with obstructive sleep apnea (Carlson et al., 1996). Enhancement by CIH of chemoreflex-induced increases in sympathetic outflow (Marcus et al., 2010) may also have contributed to the observed diminution of hypoxic bradycardia.

4.6. Plasma catecholamine concentrations

We observed no differences among the six exposure/treatment groups in plasma norepinephrine or epinephrine after 21 d of CIH or NORM. The present findings do not agree with those of previous investigators who have shown increased plasma catecholamine concentrations after 10–14 d of CIH (Kumar et al., 2006; Olea et al., 2014; Peng et al., 2006b). The intermittent hypoxia exposure paradigms are similar in the present and previous studies; however, a possible explanation for the discrepancy may be related to the length of exposure. We previously observed higher plasma catecholamines in CIH vs. NORM rats after 14 d of exposure, whereas there was no between-group difference at 21 d. Based on our previous findings (Marcus et al., 2010), we consider it likely that sympathetic outflow was elevated at 21 d of CIH exposure, but that this increase was not reflected in catecholamine levels. Taken together, the previous and present data suggest that plasma catecholamine levels rise during the early days of CIH exposure, but tend to normalize as CIH continues past 2 wk. We cannot explain, on the basis of our data, this apparent time-dependency in the effect of CIH on rates of norepinephrine release and/or removal that would result in normalization of plasma norepinephrine levels.

4.7. Experimental considerations

The within-subjects design of this study lends strength to the present findings by accounting for inter-individual differences and also the effects of time, repeated testing, and maturation on hypoxic chemosensitivity. In addition, our intermittent hypoxia paradigm is clinically relevant because it mimics the SpO₂ profile and time course (20 to 30-s desaturation and 10 to 15-s resaturation) of events characteristic of obstructive sleep apnea in the human. Recently, the time-dependent oxygen saturation profile during each intermittent hypoxia episode was shown to be an important determinant of oxidative stress during CIH exposure (Lim et al., 2014). Although the desaturation-resaturation profile we used models individual episodes of human sleep apnea and our frequency of 15 events/h is consistent with moderate-to-severe obstructive sleep apnea, the periodicity of events (one event every four min) is not commonly observed. Individuals with sleep apnea typically experience clustering, rather than even spacing, of events. In spite of the limitations of the present model, our findings are consistent with previous human studies showing augmented hypoxic ventilatory responses and oxidative stress in patients with obstructive sleep apnea (Lavie, 2009; Narkiewicz et al., 1999) and healthy humans exposed to CIH (Pialoux et al., 2009). We emphasize that our findings do not apply to other patterns of exposure intended to mimic sleep apnea or to “therapeutic” intermittent hypoxia paradigms (Navarrete-Opazo et al., 2015).

Our method of detecting nitrotyrosine in carotid body did not allow us to localize its presence to glomus cells; therefore, it is possible that the stimulatory effect of CIH on ROS production was not confined only to the carotid chemoreceptor, but also occurred in carotid body endothelial cells. This finding raises the possibility that ROS-induced endothelial dysfunction could, via “stagnant hypoxia”, affect carotid chemosensitivity apart from the direct effects of ROS on glomus cell function. An analogous effect has been observed in an experimental model of heart failure (see Schultz (Schultz et al., 2015). Our laboratory has previously shown, in cerebral and skeletal muscle resistance arteries, that CIH impairs endothelium-dependent hypoxic vasodilation (Phillips et al., 2004).

Although we demonstrate a role for oxidative stress in causing the CIH-induced increase in hypoxic chemosensitivity, we cannot pinpoint the culpable free radical oxidant(s). Superoxide anion, the primary source of ROS by virtue of its ability to generate other oxidants, was not measured directly in our study. Instead, our measurements of nitrotyrosine immunoreactivity reflect production of peroxynitrite, the rapidly-formed product of the interaction between superoxide and nitric oxide. Recently, evidence has emerged that CIH-induced sensitization of chemoreflex pathways is critically dependent on peroxynitrite (Moya et al., 2016).

We influenced CIH-induced chemoreflex sensitization via pharmacological interventions targeted to xanthine oxidase, NADPH oxidase, and angiotensin II; however, previous investigators have shown that other molecules and signaling pathways, *i.e.* endothelin, 5-HT, adenosine and inflammatory cytokines, are also involved in CIH-induced chemoreflex sensitization (Del Rio et al., 2011a; Iturriaga et al., 2014; Pawar et al., 2009; Peng et al., 2013; Peng et al., 2006a; Sacramento et al., 2015). Because most, if not all, of these molecules or processes have pro-oxidant properties, we speculate that oxidative stress is a common thread linking the many causes of CIH-induced chemoreflex sensitization (Del Rio et al., 2010).

In conclusion, we found that CIH-induced sensitization of chemoreflex control of breathing was greatly attenuated by inhibition of NADPH oxidase, xanthine oxidase, and inhibition of xanthine oxidase combined with blockade of angiotensin II receptors, presumably via antioxidant mechanisms. Because oxidative stress in carotid body and adrenal medulla play a causal role in the ventilatory abnormalities and sympathetic nervous system overactivity observed in animal models of human sleep apnea (Kumar et al., 2015; Kumar et al., 2006; Marcus et al., 2012; Peng et al., 2013; Peng et al., 2009), these findings suggest potential therapeutic targets for attenuating the adverse cardiorespiratory sequelae in individuals with this common condition. Anti-oxidant therapy may be a useful adjunct to continuous positive airway pressure (CPAP), the first-line treatment for obstructive sleep apnea, which is effective but often underutilized (Rotenberg et al., 2016) due to the cumbersome and intrusive nature of the positive pressure device.

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Highlights

- Chronic intermittent hypoxia (CIH) augmented the hypoxic ventilatory response.
- This increase was prevented by pharmacological inhibitors of superoxide production.
- Superoxide inhibition prevented the CIH-induced drop in metabolic rate.
- CIH increased carotid body nitrotyrosine production.

Figure 1.

Cardiorespiratory and metabolic responses during acute exposure to graded hypoxia in all rats (n=51) under baseline conditions prior to CIH or NORM exposure and drug treatments. $\dot{V}_E$, minute ventilation; VT, tidal volume; f_B , breathing frequency; $\dot{V}O_2$, oxygen consumption; $\dot{V}CO_2$, carbon dioxide production; SpO₂, arterial oxygen saturation. Statistically significant increases were observed in all ventilatory variables at all levels of inspired FIO₂ with the exception of VT in mild hypoxia (FIO₂, 0.15). Heart rate showed a biphasic response consisting of a small, but statistically significant, increase in mild hypoxia and substantial decreases in moderate and severe hypoxia (FIO₂, 0.12 and 0.09). Metabolic rate ($\dot{V}O_2$ and $\dot{V}CO_2$) fell progressively during the acute hypoxic exposure. *p<0.05 vs. normoxia by repeated-measures ANOVA.

Figure 2.

Effects of 7 d of drug treatment on primary outcome measures of hypoxic chemosensitivity (upper and middle panels) and metabolic rate in normoxia (lower panel) prior to initiation of CIH or NORM exposure. No treatment had effects that were significantly different from those in untreated rats.

Figure 3.

Slopes of the relationships between ventilatory variables and SpO_2 during exposure to acute, graded hypoxia after 14 and 21 d of CIH or NORM in the six experimental groups. Data are expressed as percentages of the baseline, pre-exposure values. * $p < 0.05$ vs. NORM untreated rats by repeated measures, general linear model

Figure 4.

Slopes of the relationships between ventilatory equivalent for $\dot{V}CO_2$ (upper panel) and respiratory “drive” (bottom panel) with SpO_2 during exposure to acute, graded hypoxia after 14 and 21 d of CIH or NORM in the six experimental groups. Data are expressed as percentages of the baseline, pre-exposure values. Both indicators of hypoxic chemosensitivity were increased, relative to normoxic control rats, in CIH-exposed vehicle-treated rats but not in CIH-exposed rats treated with allopurinol (Allo), allopurinol plus losartan (Allo+Los), or apocynin (Apo). * $p < 0.05$ vs. NORM untreated rats by repeated measures, general linear model

Figure 5.

Effects of exposure and drug treatment on $\dot{V}\text{CO}_2$ in the six experimental groups. $\dot{V}\text{CO}_2$ was decreased in normoxia following CIH exposure in vehicle-treated rats. In contrast, $\dot{V}\text{CO}_2$ remained stable in rats exposed to normoxia and in CIH-exposed rats treated with allopurinol (Allo), allopurinol plus losartan (Allo+Los) and apocynin (Apo). * $p < 0.05$ vs. pre-exposure baseline by repeated measures, general linear model

Figure 6.

Representative micrographs showing 3-nitrotyrosine (3-NT) immunoreactivity in carotid bodies. Left panel shows, from top to bottom, undeconvoluted images from normoxic rats (NORM) treated with allopurinol + losartan, chronic intermittent hypoxia-exposed rats (CIH) treated with vehicle, CIH rats treated with allopurinol (Allo), CIH rats treated with Allo + losartan (Los), and CIH treated with apocynin (Apo), and a negative control produced by omission of the primary antibody. Middle and right panels show the same images as in the left panel after application of a color deconvolution algorithm for quantification of 3-NT immunoreactivity. Middle panel, 3-NT immunodetection. Right panel, hematoxylin.

Figure 7.

Summary data showing the effects of vehicle, Allo, Allo+Los and Apo on nitrotyrosine immunoreactivity in the carotid body of rats exposed to CIH. * $p < 0.05$ vs. Normoxia+Allo+Los by one-way ANOVA with Dunnett post hoc tests ($n=5$ per group).

Figure 8.

Influence of exposure and drug treatments on the bradycardia produced by acute hypoxia. The change in heart rate (HR) from normoxia to acute hypoxia (FIO₂, 0.12) is shown before and after CIH or NORM in the six exposure/treatment groups. In NORM-exposed rats and in rats prior to CIH exposure, HR decreased during acute hypoxia. In contrast, HR increased at this level of hypoxic inspirate after exposure to CIH. None of drug treatments altered this pattern. *p<0.05 vs. NORM untreated rats by repeated measures, general linear model

Figure 9. Plasma concentrations of norepinephrine (upper panel) and epinephrine (lower panel) measured after 21 d of the specified exposures and drug treatments. No statistically significant differences were detected by one-way ANOVA.

Slopes of the relationships between respiratory variables and SpO₂ during acute hypoxia prior to exposure to CIH or NORM in all treatment groups. No statistically significant between-group differences were observed (1-way ANOVA).

Table 1

	NORM Untreated	NORM Allo+Los	CIH Vehicle	CIH Allo	CIH Allo+Los	CIH Apocynin	P value
$\dot{V}_E/\dot{V}CO_2/SpO_2$	-2.9±0.3	-2.9±0.4	-2.0±0.3	-2.0±0.3	-2.5±0.3	-2.8±0.4	0.175
VT/T _I $\dot{V}CO_2/SpO_2$	-0.08±0.01	-0.07±0.03	-0.05±0.01	-0.04±0.1	-0.06±0.01	-0.07±0.01	0.190
$\dot{V}_E/SpO_2$	-3.3±0.3	-3.4±0.5	-2.3±0.3	-2.5±0.3	-3.2±0.4	-3.8±0.6	0.137
VT/SpO ₂	-0.11±.001	-0.13±.002	-.009±.001	-.008±.001	-.011±.002	-.015±.003	0.192
f_b/SpO_2	-2.7±0.2	-2.8±0.2	-1.7±0.2	-2.2±0.3	-2.5±0.3	-2.8±0.4	0.077

Body mass at baseline and at the time of the 14- and 21-day hypoxic ventilatory response tests after exposure to normoxia (NORM) or chronic intermittent hypoxia (CIH) with concomitant treatments with vehicle, allopurinol, allopurinol plus losartan (allo+los), or apocynin. Means±SEM. Statistical significance ($p<0.05$): b, effect of day; c, exposure/treatment-by-day interaction

Table 2

	Baseline	14 days	21 days	P value
NORM untreated	394±7	402±7	408±6	
NORM allo+los	404±8	405±6	409±7	
CIH vehicle	389±7	415±7	425±8	0.000 ^{b,c}
CIH allopurinol	391±6	402±6	411±6	
CIH allo+los	386±3	391±7	396±8	
CIH apocynin	389±3	417±4	427±3	