Obstructive Sleep Apnea

Carotid Body Ablation Abrogates Hypertension and Autonomic Alterations Induced by Intermittent Hypoxia in Rats

Rodrigo Del Rio,* David C. Andrade, Claudia Lucero, Paulina Arias, Rodrigo Iturriaga*

Abstract—Chronic intermittent hypoxia (CIH), the main feature of obstructive sleep apnea, enhances carotid body (CB) chemosensory responses to hypoxia and produces autonomic dysfunction, cardiac arrhythmias, and hypertension. We tested whether autonomic alterations, arrhythmogenesis, and the progression of hypertension induced by CIH depend on the enhanced CB chemosensory drive, by ablation of the CB chemoreceptors. Male Sprague-Dawley rats were exposed to control (Sham) conditions for 7 days and then to CIH (5% O₂, 12 h/8 h/d) for a total of 28 days. At 21 days of CIH exposure, rats underwent bilateral CB ablation and then exposed to CIH for 7 additional days. Arterial blood pressure and ventilatory chemoreflex response to hypoxia were measured in conscious rats. In addition, cardiac autonomic imbalance, cardiac baroreflex gain, and arrhythmia score were assessed during the length of the experiments. In separate experimental series, we measured extracellular matrix remodeling content in cardiac atrial tissue and systemic oxidative stress. CIH induced hypertension, enhanced ventilatory response to hypoxia, induced autonomic imbalance toward sympathetic preponderance, reduced baroreflex gain, and increased arrhythmias and atrial fibrosis. CB ablation normalized blood pressure, reduced ventilatory response to hypoxia, and restored cardiac autonomic and baroreflex function. In addition, CB ablation reduced the number of arrhythmias, but not extracellular matrix remodeling or systemic oxidative stress, suggesting that reductions in arrhythmia incidence during CIH were related to normalization of cardiac autonomic balance. Present results show that autonomic alterations induced by CIH are critically dependent on the CB and support a main role for the CB in the CIH-induced hypertension. (Hypertension. 2016;68:436-445. DOI: 10.1161/HYPERTENSIONAHA.116.07255.) ● Online Data Supplement

Key Words: blood pressure ■ cardiac arrhythmias ■ extracellular matrix ■ hypertension ■ obstructive sleep apnea

The obstructive sleep apnea (OSA), a worldwide sleep breathing disorder that affect 9% of women and 24% of adult men, is an independent risk factor for systemic hypertension and stroke and is associated with atrial arrhythmogenesis. Chronic intermittent hypoxia (CIH), which is the principal feature of OSA, is considered the main factor for the hypertension. CIH produces autonomic dysfunction characterized by sympathetic hyperactivity, alterations of heart rate (HR) variability (HRV), and reduction of cardiac baroreflex efficiency. Although the link between OSA and hypertension is well established, the mechanisms responsible for the autonomic imbalance and the hypertension are not entirely known. CIH produces oxidative stress, inflammation, and endothelial dysfunction that contribute to the hypertension. However, a growing body of evidences suggests that the carotid body (CB), the main oxygen chemoreceptor organ, plays a crucial role in the development of autonomic alterations and hypertension after CIH. Indeed, patients with OSA and animals exposed to CIH show enhanced cardiorespiratory and sympathetic responses to hypoxia, suggesting that CIH potentiates the CB-mediated chemoreflex drive. Fletcher et al found that bilateral CB denervation before the CIH exposure prevents the development of the hypertension in rats. Despite this important result, the idea that the CB chemoreceptor contributes to the progression of cardiovascular pathologies associated with OSA was not seriously considered until the last decade. Indeed, in the last years, the proposal that the CB is involved in the progression of the CIH-induced hypertension received further attention.

Neural recordings of CB chemosensory activity have shown that CIH selectively

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enhances CB chemo sensory discharge in normoxia and in response to hypoxia.\textsuperscript{12,19,23–25} Furthermore, it has been shown that arterial blood pressure (BP) rapidly increases after 3 to 4 days after CIH exposure,\textsuperscript{14,26–28} which is consistent with the time required to potentiate the neural discharges from the CB chemoreceptor during CIH.\textsuperscript{19,24} Despite all the advances related to the contribution of CB chemoreception to the cardiovascular consequences of CIH, there are no studies showing the role played by the CB chemoreflex once the hypertensive phenotype has become expressed because of CIH exposure. It is worth noting that it is fundamental from a translational perspective because in previous studies performed by Fletcher et al.,\textsuperscript{20} the CBs were removed before the start of CIH and, therefore, before the development of systemic hypertension.

Taken together, the available evidence suggests that CIH-induced hypertension is associated with an enhanced CB chemo sensory drive. However, there is no study available intended to proof causality on the role played by the CB on the development of autonomic imbalance, cardiac arrhythmogenesis, and hypertension during exposure to CIH. Accordingly, the aim of the study was to determine whether the CB is essential on the progression of cardiovascular pathophysiology in OSA. Thus, to determine the causal contribution of the CB to the cardiovascular alterations resulting from CIH exposure, we tested whether the withdrawal of the CB chemo sensory inputs to the brainstem may restore the altered cardiorespiratory and autonomic balance and finally normalize arterial BP in conscious rat exposed to CIH. Furthermore, we studied the effects of CB ablation (CBA) on the increased cardiac arrhythmogenesis and extracellular matrix (ECM) remodeling in cardiac atrial tissue from rats exposed to CIH.

**Methods**

Experiments were performed on 22 male Sprague–Dawley rats (247.0±7.6 g) fed with standard diet ad libitum and kept on a 12-hour light/dark schedule. The protocol was approved by the Bioethical Committee of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, and was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Experimental Protocol**

Rats underwent BP radiotelemetry implantation and 7 days post-recovery were housed in individual chambers and exposed to Sham conditions for 7 days and then to CIH as previously described.\textsuperscript{7,18,24,29} At day 21 of CIH, bilateral CB ablation was performed and rats were kept in CIH for 7 more days (Figure S1 in the online-only Data Supplement).

**Selective CB Ablation**

The CBs were cryogenically ablated as previously described\textsuperscript{30–32} (Figure S2).

**Cardiorespiratory Recordings**

Arterial BP and HR measurements were performed in conscious rats as previously described.\textsuperscript{7,29} Tidal volume (\(V_T\)), respiratory frequency (\(f_R\)), and minute volume (\(V_{\text{min}}\)) were determined by unrestrained whole-body plethysmography. Peripheral chemoreflex drive was evaluated as previously described.\textsuperscript{10,13}

**Baroreflex Sensitivity**

Spontaneous baroreflex sensitivity (BRS) was measured by the sequence method.\textsuperscript{11,30} In addition, intravenous infusion of phenylephrine (25 µg/kg) and sodium nitroprusside (50 µg/kg) was used to fully determine the cardiac baroreflex function.\textsuperscript{34}

**Cardiac Autonomic Balance**

HRV was used to assess autonomic balance as previously described.\textsuperscript{12,13,19,30} In addition, we studied the effects of the \(\beta\)-adrenergic and cholinergic receptors blockers on resting HR to determine the sympathovagal control to the heart.

**Cardiac Arrhythmias**

Arrhythmias were visually inspected and scored as previously described.\textsuperscript{30,35}

**ECM Remodeling**

Hearts were harvested from anesthetized rats to determine the ECM fibrotic index, metalloproteinase-2 (MMP-2), and MMP-2 inhibitor (TIMP-2) protein expression.

**Systemic Oxidative Stress**

Plasma thiobarbituric acid reactive substances were used as a systemic oxidative stress marker.\textsuperscript{19,24}

**Data Analysis and Statistics**

Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, CA). Data were analyzed using both repeated measures ANOVA and nonrepeated measures ANOVA as needed, followed by proper post hoc analysis. Data were expressed as mean±SEM.

**Results**

CIH increased mean arterial BP by \(\approx 10\) mm Hg in 4 days, which remained elevated during the CIH exposure (Figure 1A). At day 21 of CIH, mean arterial BP was significantly increased compared with the Sham condition (109.8±2.3 versus 99.9±1.8 mm Hg, CIH versus Sham, respectively; \(P\leq 0.01\), Figure 1C). The increment of mean arterial BP during CIH was because of an elevation of systolic and diastolic arterial BP (Table 1). Remarkably, when performed in hypertensive rats, CBA promptly reduced BP toward the values observed in Sham condition (Figure 1A through 1C; Table 1). No differences in resting HR were observed among the experimental conditions (Figure 1D). CIH increased systemic oxidative stress, but CBA did not reduce the elevated MDA plasma levels (Figure S3).

Resting ventilatory variables measured in normoxia are shown in Table 1. Tidal volume (\(V_T\)) was higher during CIH compared with the Sham condition (0.55±0.02 versus 0.48±0.02 mL, CIH versus Sham, respectively; \(P<0.001\)), but no changes in respiratory frequency (\(f_R\)) was observed along the experimental conditions. During acute hypoxic challenges (\(\text{FiO}_2\) 10%), minute ventilation (\(V_{\text{min}}\)) was markedly elevated in CIH compared with the Sham mainly because of an increase in \(V_T\), but not in \(f_R\) (Figure 2B through 2D). Ablation of the CBs significantly reduced the enhanced hypoxic ventilatory response induced by exposure CIH (Figure 2).

Spontaneous BRS, assessed longitudinally in rats exposed to Sham conditions followed by 21 days of CIH, showed that BRS was being progressively reduced by CIH (Figure 3A and 3B). In the same group of animals, CBA markedly improved BRS (1.6±0.1 versus \(-2.5±0.1\) mm Hg/mL, CIH versus CBA+CIH, respectively; \(P<0.01\)). In addition, in a subset of rats, we studied the effects of intravenous infusions of phenylephrine (25 µg/kg) and sodium nitroprusside (50 µg/kg) to fully
determine the cardiac arm of the baroreflex control (Figure 3C and 3D; Table 2). We found that exposure to CIH increased the BP midpoint and reduced the overall gain ($P \leq 0.01$) of the $\Delta HR/\Delta BP$ curve when compared with the Sham condition. CBA produced a resetting of the midpoint-operating pressure toward Sham values (111.5±5.6 versus 102.1±1.7 mm Hg, CIH versus CIH+CBA, respectively; $P \leq 0.01$), but it did not restore the gain (4.5±0.9 versus 5.4±1.9 beats/mm Hg, CIH versus CIH+CBA, respectively; $P > 0.05$).

HRV showed a progressive increase of the low/high frequency ratio during CIH compared with the Sham condition, whereas the ablation of the CBs promptly restored the normal low/high frequency ratio values (Figure 4A and 4B). Intravenous infusion of atropine (1 mg/kg) and propranolol (1 mg/kg) to assess changes in HR associated with sympathovagal control showed that cardiac autonomic balance was shifted toward sympathetic predominance during CIH, and that CBA markedly reduced the enhanced cardiac sympathetic tone (Figure 4C). Although some degree of parasympathetic withdrawal occurs in CIH (Figure 4D), the ablation of the CBs did not significantly improve the parasympathetic modulation of the heart ($\Delta HR$ after atropine 40.5±10.9 versus 30.8±3.6 bpm, CIH versus CIH+CBA, respectively; $P \geq 0.05$). In addition, the low-frequency component of the systolic arterial BP variability, an indirect measure of sympathomotor tone, was reduced by CBA (Figure S3).

Arrhythmia incidence increased during CIH when compared with Sham condition (184.0±22.4 events/h versus 101.8±25.7 events/h, CIH versus Sham, respectively; $P \leq 0.01$). Histological and Western blot analyses of atrial tissue showed...
that CIH produced ECM remodeling characterized by increases in atrium fibrosis. Although not statistically significant, CIH tends to reduce MMP-2 expression without significant changes in TIMP-2 levels (Figure 5D through 5F; Figure S5). Despite ECM remodeling during CIH, increased susceptibility to cardiac arrhythmias is critically dependent on the enhanced cardiac sympathetic modulation because administration of propranolol (1 mg/kg IV) normalized the arrhythmia index to the values observed in Sham conditions (Figure 5C). CBA markedly reduced the number of cardiac arrhythmias, despite that rats continue to be exposed to CIH (Figure 5A and 5B). No significant changes in atrium fibrosis, MMP-2, and TIMP-2 levels were found in atrial tissue from rats that underwent CBA (Figure 5; Figure S5). Therefore, the beneficial effect of CBA on CIH-induced cardiac arrhythmias seems to be related to the normalization of cardiac autonomic balance.

Table 1. Effect of CBA on Basal Physiological Variables During CIH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>CIH</th>
<th>CIH+CBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>123.0±4.5</td>
<td>135.5±2.1†</td>
<td>124.2±2.1†</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>87.6±3.2</td>
<td>96.5±1.1*</td>
<td>89.2±2.2†</td>
</tr>
<tr>
<td>PP, mmHg</td>
<td>35.3±1.7</td>
<td>39.0±1.8</td>
<td>35.0±1.1</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>361.5±6.9</td>
<td>354.0±18.9</td>
<td>323.0±21.2</td>
</tr>
<tr>
<td>V₄, mL/kg</td>
<td>0.48±0.02</td>
<td>0.55±0.02*</td>
<td>0.44±0.02†</td>
</tr>
<tr>
<td>V₄, mL/kg·min</td>
<td>56.5±6.5</td>
<td>54.5±4.5</td>
<td>53.4±4.1</td>
</tr>
<tr>
<td>fᵣ, breaths/min</td>
<td>119.2±16.2</td>
<td>99.5±11.4</td>
<td>104.3±4.5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>247.0±7.6</td>
<td>291.5±10.6*</td>
<td>282.8±5.3*</td>
</tr>
</tbody>
</table>

CBA indicates carotid body ablation; CIH, chronic intermittent hypoxia; DBP, diastolic blood pressure; fᵣ, respiratory frequency; HR, heart rate; PP, pulse pressure; SBP, systolic blood pressure; VI, ventilatory minute volume; and VT, ventilatory tidal volume.

*P<0.01 vs Sham. †P<0.01 vs CIH. n=7.

Discussion

Our results confirm that the CB plays a crucial role in the progression and maintenance of the autonomic alterations and the hypertension induced by CIH in rats. CBA markedly reduced the elevated BP and restored the cardiorespiratory and autonomic balance, suggesting that the maintenance of the hypertension after CIH is critically dependent on the cyclic hypoxic stimulation of the CB chemoreflex. In addition, present results show that systemic oxidative stress persisted after CBA, but the elevated BP returned to normal values in rats that underwent CBA. Therefore, our results provide compelling evidence showing that the CB is required for the development

Figure 2. Effect of bilateral carotid body ablation (CBA) on hypoxic ventilatory response during chronic intermittent hypoxia (CIH). A, Representative plethysmographic recordings of tidal volume (V₄) in normoxia (Fₒ₂ 21%) and hypoxia (Fₒ₂ 10%) in the same rat in Sham condition (day 7), CIH (day 28), and CIH+CBA (day 36). B, The respiratory frequency (fᵣ) did not change after hypoxia in CIH and CIH+CBA. C, V₄ increased at Fₒ₂ 10%. D, V₄ was slightly increased in response to Fₒ₂ 10% in CIH and was significantly reduced after bilateral CBA. *P<0.001 vs Sham; †P<0.001 vs CIH+CBA, Tukey test after 1-way ANOVA; n=6 rats.
of hypertension during CIH, despite the increases in systemic oxidative stress.

Autonomic dysfunction has been proposed as a mechanism involved in the generation of high BP in patients with OSA and animals exposed to CIH. Cyclic episode hypoxemia-reoxygenation in patients with OSA enhances the ventilatory, cardiovascular, and sympathetic responses to hypoxia.\textsuperscript{16,17,36,37} Similarly, animal exposed to CIH show sympathetic hyperactivity and develop systemic hypertension.\textsuperscript{8–10,13,18,19,38} The autonomic dysfunction is associated with a reduction of BRS and changes in HRV in patients with OSA\textsuperscript{37,39} and animals exposed to CIH.\textsuperscript{9,12–14,40,41} Interaction of chemo- and baroreflexes plays a main role in the cardiorespiratory and autonomic homeostasis. It is well known that hypoxic chemoreflex activation evokes baroreflex inhibition attributed to central nervous system modulation at the level of the nucleus of the solitary tract.\textsuperscript{42,43} The hypertension induced by CIH is associated with reduced BRS efficiency in adult animals exposed to CIH for 1 to 3 months.\textsuperscript{9,11–14,40,41} but other studies performed in juvenile rats have shown that juvenile rats exposed to CIH for 15 days showed an increased cardiac baroreflex gain associated with a rightward shift on the operating point to higher pressures, as related to control rats.\textsuperscript{38} Zoccal et al\textsuperscript{38} proposed that the sympathetic-mediated hypertension is not secondary to a reduction in cardiac baroreflex gain, but to an enhanced respiratory–sympathetic coupling. As abovementioned, previous studies performed in adult rats\textsuperscript{9,44} exposed to CIH clearly evidenced a significant impairment of the cardiac baroreflex. The main differences among these studies are the age of the animals and the duration of the CIH exposure. Indeed, the cardiac baroreflex control of juvenile rats seems to be less affected by CIH exposure. Indeed, in juvenile rats exposed to CIH for 10 days, the development of hypertension seems not to be related to alterations on the cardiac baroreflex gain.\textsuperscript{38}

Table 2. Effect of CBA on Baroreflex Parameters After Intravenous Infusion of Phenylephrine (25 µg/kg) and Sodium Nitroprusside (50 µg/kg) During CIH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>CIH</th>
<th>CIH+CBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range, bpm</td>
<td>240.3±55.1</td>
<td>44.1±16.23*</td>
<td>42.4±9.5*</td>
</tr>
<tr>
<td>Slope, beats/ mm Hg</td>
<td>0.3±0.3</td>
<td>0.8±0.3</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>Gain, beats/ mm Hg</td>
<td>14.7±1.1</td>
<td>4.5±0.9*</td>
<td>5.1±1.9*</td>
</tr>
<tr>
<td>Midpoint of BP, mm Hg</td>
<td>97.7±3.8</td>
<td>111.5±5.6*</td>
<td>102.1±1.7†</td>
</tr>
<tr>
<td>Lower Plateau, beats</td>
<td>341.7±60.6</td>
<td>366.8±15.3</td>
<td>318.7±10.0</td>
</tr>
<tr>
<td>Upper Plateau, beats</td>
<td>582.0±53.12</td>
<td>410.9±21.4</td>
<td>360.5±17.5</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; CBA, carotid body ablation; and CIH, chronic intermittent hypoxia.

*P<0.01 vs Sham; †P<0.01 vs CIH. n=4.
Our results showed that BRS is progressively reduced by CIH. Moreover, CIH produced a significant elevation of the BP midpoint and a marked reduction of the operative range and the gain (Figure 3), which are partially recovered after CBA. A reduced baroreflex gain secondary to the increase BP during CIH may contribute to the further deterioration of the neural reflex control of BP. It is likely that the enhanced CB chemoreflex impair the central baroreflex response to the elevated BP. Our results suggest that repetitive activation of the peripheral CB-mediated hypoxic chemoreflex drive during CIH results in alterations of the baroreflex control. Nevertheless, we cannot rule out that exposure to CIH per se may also reduce BRS and reset the baroreflex midpoint to operate at a higher BP level. Further studies are needed to prove that CB chemoreceptor potentiation leads to baroreflex impairment after CIH.

Present results showing that mean arterial BP increased ≈10 mm Hg in 4 days after the beginning of CIH, agree with previous observations performed in conscious rats. A fast increment of BP in response to CIH, which occurs in few days, is difficult to attribute simply to vascular remodeling. It is likely that this hypertensive response is caused by a higher sympathetic vasoconstrictor tone to the blood vessels because of the enhanced CB chemosensory drive. Indeed, the time course of the BP elevation is consistent with the time required by CIH to significantly enhance CB chemosensory output. Compatible with this interpretation, we previously found that external carotid arteries segments harvested from rats exposed to CIH for 21 days, present moderate increases in the contractile responses to KCl and reduced NO-dependent relaxation induced by acetylcholine. Thus, it is plausible that more pronounced vascular changes may take place if CIH is prolonged for >28 days. We cannot preclude that the relative contribution of the CB to the hypertension will be diminish if the CIH stimulus is maintained for 2 to 3 more weeks, whereas the contribution of the vascular compartment may increase.

Our results support the idea that CB is involved in the onset and progression of the hypertension in patients with OSA. Indeed, it has been shown that patients with OSA display enhanced cardiorespiratory and sympathetic responses to hypoxia, suggesting the presence of an augmented CB chemoreflex drive. Animals exposed to CIH show similar enhanced hypoxic ventilatory responses to acute hypoxia. Furthermore, recording of CB chemosensory discharges have confirmed that CIH produces long-term facilitation of the CB chemosensory responses to hypoxia. Exposure of rats and cats to intermittent hypoxia for few days increases the basal CB discharges measured in normoxia and enhances the chemosensory responses to hypoxia. Recently, new experimental evidence has shown that the CB is involved in several sympathetic-mediated diseases. The selective ablation of the CB reduced BP in spontaneous hypertensive rats, reduced sympathetic hyperactivation, improved survival in heart failure experimental models, and prevented the development of...
hypertension and insulin resistance in rats fed with a high-fat diet. Thus, it is possible that the ablation of the CB will improve cardiovascular control in OSA. However, there is no available clinical information showing the effects of CB denervation in patients with OSA. OSA is characterized by the repeated episodes of airflow detention during sleep produced by the upper airway collapse, eliciting intermittent hypoxia, hypercapnia, negative intrathoracic pressure, sleep fragmentation, and microarousal. During apnea, hypoxia and hypercapnia stimulate the CB chemoreceptor eliciting acute ventilatory, sympathetic, and hypertensive responses but also contributes to the microarousal. Therefore, future studies addressing the effects of CBA on CIH-induced arrhythmias are needed.

Cardiac arrhythmogenesis is increased in patients with OSA. It has been shown that activation of the sympathetic nervous system and changes in cardiac ECM are both associated with poor cardiac rhythm maintenance. In this study, we show that rats exposed to CIH displayed increases in arrhythmia incidence and fibrosis in the atrial tissue. Remarkably, we found for the first time that the CB plays a pivotal role in the development of CIH-induced cardiac arrhythmias. The complete molecular pathways involved on the effects of CBA on cardiac arrhythmias remain to be elucidated. Nevertheless, we may speculate that the effects of CBA are likely due, at least in part, to the restoration of cardiac autonomic balance. Indeed, rats exposed to CIH and treated with propranolol showed a similar number of arrhythmic events as compared with the rats that underwent CBA. Interestingly, we found that CBA did not change cardiac fibrosis during CIH exposure. Indeed, we found that rats exposed to CIH that underwent CBA displayed similar degrees of cardiac fibrosis and expression of MMP-2 compared with CIH rats. Therefore, our results suggest that cardiac ECM remodeling is not primary involved in cardiac arrhythmogenesis induced by 28 days of CIH. Cardiac remodeling is a time-dependent process that occurs early in response to several cardiac stressors. Moreover, ECM remodeling has been shown to be a dynamic

Figure 5. Bilateral carotid body ablation (CBA) reduced chronic intermittent hypoxia (CIH)–induced cardiac arrhythmias. A, Representative recordings of heart rate (HR) tachograms obtained in the same rat at day 7 of Sham condition, day 28 of CIH condition, and day 36 after CIH+CBA. Bilateral CBA markedly reduced the number of arrhythmic events during CIH. B, Summary of the effects of CIH and CIH+CBA on arrhythmia index (events/h). C, Comparison of the effect of sympathetic blockade with propranolol (1 mg/kg IV) and CBA on the incidence of arrhythmic episodes during CIH. Note that bilateral CBA significantly reduced the arrhythmic events to the same extent compared with sympathetic blockade. D, Representative images showing Masson trichrome staining in the heart atrium from 1 Sham rat, 1 CIH rat, and 1 CIH+CBA rat. Note that CBA did not change atrium fibrosis induced by CIH exposure. E, Summary of the effects of CIH and CIH+CBA on atrium tissue fibrosis. F, Western blot of metalloproteinase-2 (MMP-2). The MMP-2 levels trend to be reduced in CIH but did not reach statistical significance (P>0.05). Bilateral CBA did not change the level of MMP-2 during CIH. *P<0.001 vs Sham; +P<0.001 vs CIH, Tukey test after 1-way ANOVA. A and B, n=6 rats. C to F, n=4 rats.
continuous process that is involved in cardiac function maintenance and arrhythmogenesis.\textsuperscript{53,54} Then, it is plausible that ECM remodeling could contribute to cardiac function deterioration in the long term during CIH exposure. Indeed, it has been shown that patients with OSA displayed an impaired cardiac function compared with healthy subject.\textsuperscript{53,55} Then we cannot rule out that cardiac fibrosis and ECM remodeling may play a role in cardiac arrhythmogenesis in long-term exposure to CIH. Future studies will be needed to address the contribution of cardiac remodeling in the development of cardiac arrhythmias in OSA.

**Perspectives**

The role played by the CB in the development and progression of the hypertension induced by OSA is a fundamental unanswered question. Present results support a main role for peripheral CB chemoreceptors in the context of OSA-induced autonomic dysfunction and hypertension. The intermittent hypoxic episodes enhance the CB responsiveness to hypoxia, which in turn potentiates the ventilatory and sympathetic responses to hypoxia, alters BRS and HRV, leading to the hypertension. Moreover, our results demonstrate that the CB plays a pivotal role in the development of CIH-induced cardiac arrhythmias. The CB has been involved in several human sympathetic-mediated diseases, and the denervation or selective ablation of the CBs has been proposed as a feasible clinical treatment for severe and resistant hypertension in humans\textsuperscript{49,50} and cardiovascular consequences of heart failure.\textsuperscript{50} Thus, it is possible that the modulation of the enhanced CB function will be of therapeutic value to develop new treatments intended to improve the cardiovascular consequences of OSA.

We previously reported that CIH induced local and systemic oxidative stress and that increase in reactive oxygen species in the CB is the key component on CB potentiation after CIH exposure.\textsuperscript{7,19,24,25} We found that CIH induced a reactive oxygen species–dependent increases of tumor necrosis factor-\(\alpha\) and interleukin–1\(\beta\) in the CB, suggesting that these proinflammatory cytokines may mediate the reactive oxygen species–induced CB potentiation.\textsuperscript{24} Accordingly, we studied the effects of ibuprofen on the increased tumor necrosis factor-\(\alpha\) and interleukin–1\(\beta\) levels in the rat CB, the potentiation of CB chemosensory, and the hypertension.\textsuperscript{25} Ibuprofen treatment, that prevents the CB cytokines upregulation and the hypertension, failed to prevent the CB chemosensory potentiation, but it reduces the c-fos–labeled neurons in the nucleus of the tractus solitarius (NTS) of rats exposed to CIH. Thus, actions of cytokines on the arterial BP in rats exposed to CIH may occur in multiple sites, including the NTS. Waki et al\textsuperscript{46} found that abnormal expression of proinflammatory genes 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 increase the arterial BP by increasing the neuronal activity in the NTS of spontaneous hypertensive rats. Our results showing that ibuprofen reduced the c-fos immunoreactivity in NTS neurons support a novel role for inflammation in the hypertension induced by CIH. Recently, McBryde et al\textsuperscript{49} found that carotid sinus denervation in spontaneous hypertensive rats reduced CD3+ cells in single-cell suspensions of whole-brain stem homogenates, suggesting that CB denervation can reduce T-cell–mediated infiltration of tissue, which is associated with several forms of hypertension. Thus, the ablation of the CBs, which improve rat survival in heart failure\textsuperscript{50} and prevent the development of insulin resistance and hypertension in rats fed with high fructose diet,\textsuperscript{50} has been proposed for the treatment of severe and resistant hypertension in humans.\textsuperscript{9,30} Our findings indicate that CB ablation is an effective means for robust and sustained sympathoinhibition, which could translate to patients with neurogenic hypertension.

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**Disclosures**

None.

**References**


What Is New?
• Using selective elimination of the carotid body (CBs) in rats with chronic intermittent hypoxia–induced hypertension, we unveiled the pivotal role played by these chemoreceptors in the maintenance of autonomic imbalance, cardiac arrhythmias, and increased arterial blood pressure because ablation of the CBs markedly normalized autonomic balance and baroreflex control of heart rate, reduced cardiac arrhythmogenesis, and finally significantly reduced arterial blood pressure.

What Is Relevant?
• Episodic intermittent hypoxia exposure during obstructive sleep apnea leads to systemic hypertension, autonomic imbalance, and increased the number of cardiac arrhythmias. Although it is well accepted that oxidative stress partially contributes to cardiac, hemodynamic, and ventilatory alterations in sleep apnea, the role of the oxygen-sensitive CB chemoreceptors in the progression of hemodynamic disturbances remains unsolved.

Novelty and Significance

Summary
We aimed to determine whether withdrawal of the CB chemosensory afferents in chronic intermittent hypoxia–hypertensive rats restored the altered cardiorespiratory, autonomic balance, and reversed the hypertension in conscious rats. We found that ablation of the CB in chronic intermittent hypoxia–induced hypertensive rats normalized arterial blood pressure, autonomic balance, and cardiac baroreflex function but did not reduce the systemic oxidative stress. In addition, CB ablation reduced the number of cardiac arrhythmias without affecting atrial extracellular matrix remodeling, suggesting that the reduction of cardiac arrhythmias was related to the improvement in cardiac autonomic balance.
Carotid Body Ablation Abrogates Hypertension and Autonomic Alterations Induced by Intermittent Hypoxia in Rats

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CAROTID BODY ABLOATION ABROGATES HYPERTENSION AND AUTONOMIC ALTERATIONS INDUCED BY INTERMITTENT HYPOXIA IN RATS

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Short running head: Carotid Body and Intermittent Hypoxia-related Hypertension

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MATERIALS

Animals

Experiments were performed on 22 male Sprague-Dawley rats (247.0 ± 7.6 g), fed with standard diet *ad libitum* and kept on a 12-h light/dark schedule (07:30-19:30). The protocol was approved by the Bioethical Committee of the Facultad de Ciencias Biológicas of Pontificia Universidad Católica de Chile, Santiago. All studies rats were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Chronic Intermittent hypoxia exposure

Unrestrained, freely moving rats housed in individual chambers (12 cm x 35 cm, 2.2 L) were exposed to CIH (5% FiO₂, 12 episodes/h, 8 h/day for 28 days. At day 21 of CIH the rats were subjected to selective carotid body ablation (CBA) and then kept in CIH for 7 more days (Figure S1). The intermittent hypoxic pattern was applied from 08:30 to 16:30 h. A computerize system based on solenoid valves controls the alternating cycles of N₂ and room air. In the Sham condition, rats were exposed to air by air flushing an equal flow of compressed air into chambers. Room temperature was kept at 22-23°C.

Selective carotid body ablation

After 21 days of CIH the CBs were cryo-destroyed as previously described¹,². Briefly, rats were anesthetized with 2% isoflurane in O₂, under sterile surgical conditions the CBs were exposed and visually identified. The CBs were destroyed using a fine tipped force cooled in liquid N₂. The effectiveness of this maneuver was confirmed by the disappearance of the CB-mediated ventilatory reflex response to NaCN (100 µg/kg i.v.) (Figure S2). We did not evaluate the carotid baroreceptor functional integrity following CB ablation in the present study. However, the effect of CB ablation on the functionality of the carotid baroreceptors was documented in previous studies¹,². In these studies, carotid sinus nerve recordings before and after ablation showed virtually no effect on the baroreceptor-like nerve activity.

Arterial blood pressure and heart rate

The arterial blood pressure (BP) measurement was performed in conscious freely moving rats using a radio-telemetry system (Data Science International, USA). Ten days before the beginning of the experiments, rats were anesthetized with 2% isoflurane in O₂, and a skin incision was made to expose the femoral artery. The tip of a pressure transmitter PA-C40 was guided into the femoral artery, and the transmitter body was placed into subcutaneous pocket. After surgery the rats received a subcutaneous injection of ketoprofen (1%) and enrofloxacin (1%). BP was
recorded after 10 days of recovery continuously for at a sample of 1 KHz. Heart rate (HR) was derived from dP/dt signal obtained from the BP recordings.

Whole body plethysmography

Tidal volume (VT), respiratory frequency (fr) and minute volume (VT x fr = Vi) were determined by unrestrained whole body plethysmography (Fine Pointe, Buxco Research Systems, USA). The rats were placed in a plexiglas chamber that was ventilated by continuous bias flow, which was adjustable by a flowmeter. Resting ventilation was recorded for 15 min while the rats breathe room air. Peripheral chemoreceptors were stimulated by allowing the rats to breathe hypoxic gas (10% O2 in balance N2) for 10 min. The ventilation during normoxia hypoxia was assessed in Sham condition (day 7), CIH (day 28) and 7 days after the CB ablation during CIH (day 36). The sample rate was 1 KHz.

Spontaneous baroreflex sensitivity (BRS)

The BRS was measured by the sequence method (Hemolab Software)3. For BRS we assessed the heart rate (HR) obtained from BP and the mean arterial BP (MABP) time series searching groups sequentially increased or decreased HR and decreased or increased MABP, respectively. The data analyzed correspond to 10 min continuous recordings when rats were resting in normoxic conditions. Only sequences with correlation coefficient r > 0.80 were selected for analysis4.

Baroreflex function determined by infusion of sodium nitroprusside and phenylephrine in conscious animals

Rats were anesthetized with 2% isoflurane in O2 and midline incision in the neck was performed to isolate a lateral branch of the jugular vein. A small incision was made and a 3Fr polyurethane catheter was guided into the jugular vein and was tunneled subcutaneously to the back of the neck and connected to a vascular access port (Instech, USA). An intravenous infusion of sodium nitroprusside (50 µg/kg, 0.1 µl/s) (Sigma-Aldrich, USA) followed by an infusion of phenylephrine (25 µg/kg, 0.1 µl/s) (Sigma-Aldrich, USA) were used to induce a decrease in BP followed by an increase in BP. The cardiac baroreflex function was analyzed using a logistic regression over the entire pressure range. The data were acquired every 2 mmHg from the threshold to the saturation points. Data was fit to the equation: HR = A/[1+exp{B(MAP-C)}]+D, where A is HR range; B is the slope coefficient; C is the pressure at the midpoint of the range (BP50); and D is the minimum HR. The peak slope (maximum gain) was determined by the first derivative of the baroreflex curve and was calculated with the equation: Gainmax= A(1) x A(2) x [1/4], where A(1) is the range and A(2) is the average slope. The mean values for each curve parameter were used to derive composite curves for each group of rats.
Autonomic balance

Heart rate variability (HRV) was used as indirect method to assess autonomic balance during the whole length of the experiment (Figure S1). HRV was analyzed using the LabChart pro 7.0 software. We used dP/dt signal obtained from BP recordings for analysis of the R-R variability. Power spectrum density (PSD) of HRV was obtained using a Fast Fourier Transform algorithm after Hann windowing with 50% overlap to avoid spectral leakage. Analysis was performed in 10 min intervals obtained when the rats were resting in normoxia. Frequency spectrum cut-off frequencies were defined as: low frequency (LF): 0.04 – 0.6 Hz and high frequency (HF) 0.6 – 2.4 Hz. The PSD was expressed as normalized units (n.u.) and LF/HFHRV ratio1,4,6. Additionally, we study sympathto-vagal control of HR using intravenous infusion of propranolol (1 mg/kg) and atropine (1 mg/kg) through the implanted cannula on the jugular vein. The change in HR was expressed as the difference compared to baseline values (ΔHR). In addition, we calculated the spectral variability of the low frequency component of the systolic blood pressure (LFsbp) variability as an index of the sympathetic vasomotor tone1,7,8. The power of the LFsbp was calculated in 10 min intervals obtained when the rats were resting in normoxia following FFT using 0.2-0.6 Hz as cut-off frequency (Figure S3).

Arrhythmia index

Heart rate time series were derived from arterial blood pressure waveforms1 obtained by radio-telemetry during one hour while the rats where resting. Irregular heartbeats were visually inspected in the same rat during sham condition (Day 7), during CIH exposure (Day 28) and during CIH exposure after the rat underwent CBA (Day 36). Additionally, we assessed the contribution of sympathetic control on arrhythmia incidence using intravenous infusion of propranolol (1 mg/kg). Arrhythmic episodes were defined as premature or delayed beats with changes greater than 3 standard deviations from the mean beat to beat interval duration. All events meeting the stated criteria were identified and recorded to determine an index of events per hour.

Cardiac remodeling

Hearts (n = 4 per group) obtained from anaesthetized Sham rats, CIH exposed rats and CIH+CBA rats were fixed in 4% buffered-PFA, embedded in paraffin and sectioned (5 µm). Short-axis atrium slices were stained with Masson’s trichrome stain for the analysis of extracellular matrix (ECM) fibrosis9. Fibrotic area was determined by quantitative morphometry using multiple digital images and analyzed offline using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). Fibrotic area was determined from the following equation: Fibrosis (%) = [Masson’s trichrome-positive blue stained area/total tissue area] x 100.
Western blot

After euthanizing the rats (n = 4 per groups), hearts were removed and quickly frozen on dry ice and stored in -80°C. The tissue was lysed in RIPA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA) and proteins concentration determined using a BCA protein assay kit (Thermo Scientific, USA). Equal amount of protein was loaded into 10% polyacrylamide nu-page Bis-Tris gel (Life Technologies, USA) for electrophoretic separation. Gels containing the proteins were then transferred to PVDF membrane (Life Technologies, USA) using Novex Electrophoretic System (room temperature). The membranes were first incubated with blocking buffer (BSA 3% in PBS) (Thermo Scientific, USA) and then incubated overnight at 4°C with a rabbit polyclonal anti- MMP-2 antibody (1:1000, Novus, USA) followed by 1h incubation with a goat anti-rabbit secondary antibody (1:2000, Thermo Scientific, USA). Membranes were then developed using chemoluminiscenct imaging systems (UVP Doc-It® Life Science Software, USA). Following stripping of the membranes (Thermo Scientific, USA) was incubated overnight at 4°C with a rabbit polyclonal anti- TIMP-2 antibody (1:500, Novus, USA) followed by 1h incubation with a goat anti-rabbit secondary antibody (1:2000, Thermo Scientific, USA). Analysis of the specific bands optical densities were performed using ImageJ Software (National Institute of Health, USA). GAPDH and β-Actin were used as the housekeeping controls (Figure S5)

Systemic oxidative stress

Thiobarbituric acid reactive substances (TBARS) were used as an oxidative stress marker. Arterial blood samples (n = 4 per group) were collected through the carotid artery and placed in heparinized ice-cold microcentrifuge tubes. Plasma was separated by centrifugation and stored at -80°C. Malondialdehyde (MDA) was used as a standard and the levels of TBARS was expressed as µM (Figure S4).

Data analysis and statistics

Data are expressed as mean ± standard error of the mean (SEM). Differences were assessed by 2-way, paired and not paired measured analysis of variance (ANOVA) test, followed Tukey or Bonferroni two tails post-hoc analysis. The level of statistical significance was set at p value <0.05. All statistical analysis was performed using GraphPad Prism 6.0 software (La Jolla, California, USA).

REFERENCES


Figure S1. Timeline of longitudinal experiments. Carotid body ablation (CBA) surgery was performed after 21 days of chronic intermittent hypoxia (CIH). HRV, heart rate variability.
Figure S2. Efficacy of bilateral carotid body ablation (CBA) on CB-mediated chemoreflex ventilatory response to NaCN (100 μg/kg i.v.). After 21 days of chronic intermittent hypoxia (CIH). CBA abolished NaCN induced ventilatory response.
Figure S3. Effects of bilateral carotid body ablation on sympatho-mediated vasomotor tone during CIH. Exposure to CIH increases the low frequency component of the systolic blood pressure (LF$_{SBP}$) variability. Remarkably, rats that underwent CBA displayed a significant reduction in the LF$_{SBP}$ compared to the values obtained in CIH rats. * p<0.001 vs. Sham; + p<0.001 vs. CIH, Tukey post hoc test after One-way ANOVA. n=7 rats.
Figure S4. Bilateral carotid body ablation did not restore increases in systemic oxidative stress during chronic intermittent hypoxia. Exposure to CIH significantly increases the plasma MDA levels compared to Sham condition. Importantly, bilateral CBA during CIH have null effects on systemic oxidative stress. * p<0.001 vs. Sham, Tukey post hoc test after One-way ANOVA. n=4 rats.
Figure S5. Effect of carotid body ablation on TIMP-2 expression during CIH. Exposure to CIH did not change the expression of TIMP-2 on cardiac atrial tissue. Ablation of the CB (CBA) during CIH tends to increase TIMP-2 expression but this did not reach statistical significance. Tukey post hoc test after One-way ANOVA. n=4 rats.