Carotid body denervation prevents fasting hyperglycemia during chronic intermittent hypoxia

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1Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland; 2Division of Gastroenterology and Hepatology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland; and 3Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland

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Shin MK, Yoo Q, Jun JC, Bevans-Fonti S, Yoo DY, Han W, Mesarwi O, Richardson R, Fu YY, Pasricha PJ, Schwartz AR, Shirahata M, Polotsky VY. Carotid body denervation prevents fasting hyperglycemia during chronic intermittent hypoxia. J Appl Physiol 117: 765–776, 2014. First published August 7, 2014; doi:10.1152/japplphysiol.01133.2013.—Obstructive sleep apnea is recurrent upper airway obstruction during sleep leading to sleep fragmentation and impaired glucose metabolism, but mechanisms are unknown. Carotid bodies orchestrate physiological responses to hypoxemia by activating the sympathetic nervous system. Therefore, we hypothesized that carotid body denervation would abolish glucose intolerance and insulin resistance induced by chronic IH. Male C57BL/6J mice underwent carotid sinus nerve dissection (CSND) or sham surgery and then were exposed to IH or intermittent air (IA) for 4 or 6 wk. Hypoxia was administered by decreasing inspired oxygen from 20.9% to 6.5% once per minute, during the 12-h light phase (9 a.m.–9 p.m.). As expected, denervated mice exhibited blunted hypoxic ventilatory responses. In sham-operated mice, IH increased fasting blood glucose, baseline hepatic glucose output (HGO), and expression of a rate-limiting hepatic enzyme of gluconeogenesis phosphoenolpyruvate carboxykinase (PEPCK), whereas the whole body glucose flux during hyperinsulinemic euglycemic clamp was not changed. IH did not affect glucose tolerance after adjustment for fasting hyperglycemia in the intraperitoneal glucose tolerance test (IPTGG). CSND prevented IH-induced fasting hyperglycemia and increases in baseline HGO and liver PEPCK expression. CSND tended to augment the insulin-stimulated glucose flux and enhanced liver Akt phosphorylation at both hypoxic and normoxic conditions. IH increased serum epinephrine levels and liver sympathetic innervation, and both increases were abolished by CSND. We conclude that chronic IH induces fasting hyperglycemia increasing baseline HGO via the CSN sympathetic output from carotid body chemoreceptors, but does not significantly impair whole body insulin sensitivity.

Sleep apnea; type 2 diabetes; hypoxic chemoreflex; hepatic glucose output; gluconeogenesis.

OBSTRUCTIVE SLEEP APNEA (OSA) is recurrent upper airway obstruction during sleep leading to sleep fragmentation and chronic intermittent hypoxia (IH) during sleep (20). OSA leads to cardiovascular morbidity and mortality (46, 47, 67, 85, 86), which have been attributed to the metabolic syndrome associated with this disorder (14). A strong independent association between OSA, insulin resistance, and type 2 diabetes has been demonstrated in multiple reports (1, 9, 17, 48, 55, 66, 68, 84). Clinical studies indicated that the development of insulin resistance is linked to IH of OSA (66, 68).

Acute exposure to sustained hypoxia leads to insulin resistance (54). Studies in healthy human volunteers have shown that acute IH also leads to insulin resistance in the intravenous glucose tolerance test (IVGTT) (44). We have previously developed a mouse model of IH, which mimics the oxyhemoglobin desaturation profile of human OSA (69, 70), and have shown that, similar to humans, both acute and chronic murine IH lead to insulin resistance (10, 26). However, mechanisms of insulin resistance in IH remain poorly understood.

Human OSA and IH in rodents activate the sympathetic nervous system (SNS) (16, 34, 51, 65, 78). Activation of the SNS leads to hyperglycemia, glucose intolerance, and insulin resistance, increasing hepatic glycogenolysis and gluconeogenesis (3, 87). In addition, catecholamines induce adipose tissue lipolysis (4). Exuberant lipolysis results in release of free fatty acids (FFA), leading to fatty liver and insulin resistance (6, 52, 81). Activation of the SNS during IH occurs through the augmented hypoxic chemoreflex mediated by the carotid body and blunted baroreflex in the carotid sinus (22, 65). We anticipated that carotid sinus nerve (CSN) dissection (CSND) would attenuate both the hypoxic chemoreflex, which would decrease SNS activity, and the baroreflex, which would increase SNS activity. CSND abolished IH-induced hypertension (38), suggesting that the hypoxic chemoreflex has a dominant effect. We hypothesized that IH leads to insulin resistance via the hypoxic chemoreflex in the carotid body and ensuing activation of effenter sympathetic innervation of the insulin sensitive organs and, therefore, CSND would prevent IH-induced insulin resistance.

METHODS

Animals and overall design. One hundred thirty-four adult male C57BL/6J mice, 6–8 wk of age (Jackson Laboratory, Bar Harbor, MA) underwent CSND (n = 60) or sham surgery (n = 64). After a 2-wk recovery animals were exposed to chronic intermittent hypoxia (IH) or intermittent air (IA) while fed a regular chow diet. (1) Forty six mice were exposed to IH or IA for 6 wk. In these mice, the intraperitoneal glucose tolerance test (IPTGG) was performed after 4 wk of exposure; pulse oximetry was measured after 5 wk of exposure (Table 1). Upon completion of the exposure, mice were bled by retroorbital puncture and euthanized under 1–2% isoflurane anesthesia after a 5-h fast. All blood draws were performed between 12 p.m. and 1 p.m. (2) Thirty mice were exposed to IH or IA for 6 wk. The left
Sham-IA, ventilation (V˙E) and the hypoxic ventilatory response (HVR) were
(28). Briefly, a gas control delivery system was designed employing
volumetric chamber. V˙E was reported as VT
transducer, Hato Ray, Puerto Rico). Calibration injections of 10, 20,
from changes in pressure (Statham Gould PM15E differential pressure
ports through which the gases enter and exit the chamber were closed
barometric plethysmography chamber to measure ventilation. The
flow of air, nitrogen, and oxygen into cages. During each cycle of IH,
percentage of O2 decreased from ~21% to ~6–7% over a 30-s period,
rapid return to ~21% over the subsequent 30-s period. This regimen of IH induces
acetylsalicylic acid (ASA) administered to prevent infection and buprenorphine at 0.05
postoperative period (3 days), penicillin G at 1,000 U sc. twice per day
at the points of branching from the glossopha-}
and body temperature was maintained at 37°C. The CSNs were
bilateral dissection at the points of branching from the glossopharyngeal nerve to the cranial pole of the carotid body. During the early
postoperative period (3 days), penicillin G at 1,000 U sc. twice per day
was administered to prevent infection and buprenorphine at 0.05
mg·kg⁻¹·day⁻¹ sc was administered to prevent discomfort. Sham surgery
was performed in a similar manner except that the CSNs were
not severed.

Minute ventilation and the hypoxic ventilatory response. Minute
ventilation (V˙E) and the hypoxic ventilatory response (HVR) were
measured prior to IH, after 2 or 4 wk of IH as previously described by
our laboratory (59). The animals were placed in a whole body
barometric plethysmography chamber to measure ventilation. The
ports through which the gases enter and exit the chamber were closed
to produce a constant chamber volume. Once the chamber is at
constant volume, VT (tidal volume) and F (frequency) were measured
from changes in pressure (Statham Gould PM15E differential pressure
transducer, Hato Ray, Puerto Rico). Calibration injections of 10, 20,
and 30 µl of room air were made with the animal inside the constant
volume chamber. VT was reported as V˙T × f; V˙E was measured during
quiet wakefulness at baseline, 21% O2 (15 min × 2), and in response
to 10% O2.

Intermittent hypoxia. IH was performed as previously described
(28). Briefly, a gas control delivery system was designed employing
programmable solenoids and flow regulators, which controlled the
flow of air, nitrogen, and oxygen into cages. During each cycle of IH,
the percentage of O2 decreased from ~21% to ~6–7% over a 30-s period,
followed by a rapid return to ~21% over the subsequent 30-s period.

Table 1. Basic characteristics of C57BL/6J mice exposed to chronic intermittent hypoxia (CIH) or intermittent air (IA) after
carotid sinus nerve dissection (CSND) or sham surgery

<table>
<thead>
<tr>
<th></th>
<th>IA</th>
<th>CIH</th>
<th>CSND</th>
<th>IA</th>
<th>CIH</th>
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<tr>
<td>n</td>
<td>14</td>
<td>10</td>
<td>9</td>
<td>13</td>
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<td>Age, wk</td>
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<td>IH or IA</td>
<td>6–8</td>
<td>6–8</td>
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<td>Surgery</td>
<td>18</td>
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<tr>
<td>Body weight, g</td>
<td></td>
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<tr>
<td>Day 0 of IH or IA</td>
<td>28.8 ± 1.4</td>
<td>28.9 ± 2.3</td>
<td>28.8 ± 1.7</td>
<td>28.9 ± 2.1</td>
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<tr>
<td>Day 42 of IH or IA</td>
<td>26.3 ± 1.5*</td>
<td>25.7 ± 1.6*</td>
<td>27.0 ± 1.8</td>
<td>26.2 ± 1.8*</td>
<td></td>
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<tr>
<td>Food intake, g/day</td>
<td>2.5 ± 0.4</td>
<td>2.6 ± 0.6</td>
<td>2.7 ± 0.5</td>
<td>2.8 ± 0.6</td>
<td></td>
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<tr>
<td>Epididymal fat, g</td>
<td>0.56 ± 0.02</td>
<td>0.42 ± 0.02$</td>
<td>0.50 ± 0.02$</td>
<td>0.52 ± 0.04</td>
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<tr>
<td>Plasma epinephrine, ng/ml</td>
<td>1.14 ± 0.15</td>
<td>1.95 ± 0.23$</td>
<td>1.08 ± 0.12</td>
<td>1.20 ± 0.27</td>
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<tr>
<td>Plasma norepinephrine, ng/ml</td>
<td>6.98 ± 1.28</td>
<td>6.80 ± 0.71</td>
<td>8.47 ± 0.46†</td>
<td>7.46 ± 1.86‡</td>
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<tr>
<td>Plasma corticosterone, ng/ml</td>
<td>72.0 ± 9.4</td>
<td>78.0 ± 19.1</td>
<td>65.5 ± 7.4</td>
<td>73.9 ± 8.7</td>
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<tr>
<td>Plasma leptin, ng/ml</td>
<td>5.4 ± 0.6</td>
<td>5.9 ± 0.2</td>
<td>5.9 ± 0.4</td>
<td>5.0 ± 0.5</td>
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<tr>
<td>Plasma adiponectin, ng/ml</td>
<td>5.2 ± 0.4</td>
<td>5.9 ± 0.9</td>
<td>5.9 ± 0.4</td>
<td>6.3 ± 1.4</td>
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<tr>
<td>Fasting plasma triglycerides, mg/dl</td>
<td>26.9 ± 2.4</td>
<td>30.1 ± 1.8</td>
<td>31.1 ± 1.1</td>
<td>29.9 ± 3.5</td>
<td></td>
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<tr>
<td>Fasting plasma insulin, mmol/L</td>
<td>0.36 ± 0.03</td>
<td>0.28 ± 0.04</td>
<td>0.30 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td></td>
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<tr>
<td>Fasting plasma GLP-1, pmol/L</td>
<td>1-2.3%</td>
<td>7.9% ± 9.7%</td>
<td>8.69 ± 2.7</td>
<td>89.41 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Liver triglyceride, mg/g of tissue</td>
<td>1.1 ± 29.9</td>
<td>7.1 ± 1.3</td>
<td>6.3 ± 0.5</td>
<td></td>
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</table>

Values are means ± SE. *P < 0.01, #P < 0.001 for the difference between day 0 and day 42, respectively. †: P < 0.01 and P < 0.05 for the effects of CIH and CSND, respectively. ‡P = 0.056 for the effect of CSND. §P < 0.05 for the effect of CIH.

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aged throughout the observation period of IH; nadirs and peaks of SpO₂ were also identified and averaged.

**Intraperitoneal glucose tolerance test.** Intraperitoneal glucose tolerance test (IPGTT) was performed during week 4 of IH or IA exposures in unanesthetized animals. IPGTT was performed after a 5-h fast by injecting 1 g/kg glucose intraperitoneally. Glucose levels were measured by tail-snip technique using a hand-held glucometer (Accu-Check Aviva, Roche, Indianapolis, IN) at baseline and at 10, 20, 30, 60, and 120 min after glucose injection. The homeostasis model assessment (HOMA) index was calculated as fasting serum insulin (μU/ml) × fasting blood glucose (mmol/l)/22.5.

**Hyperinsulinemic euglycemic clamp.** The hyperinsulinemic euglycemic clamp was performed in conscious mice during week 6 of exposure to IH or IA as previously described (26, 31, 32, 76). Briefly, under 1–2% isoflurane anesthesia, catheters (MRE025 Braintree Scientific) were chronically implanted in the left femoral artery and vein for measurement of blood glucose and infusion of solutions. The catheters were perfused throughout the recovery period by an infusion pump with a sterile saline solution containing heparin (20 U/ml). Animals were allowed 72 h to recover from surgery. IH or IA exposures were continued during recovery and the clamp. The baseline hepatic glucose output was first determined by infusing \[^3\text{H}\]glucose (10 μCi bolus + 0.1 μCi/min; NEN Life Science Products) for 80 min and then acquiring a 100-μl plasma sample to measure \[^3\text{H}\]glucose level. Blood was then centrifuged at 10,000 g and supernatant collected. Red blood cells were resuspended in heparinized saline and reinjected into the mouse. Subsequently a 120-min hyperinsulinemic-euglycemic clamp was conducted with continuous infusion of human insulin (20 mU·kg⁻¹·min⁻¹; Novo Nordisk, Princeton, NJ). Blood samples were collected from the femoral artery catheter at 10-min intervals with an Accu-Chek Aviva glucometer for the immediate measurement of glucose concentration, and 50% glucose was infused at variable rates through the femoral venous catheter to maintain blood glucose at ~100–125 mg/dl. Insulin-stimulated whole body glucose flux was estimated using continuous infusion of \[^3\text{H}\]glucose (0.1 μCi/min) throughout the clamp. The hepatic glucose output during the clamp was calculated by subtracting the cold glucose infusion rate from the insulin-stimulated whole body glucose flux (31, 32).

**Insulin signaling.** The animals underwent CSND or sham surgery and after recovery for 2 wk were exposed to IH or IA for 4 wk. At the end of the exposure, the animals were injected with insulin at 5 U/kg or normal saline intraperitoneally and euthanized under 1–2% isoflurane 15 min after injection. Liver tissue, skeletal muscle (quadriceps), and epididymal fat were collected, snap-frozen in liquid nitrogen, and stored at -80°C. Insulin signaling was assessed by measuring phosphorylated Akt (pAkt) to total Akt ratios after insulin injection. The tissues were homogenized in the whole lystate buffer. SDS-PAGE and Western blot were performed using Bio-Rad precast gel system. Protein (30 μg) was applied per lane. For total Akt and pAkt (Ser473) measurements, we used rabbit polyclonal antibodies from Cell Signaling Technology (Danvers MA), cat no. 9272 and no. 9271, respectively, diluted 1:2,000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detected with G9295 antibody from Sigma was used as a loading control. The secondary antibody was goat anti-rabbit-horseradish peroxidase conjugate from Bio-Rad (Hercules, CA). Optical density of Akt and pAkt (60 kDa) was measured with ImageJ Software.

**Biochemical assays.** Plasma insulin, adiponectin, and leptin were measured with ELISA kits from Millipore (Billerica, MA). Plasma epinephrine and norepinephrine were measured with ELISA kits from Rocky Mountain Diagnostics (Colorado Springs, CO). Fasting plasma total cholesterol, free fatty acids (FFA), and triglycerides were measured with kits from Wako Diagnostics (Richmond, VA). Glycerol was measured with a colorimetric assay from Sigma-Aldrich (St. Louis, MO). Corticosterone and glucagon were determined with ELISA kits from R&D Systems, (Minneapolis, MN).

**Tyrosine hydroxylase (TH) staining of liver tissue.** After 1 day of IH or IA, animals were euthanized as described above, and the right lobe of the liver was collected, fixed in 4% formaldehyde overnight, and then immersed in 2% Triton-X 100 solution for 2 days at 15°C for permeabilization as we have previously described (18). The tissue was labeled with a polyclonal rabbit anti-mouse TH (Millipore, AB152, Billerica, MA) and an Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody (Invitrogen) to reveal sympathetic fibers and propidium iodide (Invitrogen) to label the nuclei. High-resolution, 3D-confocal imaging of the optical-cleared specimens was performed with a Zeiss LSM 710 NLO confocal microscope (Carl Zeiss, Jena, Germany). Tissue samples were analyzed using a Zeiss LSM 710 NLO confocal microscope (Carl Zeiss, Jena, Germany).

**Figs. 1 & 2.** Figures 1 and 2 are images from the study. Figure 1 shows the minute ventilation per body weight (V̇E; A), tidal volume per body weight (V̇T; B), and respiratory rate per s (F; C) measured in C57BL/6J mice subjected to carotid sinus nerve dissection (CSND) or sham surgery during acute exposure to hypoxia (10% O₂ for 15 min) or room air (20.9% O₂) in a whole body barometric plethysmography chamber. *P < 0.01 for the difference with 20.9% O₂ and with CSND. Representative tracings are shown in D.
Germany). After 6 wk of IH or IA, animals were euthanized as described above, and the right lobe of the liver was collected, fixed in 4% formaldehyde, and embedded in paraffin. Paraffin-embedded tissue was sectioned at 3- to 5-μm thickness and stained with primary antibodies against TH and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Grand Island, NY) and with smooth muscle actin antibody (Sigma) and Alexa Fluor 647 anti-rabbit IgG (Invitrogen), examined with a Leica TCS SP5 microscope and analyzed with the accompanying Leica Application Suite software (Leica Microsystems CMS, Mannheim, Germany).

Real-time PCR. Total RNA was extracted from liver using Trizol (Life Technologies, Rockville, MD), and cDNA was synthesized using Advantage RT for PCR kit from Clontech (Palo Alto, CA). Real-time reverse-transcriptase PCR (RT-PCR) was performed with

Fig. 2. Oxyhemoglobin saturation (SpO₂, A) during the intermittent hypoxia (IH) cycle in C57BL/6J mice subjected to CSND or sham surgery. IA, intermittent air. B: 24-h average mean blood pressure in CSND and sham-operated mice before and during exposure to IH. Mean blood pressure (BP; C) and heart rate (D) prior to and immediately after phenylephrine (PE) administration. *P < 0.001 between SpO₂ during the nadir and peak of the hypoxic cycle; †P < 0.001 for the effect of IH; ‡P < 0.001 and ¶P < 0.05, respectively, for the effect of CSND; #P < 0.001 for the effect of PE.

Fig. 3. Effects of CSND on fasting blood glucose (A), fasting serum insulin (B), the homeostatic model assessment index of insulin resistance (HOMA-IR; C), and fasting serum glucagon (D) in C57BL/6J mice exposed to intermittent hypoxia (IH) or intermittent air (IA). *P < 0.001 for the difference with IA-sham, P < 0.01 for the difference with IH-CSND and IA-CSND.
primers from Invitrogen (Carlsbad, CA) and Taqman probes from Applied Biosystems (Foster City, CA). The sequences of primers and probes for mouse 18S were previously described (41). Mouse phospho-enolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G-6-Pase) mRNA have been measured with the Applied Biosystems pre-made primers and probes. The mRNA expression levels were referenced to 18S rRNA and the values were derived according to the $2^{-\Delta\Delta CT}$ method (73).

Statistical analysis. All values are reported as means ± SE. Statistical significance for all comparisons was determined by two-way ANOVA test with Bonferroni post hoc correction for multiple comparisons. For IPGTT and ITT, we performed a repeated-measures ANOVA test, and significance was determined using Tukey’s post hoc test. A $P$ value of $<0.05$ was considered significant.

RESULTS

At the onset of the IH exposure, mice subjected to CSND and sham surgery had identical body weight (Table 1). By the end of the experiment, chronic IH resulted in weight loss. Similar weight loss by design occurred in the control IA groups, which were weight matched to the hypoxic animals by varying food intake. In sham-operated mice, chronic IH decreased the size of epididymal fat pads and increased liver fat, which was consistent with our previously published data (12).
CSND prevented loss of epididymal fat and hepatic fat accumulation. IH increased levels of plasma epinephrine in sham-operated mice, and this increase was abolished by carotid body denervation. CSND increased plasma norepinephrine levels, whereas IH had no effect (Table 1).

In sham-operated mice, acute exposure to 10% O₂ induced a 1.8-fold increase in V˙E (Fig. 1A) due to increases in V_T, whereas the respiratory rate was unchanged (Fig. 1, B–D). There was no significant change in V˙E from the pre-IH level after 2 and 4 wk of IH. After IH for 4 wk, sham-operated mice continued to demonstrate the robust HVR raising V˙E from 3.0 ± 0.2 ml·min⁻¹·g body weight⁻¹ in room air to 4.2 ± 0.3 ml·min⁻¹·g body wt⁻¹ in 10% O₂ (P < 0.05). CSND abolished the HVR (Fig. 1). After IH for 4 wk, CSND mice had identical V˙E to the sham-operated mice (3.0 ± 0.3 ml·min⁻¹·g body weight⁻¹), but there was no significant hyperventilation in 10% O₂ (3.5 ± 0.3 ml·min⁻¹·g body weight⁻¹).

Both CSND and sham-operated mice exhibited similarly severe oxyhemoglobin desaturations during IH (Fig. 2A). At baseline normoxic conditions carotid body denervation did not affect blood pressure. IH caused hypertension in both CSND and sham-operated mice on day 1 and day 7 of exposure (Fig. 2B). In CSND mice, blood pressure returned to the normoxic baseline by day 14, whereas sham-operated mice remained hypertensive. Phenylephrine induced similar increases in blood pressure and reflex bradycardia in both CSND and sham-operated mice at baseline normoxic conditions (Fig. 2, C and D). In sham-operated mice, IH for 14 days did not significantly modify the effect of phenylephrine on blood pressure and heart rate (Fig. 2D). In contrast, in CSND mice IH augmented the

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**Fig. 6. Effect of CSND on Akt Ser 473 phosphorylation (p-Akt) in the liver, skeletal muscle, and epididymal fat of C57BL/6J exposed to chronic IH or IA.** Mice were euthanized at baseline (time 0) or 15 min after insulin injection (time 15). Representative samples and quantitative analysis of liver (A and B), skeletal muscle (C and D), and epididymal fat (E and F) from mice subjected to sham surgery or CSND and exposed to IA or IH are shown. p-Akt optical density (OD) was divided by total Akt and all values were normalized to p-Akt/Akt in IA mice at time 0. Glyceroldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. On A, C, and E, lanes 1 and 2 represent mice exposed to IA at time 0; lanes 3 and 4 represent mice exposed to IA at time 15; lanes 5 and 6 represent mice exposed to IH at time 0, and lanes 7–9 represent mice exposed to IH at time 15. *P < 0.001 for the effect of insulin (time 0 vs. time 15); † and ‡: P < 0.01 and P < 0.05, respectively, for the effect of IH.
phenylephrine effect on blood pressure, whereas reflex bradycardia did not change (Fig. 2, C and D), suggesting that CSND blunted the baroreflex during IH.

IH increased liver triglyceride content and plasma total cholesterol levels, and these effects were abolished by CSND (Table 1). Fasting plasma levels of free fatty acids, triglycerides, glycerol, corticosterone, leptin, and adiponectin were not changed by our interventions. In sham-operated mice, IH increased fasting blood glucose level without change in plasma insulin, resulting in a 1.7-fold increase in the HOMA-IR (Fig. 3, A–C). CSND prevented an IH-induced increase in fasting blood glucose. CSND led to a 1.7-fold decrease in fasting plasma insulin in normoxic sham-operated mice and a 2-fold decrease in hypoxic sham-operated mice, which resulted in 2.6-fold and 3.4-fold decreases in the HOMA-IR, respectively. IH did not affect plasma glucagon level, but CSND markedly decreased it (Fig. 3D).

Chronic IH caused elevation in blood glucose levels throughout the IPGTT, which was prevented by carotid body denervation (Fig. 4, A and B). However, there were no differences in the area under curve between the groups after adjustment for fasting glucose levels at time 0 (Fig. 4C). In sham-operated fasting mice, IH induced an 84% increase in baseline hepatic glucose output, which was abolished by CSND (Fig. 5A). During the hyperinsulinemic euglycemic clamp, plasma insulin level was raised to 146 ± 12.7 μU/ml, which is equivalent to 5.1 ± 0.4 ng/ml, without significant differences between the groups. IH did not affect the insulin-stimulated whole body glucose flux (Fig. 5B). CSND induced a trend to an increase in insulin-stimulated glucose flux, regardless of the exposure. Hepatic glucose output during the clamp was completely suppressed in all groups of mice.

In the liver, CSND increased insulin-induced Akt phosphorylation, regardless of the presence of hypoxia (P = 0.001, Fig. 6, A and B). In skeletal muscle, chronic IH decreased Akt phosphorylation, and this effect was abolished by CSND (Fig. 6, C and D). In epididymal fat, IH decreased Akt phosphorylation, but CSND had no effect (Fig. 6, E and F).

Expression of key enzymes of gluconeogenesis, PEPCK and G-6-Pase, was assessed in the liver (Fig. 7). IH had a strong trend to increase PEPCK mRNA levels (P = 0.057), which was abolished by CSND. Carotid body denervation decreased hepatic PEPCK gene expression during IH by 3-fold (P < 0.001), whereas normoxic mice were not affected (Fig. 7A). Neither chronic IH nor CSND affected liver G-6-Pase gene expression (Fig. 7B).

Given that IH had the most striking effect on fasting hepatic glucose output, we determined the effect of IH on sympathetic innervation in the liver. TH staining, which is a conventional method to measure sympathetic innervation in different organs (18, 50), showed sympathetic fibers surrounding blood vessels in the hepatic triads (Fig. 8). There was no TH staining within the hepatic lobules, which is consistent with a classical concept of perivascular liver sympathetic innervation in mice and rats confined to the hepatic triads (43, 49). There was no significant change in the liver sympathetic innervation after 1 day of IH. Next we examined the effect of chronic IH on sympathetic innervation in the liver and the role of the carotid bodies. As in the 3D images (Fig. 8), TH staining was confined to the hepatic triads, where sympathetic nerves were adjacent to smooth muscle of the blood vessel wall (Fig. 9). In contrast to 1 day

**DISCUSSION**

OSA has been independently associated with insulin resistance and type 2 diabetes mellitus, which were attributed to the hallmark manifestation of OSA, chronic IH during sleep. We aimed to examine a potential role of the carotid body signaling in dysregulation of glucose metabolism in our mouse model of chronic IH. The main finding of the study was that CSND prevented IH-induced perturbations of hepatic glucose metabolism. Specifically, we have shown that IH induced fasting hyperglycemia, increasing baseline hepatic output and expression of the rate-limiting enzyme of hepatic gluconeogenesis, PEPCK, which were attenuated by CSND. We report several other novel findings. First, IH augmented sympathetic innervation to the liver and increased plasma epinephrine, and both increases were abolished by CSND. Second, IH inhibited
insulin signaling in skeletal muscle and epididymal adipose tissue, but did not affect glucose tolerance and insulin resistance measured by the whole body glucose flux during the hyperinsulinemic euglycemic clamp. Third, CSND trended to improve whole body insulin sensitivity, decreased plasma insulin and glucagon levels, activated insulin signaling in the liver, and prevented an IH-mediated decrease in insulin signaling in skeletal muscle. In the discussion below we will elaborate on mechanisms of fasting hyperglycemia during IH and implications of our work.

**Carotid body and SNS efferent output in IH.** Carotid bodies are richly vascularized organs, which reside bilaterally at the bifurcation of the carotid arteries (53, 60). They are major sensors for hypoxia and sensory discharges from the carotid bodies, which are transmitted by the CSN to the nucleus tractus solitarius in the medulla, resulting in the activation of medullary neurons regulating spinal preganglionic sympathetic activity (22, 65, 77). Human OSA and rodent chronic IH activate carotid bodies augmenting acute responses to hypoxia and increasing baseline sensory activity (63). IH also increases cervical, renal, splanchnic, thoracic, and lumbar sympathetic nerve activity (8, 23, 25, 45, 65), which may represent the efferent limb of the carotid chemoreflex. We employed the CSND model to examine the role of hypoxic chemoreflex in metabolic complications of IH. In our experiments, CSND abolished the HVR (Fig. 1), indicating that the procedure successfully reduced the carotid body output. IH did not augment the hypoxic chemoreflex. The heterogeneity of HVR changes in IH has been noted previously and has been attributed to differences in patterns and duration of the IH stimulus (61). Oxidative stress and reactive oxygen species play a critical role in the HVR augmentation in the carotid body (62, 64). Our regimen of IH leads to relatively modest oxidative stress (29, 69), which may explain the lack of changes in the HVR. Nevertheless, the difference in the HVR between sham and CSND mice persisted throughout IH exposure, suggesting that the hypoxic chemoreflex is a probable mechanism of the increased SNS efferent output during IH.

CSN provides sensory innervation to both carotid bodies and carotid sinus baroreceptors (35, 53, 60). An important question is whether we can associate CSND-mediated metabolic changes with the chemoreflex. Carotid body denervation and

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Fig. 8. Representative 3-D images of sympathetic fibers surrounding major vessels in the liver stained with tyrosine hydroxylase (TH, green) antibody (×100) after 1 day of IH or control conditions. TH is in green; nuclei are in red.
baroreceptor denervation have opposite effects on sympathetic activity: the former induces inhibition, and the latter leads to excitation, although each sensor may differentially influence the sympathetic outflow to different organs (60). In sham-operated mice, we did not observe blunting of the baroreflex previously reported in IH (24, 42). In contrast, CSND markedly augmented phenylephrine-induced hypertension during IH with the same degree of the negative chronotropic response indicating diminished baroreflex (Fig. 2, C and D). Despite the decreased baroreflex, 24-h blood pressure recording showed that CSND reversed IH-induced hypertension (Fig. 2B), which is also consistent with earlier findings in rats (38). This paradox indicates that carotid sinus baroreceptors do not play a major role in IH-induced hypertension. Taken together, our data suggest that CSN is implicated in IH-induced hypertension due to the hypoxic chemoreflex in the carotid bodies rather than via the blunted baroreflex in the carotid sinus. We further conclude that the augmented carotid body sensory output during IH increased sympathetic innervation of the liver (Fig. 9), resulting in increased hepatic glucose output (Fig. 10).

IH increased plasma epinephrine level in sham-operated mice (Table 1). The adult adrenal medulla is insensitive to acute hypoxia (80), but IH increases epinephrine efflux from the adrenal medulla (36, 38). Adrenalectomy prevented elevation of plasma catecholamines and IH-induced hypertension (2, 38). Systemic treatment of rats with antioxidants prevented acute hypoxia-induced catecholamine efflux in rats exposed to IH (36). However, it remains unclear whether IH exerts a direct effect on adrenals or whether this effect is mediated by the carotid bodies. Adrenal medulla has rich pre- and postganglionic sympathetic innervation (30, 56, 74, 75). Our data showed that the IH-induced increase in plasma epinephrine was prevented by CSND (5, 36, 38, 77). All of above suggest that, in the IH environment, the hypoxic chemoreflex may lead to adverse metabolic outcomes via the increase in the epinephrine efflux by adrenal medulla (Fig. 10).

**SNS and dysregulation of glucose metabolism during IH.** We have consistently shown in the past that fasting hyperglycemia is one of the most reproducible metabolic effects of IH, which suggests that increased hepatic glucose output is a consequence of IH (11, 13, 40, 72) (58). Acute IH for 9 h trended to increase the baseline hepatic glucose output measured by radioactive glucose infusion (26). Our present data showed that IH-induced fasting hyperglycemia was associated

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**Fig. 9. Effect of CSND on liver TH in C57BL/6j mice exposed to IH or IA control conditions.** A: representative slides of liver tissue stained for TH and smooth muscle actin (SMA). Immunofluorescence, ×100; white bars represent 25 μm. B: fold change in liver TH level compared with the IA-sham group. *P < 0.05 for the difference with IA-sham, IH-CSND, and IA-CSND.

**Fig. 10. Putative role of carotid bodies in fasting hyperglycemia in IH.** IH stimulates the carotid body chemoreflex, which activates efferent sympathetic nervous system (SNS). SNS activation increases glucagon secretion in the pancreas and epinephrine efflux by the adrenal medulla. Increased SNS innervation in the liver, glucagon, and epinephrine enhance hepatic gluconeogenesis, which causes fasting hyperglycemia.
with upregulation of hepatic gluconeogenesis, which was evident from an increase in expression of PEPCK, a transcriptionally controlled rate-limiting enzyme of gluconeogenesis (Fig. 7A). IH did not affect Akt phosphorylation in the liver (Fig. 6A), suggesting that the hepatic effect of IH was insulin-independent, probably due to the direct impact of catecholamines on gluconeogenesis.

CSND prevented fasting hyperglycemia, an increase in baseline hepatic glucose production, and upregulation of the key enzyme of gluconeogenesis, PEPCK, which suggests downregulation of gluconeogenesis. CSND-mediated decreases in hepatic sympathetic innervation could downregulate gluconeogenesis directly, via increased sympathetic innervation and increased epinephrine efflux by the adrenal medulla, and indirectly, via a decrease in glucagon levels (Fig. 3D) (27, 83) and enhanced insulin signaling in the liver (Fig. 6A). CSND abolished IH-induced triglyceride accumulation in the liver and decreased epididymal fat pads (Table 1), which suggests that CSND may improve insulin signaling by suppressing the influx of free fatty acids to the liver that resulted from catecholamine-mediated adipose tissue lipolysis (52, 81). The lack of change of circulating fatty acids and glycerol measured at a single time point in our study did not negate previous findings of accelerated lipolysis in chronic IH (28), which could occur earlier in the time course. In humans, sympathetic inhibition with the centrally acting α2 agonist clonidine prevented hypoxia-induced insulin resistance (57). Our data implicate the carotid body chemoreflex in the development of hyperglycemia during IH.

IH decreased Akt phosphorylation in epididymal adipose tissue and skeletal muscle, whereas insulin resistance was not affected. Acute IH (1 day) is known to induce systemic insulin resistance (26, 37), which was diminished but still persistent after 4 wk of exposure (37). It is conceivable that chronic IH activates protective mechanisms in different tissues compensating for downregulation of insulin signaling.

The effects of carotid denervation on systemic insulin resistance independent of hypoxia have been reported (71). Our data suggest that a beneficial impact of CSND on insulin resistance occurs in liver and skeletal muscles because CSND enhanced insulin signaling in these tissues while adipose tissue was not affected (Fig. 6).

Fasting hyperglycemia in IH did not result in a significant compensatory increase of plasma insulin, which suggests that IH may suppress insulin secretion by the pancreatic β cells. Indeed, inhibitory effects of IH on insulin secretion have been previously detected by intravenous glucose tolerance test in mice, rats, and humans (15, 37, 44). CSND simultaneously decreased fasting insulin and glucose levels (Fig. 3), suggesting that the CN5 improved insulin resistance rather than directly affected the pancreatic endocrine function.

Limitations of the study. Our study had several limitations. First, we did not measure tissue catecholamine content and could not determine whether CSND prevents an increase in hepatic output via sympathetic liver nerves or circulating catecholamines. In future studies these measurements could assess SNS activity in different organs. Second, we were unable to perform telemetry and phenylephrine infusions during the entire 4- to 6-wk exposure to IH. However, our data suggest that IH induced hypertension within the first 24 h of exposure, and this hypertension persisted for 2 wk. Third, the hyperinsulinemic euglycemic clamp has been performed using a high dose of insulin completely suppressing the hepatic glucose output. It is conceivable that at a lower concentration of insulin, we would be able to detect effects of IH and CSND on the hepatic glucose output during the clamp (33). Fourth, we did not perform the intravenous glucose tolerance test, which would determine the role of the carotid bodies in the β-cell function. However, concomitant decreases in fasting glucose and insulin after CSND suggest improvement in insulin resistance during IH rather than a direct effect on insulin secretion. Fifth, C56BL/6J mice used in our experiments are more susceptible to both metabolic abnormalities and ventilatory instabilities during hypoxia, compared with other mouse strains (7, 21, 79), and our findings may not be generalizable. However, we have reported fasting hyperglycemia and metabolic abnormalities during IH in other strains of mice (40). Several studies indicate that the carotid bodies are involved in regulation of glucose metabolism in rats (19, 71, 82). It is conceivable that the carotid bodies mediate variations in the metabolic phenotypes between different mouse strains.

Conclusions and clinical implications. We have shown that the carotid bodies play a major role in the development of fasting hyperglycemia during chronic IH (Fig. 10). Our findings suggest that sympathetic blockers can be tested as adjunct therapy of type 2 diabetes in OSA.

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