

# Hypoxia-inducible factor 2 $\alpha$ (HIF-2 $\alpha$ ) heterozygous-null mice exhibit exaggerated carotid body sensitivity to hypoxia, breathing instability, and hypertension

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Cardiorespiratory functions in mammals are exquisitely sensitive to changes in arterial O<sub>2</sub> levels. Hypoxia-inducible factors (e.g., HIF-1 and HIF-2) mediate transcriptional responses to reduced oxygen availability. We demonstrate that haploinsufficiency for the O<sub>2</sub>-regulated HIF-2 $\alpha$  subunit results in augmented carotid body sensitivity to hypoxia, irregular breathing, apneas, hypertension, and elevated plasma norepinephrine levels in adult *Hif-2 $\alpha$ <sup>+/-</sup>* mice. These dysregulated autonomic responses were associated with increased oxidative stress and decreased mitochondrial electron transport chain complex I activity in adrenal medullae as a result of decreased expression of major cytosolic and mitochondrial antioxidant enzymes. Systemic administration of a membrane-permeable antioxidant prevented oxidative stress, normalized hypoxic sensitivity of the carotid body, and restored autonomic functions in *Hif-2 $\alpha$ <sup>+/-</sup>* mice. Thus, HIF-2 $\alpha$ -dependent redox regulation is required for maintenance of carotid body function and cardiorespiratory homeostasis.

blood pressure | control of ventilation | catecholamines

Hypoxia-inducible factors (HIFs) mediate transcriptional responses to reduced O<sub>2</sub> availability (1). HIF-1, the first identified member of the HIF family, is comprised of an O<sub>2</sub>-regulated  $\alpha$  subunit and a constitutively expressed  $\beta$  subunit (2). Complete deficiency of HIF-1 $\alpha$  in *Hif-1 $\alpha$ <sup>-/-</sup>* mice results in embryonic lethality at midgestation with major malformations of the central nervous system, heart, and vasculature (3). *Hif-1 $\alpha$ <sup>+/-</sup>* mice, which are partially deficient in HIF-1 $\alpha$  expression, develop normally and are indistinguishable from their WT littermates in the sedentary state with respect to autonomic functions, including blood pressure (BP), ventilatory responses to hypoxia or hypercapnia, and plasma catecholamine levels (4). However, *Hif-1 $\alpha$ <sup>+/-</sup>* mice exhibit impaired O<sub>2</sub> sensing by the carotid body (4, 5), the primary sensory organ for detecting hypoxemia (6, 7), and altered physiological adaptations to chronic hypoxia (5, 8).

The HIF-2 $\alpha$  subunit, also known as endothelial PAS domain protein-1 (EPAS-1), shares 48% amino acid sequence identity with HIF-1 $\alpha$  (9). As in the case of HIF-1 $\alpha$ , continuous hypoxia leads to HIF-2 $\alpha$  accumulation and subsequent dimerization with HIF-1 $\beta$ . Transcriptional activation by HIF-2 regulates some target genes in common with HIF-1 as well as other genes that are uniquely regulated by HIF-2 (10–12). Homozygous deficiency of HIF-2 $\alpha$  is often lethal with the surviving *Hif-2 $\alpha$ <sup>-/-</sup>* mice exhibiting multiple organ pathology and increased oxidative stress as a result of impaired induction of genes encoding major antioxidant enzymes (AOEs) (12).

*Hif-2 $\alpha$ <sup>+/-</sup>* mice, like *Hif-1 $\alpha$ <sup>+/-</sup>* mice, are phenotypically indistinguishable from WT littermates under sedentary conditions, but exhibit impaired responses to chronic hypoxia such as impaired pulmonary vascular remodeling, erythropoiesis, and retinal neovascularization (13–15). Although the carotid body is

a prominent site of HIF-2 $\alpha$  expression (16), the effects of *Hif-2 $\alpha$*  haploinsufficiency on chemoreceptor responses to hypoxia have not been examined. Given the structural and functional similarities between HIF-1 $\alpha$  and HIF-2 $\alpha$ , we hypothesized that *Hif-2 $\alpha$*  haploinsufficiency would alter the carotid body response to acute hypoxia and the ensuing autonomic responses. To test this possibility, carotid body and ventilatory responses to hypoxia, BP, and plasma catecholamine levels were measured in adult *Hif-2 $\alpha$ <sup>+/-</sup>* and *Hif-2 $\alpha$ <sup>+/+</sup>* mice.

## Results

**Augmented Carotid Body Response to Hypoxia in *Hif-2 $\alpha$ <sup>+/-</sup>* Mice.** HIF-2 $\alpha$  expression in carotid bodies from *Hif-2 $\alpha$ <sup>+/+</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice was analyzed by immunofluorescence. Sections were stained for tyrosine hydroxylase (TH), a marker of glomus cells, which are the putative O<sub>2</sub>-sensing cells in the carotid body, and HIF-2 $\alpha$  (Fig. 1A, Left, Center). Many glomus cells expressed HIF-2 $\alpha$  in both groups of mice as evidenced by colocalization with TH (Fig. 1A, Right). HIF-2 $\alpha$  was also expressed in other carotid body cells, some of which appeared to be endothelial cells (Fig. 1A, Middle, Right). The intensity of HIF-2 $\alpha$  expression was decreased in *Hif-2 $\alpha$ <sup>+/-</sup>* mice. Morphometric analysis was performed to assess the effect of partial deficiency of HIF-2 $\alpha$  on carotid body structure. There were no significant differences between genotypes with respect to total volume of the carotid bodies, number of glomus cells per section, or ratio of glomic volume to total volume (Fig. 1B–D).

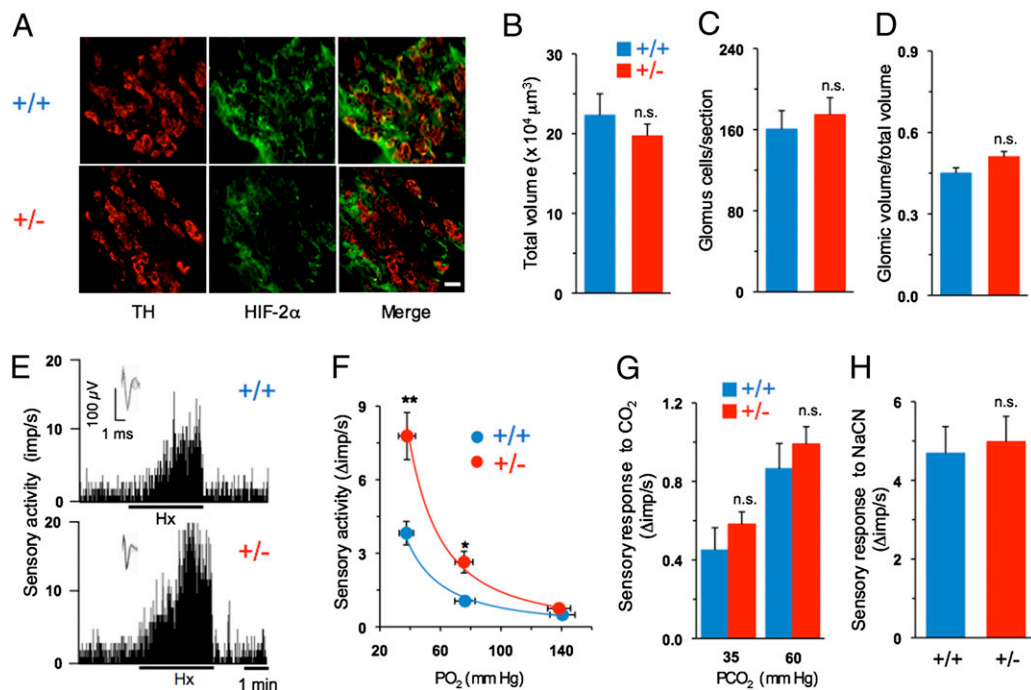
To assess the impact of partial HIF-2 $\alpha$  deficiency on the carotid body response to hypoxia, carotid bodies were isolated and single-unit sensory discharge was recorded from the carotid sinus nerve. Examples of carotid body sensory responses to hypoxia from both groups of mice and plots of mean data for graded hypoxia are shown in Fig. 1E and F, respectively. Carotid bodies from *Hif-2 $\alpha$ <sup>+/-</sup>* mice manifested a significantly greater magnitude of hypoxic sensory response compared with WT littermates. In contrast, chemoreceptor responses to hypercapnia or cyanide, which are also potent carotid body stimulants, were comparable between genotypes (Fig. 1G and H), indicating a selective alteration in the response to hypoxia in *Hif-2 $\alpha$ <sup>+/-</sup>* mice.

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**Fig. 1.** Analysis of carotid body structure and function. (A) Carotid body sections from *Hif-2α*<sup>+/+</sup> and *Hif-2α*<sup>+/-</sup> littermate mice (denoted by <sup>+/+</sup> and <sup>+/-</sup>, respectively) were stained with antibodies specific for TH, a marker of glomus cells, and HIF-2α. (Scale bar, 20 μm.) (B–D) Analysis (mean ± SEM) of total volume of the carotid body, number of glomus cells per section, and ratio of glomic volume/total volume from *Hif-2α*<sup>+/+</sup> and *Hif-2α*<sup>+/-</sup> mice (*n* = 3 mice each). (E) Sensory response of isolated carotid bodies to hypoxia (Hx; PO<sub>2</sub> ~ 40 mm Hg). Integrated carotid body sensory activity presented as impulses per second (imp/s). Inset: Superimposed action potentials from the single fiber. (F) Carotid body responses to graded hypoxia from *Hif-2α*<sup>+/+</sup> and *Hif-2α*<sup>+/-</sup> mice are shown as the difference in response between baseline and hypoxia (Δ imp/s). Data are mean ± SEM of 22 (*Hif-2α*<sup>+/+</sup>) and 16 (*Hif-2α*<sup>+/-</sup>) fibers from eight mice of each genotype. (G) CO<sub>2</sub> response (mean ± SEM) from 15 (*Hif-2α*<sup>+/+</sup>) and 18 (*Hif-2α*<sup>+/-</sup>) fibers from seven mice in each group. (H) Carotid body response to 3 μg/mL cyanide (mean ± SEM) from 21 (*Hif-2α*<sup>+/+</sup>) and 15 (*Hif-2α*<sup>+/-</sup>) fibers from six mice in each group. \**P* < 0.05; \*\**P* < 0.01; n.s., not significant.

### *Hif-2α*<sup>+/-</sup> Mice Exhibit Irregular Breathing, Apnea, and Augmented Hypoxic Ventilatory Response.

Breathing was monitored by plethysmography in unsedated *Hif-2α*<sup>+/+</sup> and *Hif-2α*<sup>+/-</sup> mice. While breathing 21% O<sub>2</sub>, *Hif-2α*<sup>+/-</sup> mice exhibited irregular breathing with brief periods of hyperventilation followed by hypoventilation, whereas WT littermates exhibited a regular breathing pattern (Fig. 2A). Analysis of breath-to-breath (BB) interval (BB<sub>*n*</sub>) versus the subsequent interval (BB<sub>*n*+1</sub>) for 500 breaths in a mouse of each genotype during room air breathing is presented as Poincaré plots in Fig. 2B. Variability of BB intervals was greater in *Hif-2α*<sup>+/-</sup> than in *Hif-2α*<sup>+/+</sup> mice. Analysis of the SD of BB intervals (17) showed that SD1 (representing the *y* axis) and SD2 (representing the *x* axis) were significantly greater in *Hif-2α*<sup>+/-</sup> compared with *Hif-2α*<sup>+/+</sup> mice [SD1, 45.5 ± 4.4 ms (*Hif-2α*<sup>+/+</sup>) vs. 75.9 ± 9.4 ms (*Hif-2α*<sup>+/-</sup>), *P* < 0.01; SD2, 91 ± 10.6 ms (*Hif-2α*<sup>+/+</sup>) vs. 153 ± 18.9 ms (*Hif-2α*<sup>+/-</sup>), *P* < 0.01; *n* = 8 mice each]. In addition, *Hif-2α*<sup>+/-</sup> mice exhibited apnea (i.e., cessation of breathing) following sighs, a phenotype that was absent in *Hif-2α*<sup>+/+</sup> mice (Fig. 2C). The incidence of postsigh apnea (duration of more than three breaths) was sevenfold higher in *Hif-2α*<sup>+/-</sup> compared with *Hif-2α*<sup>+/+</sup> mice (Fig. 2D).

Hypoxic ventilatory response (HVR) represents a major reflex triggered by the carotid body. HVR was determined in both genotypes by monitoring responses of the respiratory rate (RR) and tidal volume (V<sub>T</sub>) to hypoxia (i.e., 12% inspired O<sub>2</sub>), with sighs, sniffs, and movement-induced changes in breathing excluded from the analysis. *Hif-2α*<sup>+/-</sup> mice exhibited a significantly increased magnitude of HVR compared with WT littermates (Fig. 2E). The enhanced HVR in *Hif-2α*<sup>+/-</sup> mice was a result of significant elevations in RR as well as V<sub>T</sub> compared with control mice (Table S1). There were no significant differences between genotypes with respect to O<sub>2</sub> consumption (V<sub>O2</sub>) or CO<sub>2</sub> (V<sub>CO2</sub>)

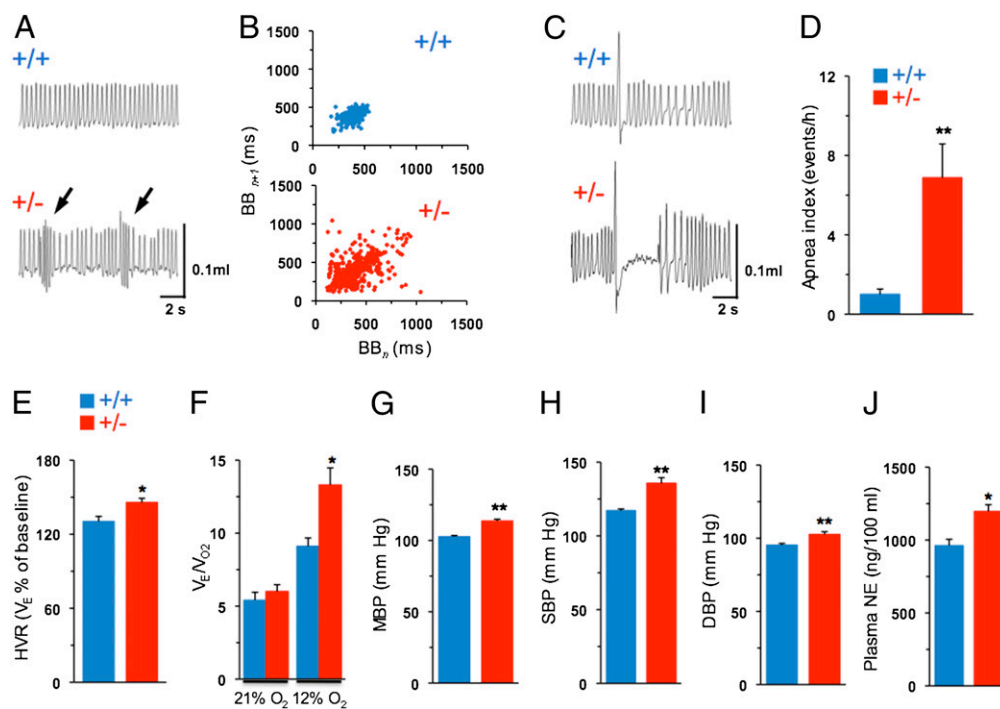
production during hypoxia (Table S1). The ratio of minute ventilation to O<sub>2</sub> consumption (V<sub>E</sub>/V<sub>O2</sub>), which is a measure of convective requirement, was significantly increased during hypoxia in *Hif-2α*<sup>+/-</sup> compared with *Hif-2α*<sup>+/+</sup> mice (Fig. 2F). In striking contrast, ventilatory responses to hypercapnia (i.e., 5% inspired CO<sub>2</sub>) were comparable between *Hif-2α*<sup>+/-</sup> and *Hif-2α*<sup>+/+</sup> mice (Table S1).

### Elevated BP and Plasma Norepinephrine Levels in *Hif-2α*<sup>+/-</sup> Mice.

Arterial BP was measured in unsedated *Hif-2α*<sup>+/+</sup> and *Hif-2α*<sup>+/-</sup> mice. Mean BP was significantly increased in *Hif-2α*<sup>+/-</sup> (115 ± 1 mm Hg) compared with *Hif-2α*<sup>+/+</sup> (103 ± 0.9 mm Hg) mice (*P* < 0.01; Fig. 2G), which was a result of increases in systolic and diastolic pressure (Fig. 2H and I). Plasma norepinephrine (NE) levels were also significantly elevated in *Hif-2α*<sup>+/-</sup> mice compared with WT littermates (Fig. 2J).

### Changes in Antioxidant Enzymes, Oxidative Stress, and Mitochondrial Electron Transport Chain Activity in *Hif-2α*<sup>+/-</sup> Mice.

HIF-2 is a potent transcriptional activator of genes encoding AOE (12). Analysis of mRNAs encoding AOE in carotid bodies was technically difficult because of the limited availability of the tissue (mouse carotid body wet weight is 20–30 μg). However, the adrenal medulla, which also expresses high levels of HIF-2α (16), was chosen as an alternative tissue source. HIF-2α protein expression in the adrenal medulla was decreased by approximately 50% in *Hif-2α*<sup>+/-</sup> mice compared with WT littermates, whereas HIF-1β protein levels were similar (Fig. 3A). Analysis of AOE gene expression in adrenal medulla revealed that mRNAs encoding catalase (*Cat*) and superoxide dismutase (*Sod1*, *Sod2*) were significantly decreased in *Hif-2α*<sup>+/-</sup> mice, with *Sod2* mRNA expression most severely reduced compared with WT littermates (Fig. 3B). Quantitative real-



**Fig. 2.** Breathing and BP in *Hif-2 $\alpha$ <sup>+/+</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice. (A) Breathing was monitored by whole-body plethysmography in unsedated mice. Example of irregular breathing with brief periods of hyperventilation (arrows) followed by hypoventilation in *Hif-2 $\alpha$ <sup>+/-</sup>* and stable breathing in *Hif-2 $\alpha$ <sup>+/+</sup>* mice. (B) Poincaré plots of  $BB_{n+1}$  versus  $BB_n$  for 500 breaths in a single mouse of each genotype. (C) Example of post-sigh apnea in *Hif-2 $\alpha$ <sup>+/-</sup>* and its absence in *Hif-2 $\alpha$ <sup>+/+</sup>* mice. (D) Mean  $\pm$  SEM data for apnea index (number of post-sigh apneas more than three breaths in duration). (E) HVR in *Hif-2 $\alpha$ <sup>+/+</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice (mean  $\pm$  SEM). Data presented are percent change in minute ventilation ( $V_E$ ) from baseline. (F) Ratio of minute ventilation to  $O_2$  consumption ( $V_E/V_{O_2}$ ) during 21% and 12%  $O_2$  breathing (mean  $\pm$  SEM). (G–J) Mean (MBP), systolic (SBP), and diastolic (DBP) BP and plasma NE levels in *Hif-2 $\alpha$ <sup>+/+</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice. Data presented as mean  $\pm$  SEM from eight mice in each group. \*\* $P < 0.01$ ; \* $P < 0.05$ .

time RT-PCR assay demonstrated that *Sod2* mRNA levels were also significantly decreased in carotid bodies from *Hif-2 $\alpha$ <sup>+/-</sup>* compared with *Hif-2 $\alpha$ <sup>+/+</sup>* mice (Fig. 3C).

*Sod2* encodes manganese superoxide dismutase (MnSOD), a major AOE in mitochondria. Analysis of mitochondrial fractions of adrenal medullae showed approximately 60% reduction in MnSOD enzyme activity in *Hif-2 $\alpha$ <sup>+/-</sup>* compared with WT littermates (Fig. 3D). The reduced MnSOD activity was associated with significantly increased levels of cytosolic and mitochondrial reactive oxygen species (ROS), as evidenced by significantly decreased aconitase activity (Fig. 3E and F), a sensitive biomarker of ROS (18). Mitochondrial electron transport chain (ETC) complex I activity, which is also a target for ROS (19, 20), was significantly decreased in *Hif-2 $\alpha$ <sup>+/-</sup>* mice, whereas ETC complex III activity was unaffected (Fig. 3G).

**Antioxidant Treatment Restores Carotid Body Response to Hypoxia and Cardiorespiratory Functions in *Hif-2 $\alpha$ <sup>+/-</sup>* Mice.** We next investigated whether preventing oxidative stress would correct autonomic functions and hypoxic sensitivity of the carotid body in *Hif-2 $\alpha$ <sup>+/-</sup>* mice. To this end, mice from both genotypes were treated for 2 wk with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a potent membrane permeable antioxidant. Antioxidant treatment normalized cytosolic and mitochondrial aconitase as well as complex I activities in *Hif-2 $\alpha$ <sup>+/-</sup>* mice (Fig. 4A–C), confirming reversal of the increased oxidative stress. More importantly, the augmented hypoxic sensory response of the carotid body was absent in antioxidant-treated *Hif-2 $\alpha$ <sup>+/-</sup>* mice (Fig. 4D and E). The phenotype of irregular breathing, post-sigh apneas, augmented HVR, elevated BP, and increased plasma NE levels was completely absent in antioxidant-treated *Hif-2 $\alpha$ <sup>+/-</sup>* mice (Fig. 5). Antioxidant treatment, however,

had no significant effects on carotid body response to hypoxia, HVR, or BP in WT littermate mice ( $P > 0.05$ ).

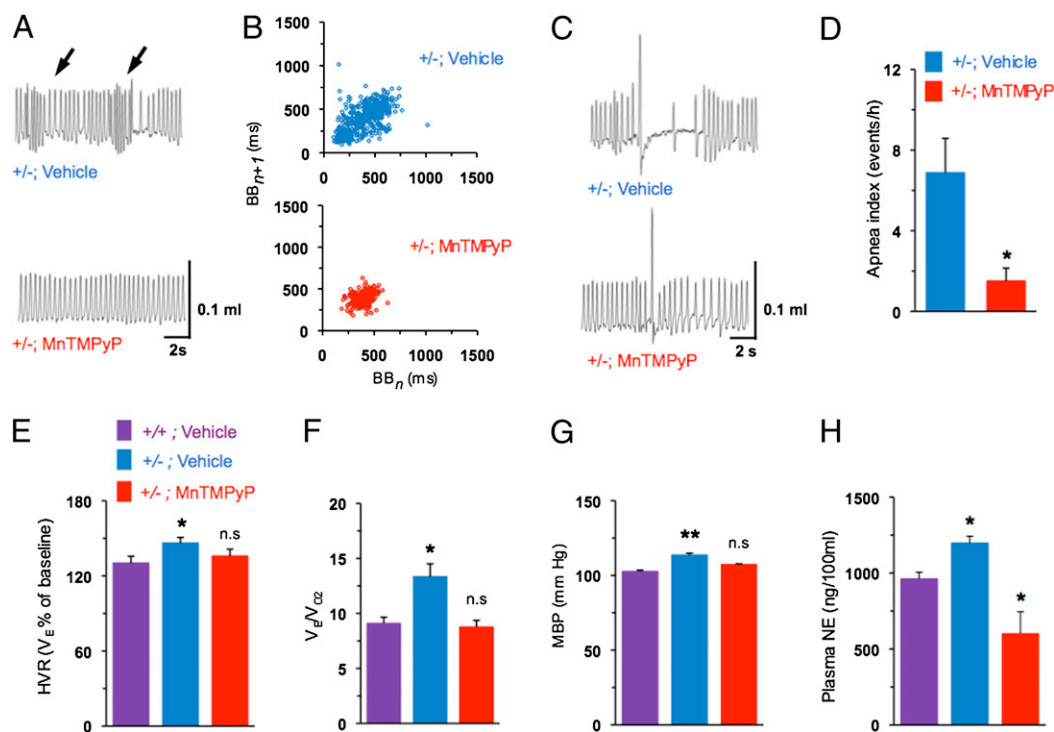
## Discussion

The present study demonstrates that mice with heterozygous deficiency of HIF-2 $\alpha$  exhibit striking augmentation of the carotid body response to acute hypoxia and equally striking abnormalities in breathing as well as hypertension with elevated plasma NE levels. Although HIF-1 $\alpha$  and HIF-2 $\alpha$  are paralogues (1, 9, 10) that share some common functions, *Hif-1 $\alpha$ <sup>+/-</sup>* mice exhibit neither hypertension nor abnormal breathing patterns (4). Moreover, carotid body sensitivity to hypoxia is severely impaired in *Hif-1 $\alpha$ <sup>+/-</sup>* mice (4, 5), a finding that is diametrically opposed to the enhanced carotid body response to hypoxia in *Hif-2 $\alpha$ <sup>+/-</sup>* mice. The striking differences between *Hif-1 $\alpha$ <sup>+/-</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice suggest that HIF-2 plays an antagonistic rather than redundant role to that of HIF-1 in regulating oxygen sensing by the carotid body and cardiorespiratory homeostasis.

A major finding of the present study is that *Hif-2 $\alpha$ <sup>+/-</sup>* mice displayed marked ventilatory instability while breathing room air and increased incidence of post-sigh apneas. Periods of hyperventilation followed by hypoventilation are regarded as a hallmark of instability of the ventilatory control system (21). Hyperactive carotid body function has been implicated in triggering irregular breathing patterns (22). Indeed, carotid bodies from *Hif-2 $\alpha$ <sup>+/-</sup>* mice showed striking enhancement of ventilatory responses to hypoxia, a well established reflex response triggered by the carotid body. The observed effects were selective to hypoxia because carotid body and ventilatory responses to  $CO_2$  in *Hif-2 $\alpha$ <sup>+/-</sup>* mice were comparable to those in WT littermates. Although HIF-2 $\alpha$  expression was reported previously in glomus cells of the carotid body (16, 23), its functional role has not been elu-







**Fig. 5.** Antioxidant treatment restores cardiorespiratory functions in *Hif-2 $\alpha$ <sup>+/-</sup>* mice. (A) Representative tracings of breathing in *Hif-2 $\alpha$ <sup>+/-</sup>* mice treated with vehicle or antioxidant MnTMPyP. Arrows denote periods of hyperventilation. (B) Poincaré plots of  $BB_{n+1}$  versus  $BB_n$  for 500 breaths in vehicle- or MnTMPyP-treated *Hif-2 $\alpha$ <sup>+/-</sup>* mice. (C and D) Examples showing post-sigh apnea in vehicle-treated *Hif-2 $\alpha$ <sup>+/-</sup>* mouse and its absence in MnTMPyP-treated *Hif-2 $\alpha$ <sup>+/-</sup>* mouse and apnea index (mean  $\pm$  SEM). (E and F) HVR and ratio of minute ventilation to  $O_2$  consumption ( $V_E/V_{O_2}$ ) during 21% and 12%  $O_2$  breathing (mean  $\pm$  SEM) in *Hif-2 $\alpha$ <sup>+/-</sup>* mice treated with vehicle or MnTMPyP. (G and H) Mean BP (MBP) and plasma NE levels in *Hif-2 $\alpha$ <sup>+/-</sup>* mice treated with vehicle or MnTMPyP (mean  $\pm$  SEM,  $n = 6$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; n.s., not significant).

In humans, exaggerated carotid body response to hypoxia, elevated BP, and increased sympathetic activity occur as a consequence of recurrent apneas (i.e., repetitive brief cessations of breathing), which cause chronic intermittent hypoxia (CIH). Oxidative stress mediates cardiorespiratory responses and sleep disorders that occur in response to CIH (27, 28). We recently demonstrated that exposure of rats and cultured cells to CIH leads to depletion of HIF-2 $\alpha$  and that restoring HIF-2 $\alpha$  levels prevents oxidative stress and its pathological sequelae (29). In striking contrast, CIH leads to increased accumulation of HIF-1 $\alpha$  (4, 30, 31) and, remarkably, *Hif-1 $\alpha$ <sup>+/-</sup>* mice subjected to CIH do not exhibit the oxidative stress, increased catecholamines, hypertension, or carotid body changes that are observed in WT mice (4). Taken together, these results suggest that functional antagonism between HIF-1 and HIF-2 plays a fundamental role in redox regulation and the maintenance of cardiorespiratory homeostasis.

## Materials and Methods

**Preparation of Mice.** Experiments were approved by the institutional animal care and use committee of the University of Chicago and performed by individuals blinded to genotype of male, age-matched *Hif-2 $\alpha$ <sup>+/+</sup>* and *Hif-2 $\alpha$ <sup>+/-</sup>* mice (12).

**Carotid Body Sensory Activity.** Sensory activity from carotid bodies *ex vivo* was recorded as described (4). Clearly identifiable action potentials (two to three active units) were recorded from one of the nerve bundles with a suction electrode and "single" units were selected based on the height and duration of the individual action potentials by using a spike discrimination program (Spike Histogram Program; AD Instruments). The  $PO_2$  and  $PCO_2$  of the superfusion medium were determined by a blood gas analyzer (ABL 5; Radiometer).

**Measurements of Respiratory Variables and BP.** Ventilation was monitored by whole-body plethysmograph, and  $O_2$  consumption and  $CO_2$  production were determined by the open-circuit method in unsedated mice (4, 5). Baseline ventilation was recorded for 2 h while the mice breathed 21%  $O_2$ . Hypoxic (12%  $O_2$  with the balance  $N_2$ ) and hyperoxic hypercapnia (5%  $CO_2/95\%$   $O_2$ ) gas challenges were given for 5 min.  $O_2$  consumption and  $CO_2$  production were measured at the end of the 5-min hypoxic challenge. All recordings were made at an ambient temperature of  $25 \pm 1^\circ C$ . BP was monitored by tail cuff method in unsedated mice by using a noninvasive BP system (AD Instruments) (5).

**Immunohistochemistry.** Carotid bodies were harvested from anesthetized mice (urethane, 1.2 g/kg i.p.) perfused with heparinized saline solution followed by 4% paraformaldehyde. Fixation of carotid bodies, immunohistochemical analysis of HIF-2 $\alpha$  and TH, and morphometric analysis were performed as described (5).

**Western Blot Analysis.** Adrenal medullae were harvested from anesthetized mice and HIF-2 $\alpha$  and HIF-1 $\beta$  proteins were analyzed by immunoblot assay (29). The following antibodies (Novus) were used: anti-HIF-2 $\alpha$  polyclonal (1:500 dilution) and anti-HIF-1 $\beta$  monoclonal H1 $\beta$ 234 (1:1,500 dilution).

**Enzyme Activities.** Aconitase enzyme activity was determined in cytosolic and mitochondrial fractions of adrenal medulla (32). MnSOD activity was measured by using a superoxide dismutase assay kit (WST; S311-10; Dojondo Molecular Technologies) (28). Complex I (NADH-ubiquinone reductase) and complex III (ubiquinol-ferricytochrome c oxidoreductase) activities were measured as described (31).

**Measurement of Plasma NE Levels.** Blood samples were collected from anesthetized mice by cardiac puncture and plasma NE was determined by HPLC combined with electrochemical detection using dihydroxybenzylamine as an internal standard (4).

**Gene Expression Analysis.** AOE mRNA expression was analyzed in adrenal medullae by RT Profiler PCR Array for Oxidative Stress (SA Biosciences). The relative expression was determined by the  $\Delta\Delta Ct$  method whereby, first, the level of gene of interest (GOI) is normalized to a housekeeping gene (HKG):

$$2^{-\Delta Ct} = 2^{-[Ct(GOI) - Ct(HKG)]} \quad [1]$$

where GOI is the gene of interest and HKG is the housekeeping gene. Fold change in gene expression is then determined as follows:

$$2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct(+/-) - \Delta Ct(+/-)]} \quad [2]$$

Sod2 mRNA was analyzed in the carotid bodies by quantitative real-time RT-PCR with mouse-specific primers detecting Sod2 mRNA or 18S rRNA with SYBR as a fluorogenic binding dye.

**Chemicals.** MnTMPyP was obtained from Alexis Biochemicals.

**Data Analysis.** In unsedated mice RR (breaths/min),  $V_T$  ( $\mu\text{L}$ ), minute ventilation [ $V_E$  (mL/min) =  $RR \times V_T$ ],  $O_2$  consumption [ $VO_2$  (mL/min)], and  $CO_2$  production

[ $VCO_2$  (mL/min)] were analyzed. Breathing variability was calculated by Poincaré plots and analysis of SD1 and SD2 of BB intervals as described (33). Respiratory variables (RR and  $V_T$ ) were averaged for at least 20 consecutive breaths over a period of 5 min of inspired  $O_2$  and  $CO_2$  challenge.  $V_T$ ,  $V_E$ ,  $VO_2$ , and  $VCO_2$  were normalized to body weight. Carotid body sensory activity (discharge from single units) was averaged during 3 min of baseline and during the 3 min of gas challenge and expressed as impulses per second. Biochemical and mRNA measurements were performed in three to five independent experiments, each in triplicate. Data are presented as mean  $\pm$  SEM. Statistical significance was assessed by ANOVA or two-way ANOVA with repeated measures followed by Tukey test.  $P$  values lower than 0.05 were considered significant.

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