# Mutual antagonism between hypoxia-inducible factors 1α and 2α regulates oxygen sensing and cardio-respiratory homeostasis

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**Breathing and blood pressure are under constant homeostatic regulation to maintain optimal oxygen delivery to the tissues. Chemosensory reflexes initiated by the carotid body and catecholamine secretion from the adrenal medulla are the principal mechanisms for maintaining respiratory and cardiovascular homeostasis; however, the underlying molecular mechanisms are not known. Here, we report that balanced activity of hypoxia-inducible factor-1 (HIF-1) and HIF-2 is critical for oxygen sensing by the carotid body and adrenal medulla, and for their control of cardio-respiratory function. In *Hif2α*$^{+/-}$ mice, partial HIF-2α deficiency increased levels of HIF-1α and NADPH oxidase 2, leading to an oxidized intracellular redox state, exaggerated hypoxic sensitivity, and cardio-respiratory abnormalities, which were reversed by treatment with a HIF-1α inhibitor or a superoxide anion scavenger. Conversely, in *Hif1α*$^{+/-}$ mice, partial HIF-1α deficiency increased levels of HIF-2α and superoxide dismutase 2, leading to a reduced intracellular redox state, blunted oxygen sensing, and impaired carotid body and ventilatory responses to chronic hypoxia, which were corrected by treatment with a HIF-2α inhibitor. None of the abnormalities observed in *Hif1α*$^{+/-}$ mice or *Hif2α*$^{+/-}$ mice were observed in *Hif1α*$^{+/-}$;*Hif2α*$^{+/-}$ mice. These observations demonstrate that redox balance, which is determined by mutual antagonism between HIF-α isoforms, establishes the set point for hypoxic sensing by the carotid body and adrenal medulla, and is required for maintenance of cardio-respiratory homeostasis.**

blood pressure regulation | ventilatory adaptation | reactive oxygen species | Nox2 | Sod2

Vertebrate organisms have evolved complex respiratory and cardiovascular systems that are designed to ensure optimal $O_2$ delivery to each cell. $O_2$ is the most critical environmental substrate for survival because it enables sufficient ATP production, which is essential to maintain the structure and function of complex organisms (1). Perturbations in $O_2$ availability also affect cellular redox homeostasis. Thus, $O_2$, energy, and redox homeostasis are inextricably linked to cardio-respiratory function. The ability of mammals to maintain homeostasis in response to changes in $O_2$ supply or demand critically depends on proper regulation of breathing and blood pressure.

Eight decades ago, the carotid body (CB) was identified as the sensory organ that monitors arterial blood $O_2$ levels and stimulates breathing in response to hypoxemia, thereby ensuring adequate $O_2$ availability (2). In response to stress, catecholamine secretion by the adrenal medulla (AM) initiates physiological responses to overcome stress, a phenomenon referred to as the "fight-or-flight" response. Considerable evidence suggests that hypoxia, by directly acting on AM chromaffin cells, stimulates catecholamine secretion in neonates (3–6) and in adults (7), which increases blood pressure and facilitates $O_2$ delivery to tissues. Thus, the CB chemosensory reflex and AM catecholamine secretion are principal regulators of cardio-respiratory function during hypoxia. Despite extensive physiological studies, the molecular mechanisms that establish the set point at which the CB and AM respond to hypoxia with changes in breathing and blood pressure have not been identified.

The identification of hypoxia-inducible factor 1 (HIF-1), and subsequent identification of HIF-2, provided important insights into the molecular mechanisms underlying responses to hypoxia (8). HIF-1 and HIF-2 are heterodimers comprised of an $O_2$-regulated HIF-1α or HIF-2α subunit and a constitutively expressed HIF-1β subunit. HIF-1α is expressed in all cells of all metazoan species, whereas HIF-2α is only expressed in certain cell types of vertebrate species (9). Complete deficiency of either HIF-1α or HIF-2α results in embryonic lethality, whereas mice with partial deficiency of HIF-1α or HIF-2α develop normally (10, 11).

Both HIF-1α and HIF-2α are expressed in the CB (12, 13). Although HIF-1α and HIF-2α are paralogs that share some common functions (14), CB responses to hypoxia are impaired in *Hif1α*$^{+/-}$ mice (15), whereas they are exaggerated in *Hif2α*$^{+/-}$ mice (16). The mechanisms underlying the contrasting responses of the CB to hypoxia in *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice are not known. The AM also expresses both HIF-1α and HIF-2α (13), but their roles in catecholamine secretion in response to hypoxia have not been examined. Recent studies revealed that HIF-1 and HIF-2 mediate expression of gene products with opposing functions in the CB. HIF-1 regulates NADPH oxidase 2 (Nox2),

## Significance

**The carotid body (CB) chemosensory reflex and catecholamine secretion by the adrenal medulla (AM) are principal regulators of cardio-respiratory function during hypoxia, but the molecular mechanisms by which the CB and AM respond to hypoxia with changes in breathing and blood pressure are unknown. Hypoxia-inducible factor-1 (HIF-1) and HIF-2 mediate adaptive transcriptional responses to hypoxia. Herein, we demonstrate that a mutual functional antagonism between HIF-1 and HIF-2 plays a critical role in $O_2$ sensing by establishing a dynamic balance that determines the proper redox set point in the CB and AM, which is essential for maintenance of cardio-respiratory homeostasis.**

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a pro-oxidant enzyme (17), whereas HIF-2 regulates superoxide dismutase 2 (Sod2), an antioxidant enzyme (11, 18), which suggests that balance between HIF-α isoforms might be important for maintaining cellular redox homeostasis. Based on these studies, we tested the hypothesis that functional antagonism between HIF-1α and HIF-2α plays a critical role in $O_2$ sensing by regulating redox state in the CB and AM, which in turn is critical for regulation of cardio-respiratory homeostasis.

## Results

**Increased HIF-1α Expression in the CB and AM of *Hif2α*$^{+/-}$ Mice.** HIF-1α expression was analyzed in CBs from *Hif2α*$^{+/-}$ and WT mice by immunofluorescence. CB sections were stained for HIF-1α and chromogranin A (CGA), an established marker of glomus cells, which are the primary $O_2$ sensors in the CB (reviewed in ref. 19). HIF-1α expression was increased in glomus cells of *Hif2α*$^{+/-}$ mice compared with WT mice (Fig. 1*A*). Increased HIF-1α protein levels in the AM from *Hif2α*$^{+/-}$ mice were also demonstrated by immunoblot assay (Fig. 1*B*). The increased HIF-1α protein levels in CB and AM were not because of increased HIF-1α mRNA levels (Fig. S1 *A* and *B*). Digoxin is a potent inhibitor of HIF-1α protein expression (20) and *Hif2α*$^{+/-}$ mice treated with digoxin (1 mg per kg per d i.p. for 3 d) no longer exhibited increased HIF-1α levels in the CB (Fig. 1*A*) and AM (Fig. 1*B*).

To determine the functional significance of increased HIF-1α levels, CB and AM responses to hypoxia were determined in vehicle- and digoxin-treated *Hif2α*$^{+/-}$ mice, as described previously (16, 21). Compared with WT littermates, vehicle-treated *Hif2α*$^{+/-}$ mice exhibited an augmented CB sensory activity and enhanced catecholamine secretion from AM chromaffin cells in response to hypoxia (Fig. 1 *C* and *D*; quantified in Fig. S1 *C* and *D*). *Hif2α*$^{+/-}$ mice exhibited elevated blood pressure and plasma catecholamine levels (Fig. 1 *E* and *F*) as well as breathing instability, which was manifested by periods of hypoventilation followed by hyperventilation, and an increased incidence of postsigh apnea (cessation of breathing for a duration greater than three breaths) (Fig. 1 *G–I*; quantified in Fig. S1 *E–G*). Bilateral sectioning of the carotid sinus nerve corrected the respiratory abnormalities in *Hif2α*$^{+/-}$ mice (Fig. S1 *H–L*), implicating the hyperactive CB chemosensory reflex. The exaggerated hypoxic sensitivity of the CB and AM, as well as the cardio-respiratory abnormalities, were also corrected in digoxin-treated *Hif2α*$^{+/-}$ mice (Fig. 1 *C–I*), in which HIF-1α levels were similar to untreated WT littermates (Fig. 1 *A* and *B*).

**Increased HIF-2α Expression in the CB and AM of *Hif1α*$^{+/-}$ Mice.** Compared with WT mice, HIF-2α expression was increased in CB glomus cells (Fig. 2*A*) and the AM (Fig. 2*B*) from *Hif1α*$^{+/-}$ mice. The increased HIF-2α protein levels in the CB and AM were not caused by increased mRNA levels (Fig. S2 *A* and *B*). Treating *Hif1α*$^{+/-}$ mice with 2-methoxyestradiol (2ME2; 200 mg per kg per d i.p. for 3 d), which reduced HIF-2α protein levels in rat pheochromocytoma PC12 cell cultures (Fig. S2*C*), normalized HIF-2α protein levels in the CB (Fig. 2*A*) and AM (Fig. 2*B*). The increased HIF-2α expression in *Hif1α*$^{+/-}$ mice was associated with impaired CB and AM chromaffin cell responses to hypoxia, which were normalized following 2ME2 treatment (Fig. 2 *C* and *D*; quantified in Fig. S2 *D* and *E*).

Exposure to chronic hypoxia induces increased CB sensitivity to low $PO_2$, which mediates ventilatory acclimatization that is manifested by an augmented response to subsequent acute hypoxia (reviewed in ref. 19). We previously reported that WT mice exposed to chronic hypobaric hypoxia (0.4 atmospheres for 72 h) manifested an augmented ventilatory response to a subsequent

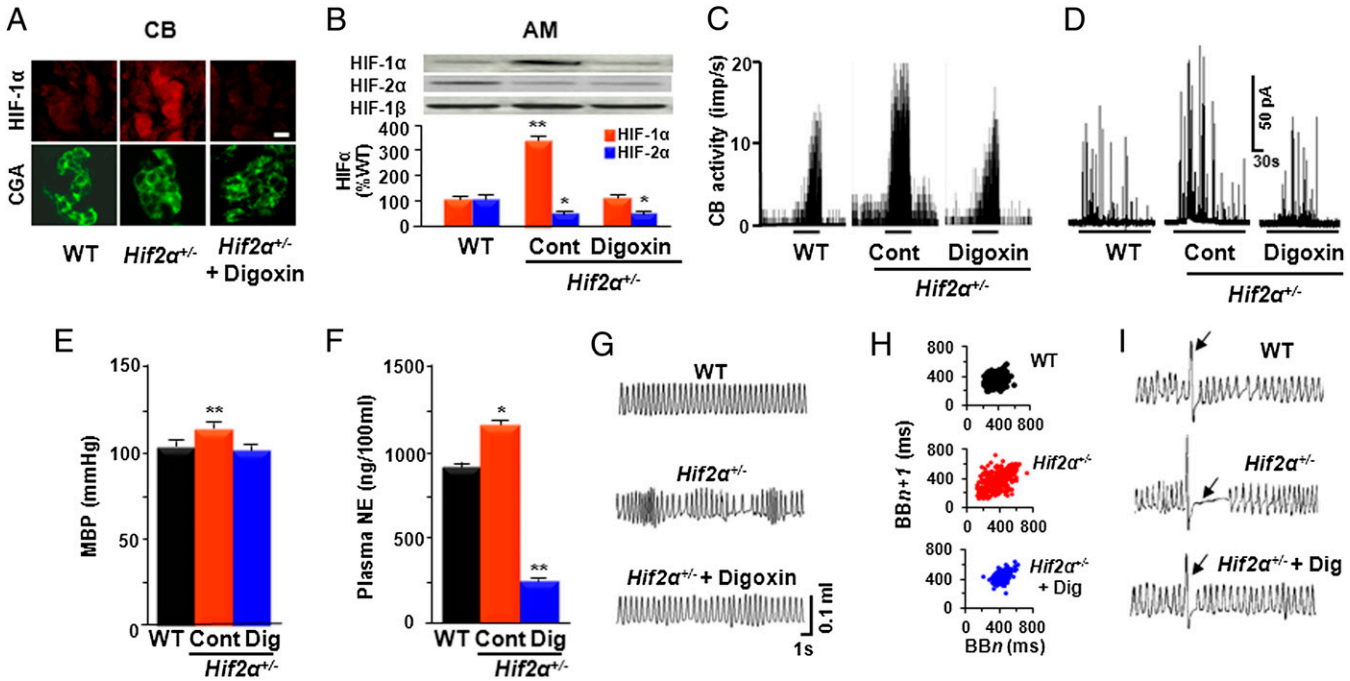


**Fig. 1.** Analysis of *Hif2α*$^{+/-}$ mice. Three groups were studied: WT mice (*Hif2α*$^{+/+}$), control (Cont) *Hif2α*$^{+/-}$ mice, and *Hif2α*$^{+/-}$ mice treated with digoxin (Dig). (*A*) The expression of HIF-1α and CGA in glomus cells of the CB was analyzed by immunofluorescence. (Scale bar, 20 μm.) (*B*) Representative immunoblots (*Upper*) and densitometric analysis (*Lower*) of HIF-1α (red bars) and HIF-2α (blue bars) protein levels in the AM relative to untreated WT mice are shown. (*C*) CB responses to hypoxia ($PO_2$ ~40 mmHg for 3 min; black bar under tracing). Integrated sensory activity is presented as impulses per second [CB activity (imp/s)]. (*D*) Catecholamine secretion from AM chromaffin cells in response to hypoxia ($PO_2$ ~40 mmHg; black bar under tracing) was measured by carbon fiber amperometry. (*E* and *F*) Mean blood pressure (MBP; *E*) and plasma norepinephrine (NE) levels (*F*) were determined. (*G–I*) Breathing was analyzed, including breathing pattern (*G*), Poincarè plots of breath-to-breath (BB) interval in milliseconds (*H*), and postsigh apnea (at arrows; *I*). Data in bar graphs are presented as mean ± SEM, $n$ = 6–8 mice each; $^*P < 0.05$, $^{**}P < 0.01$.

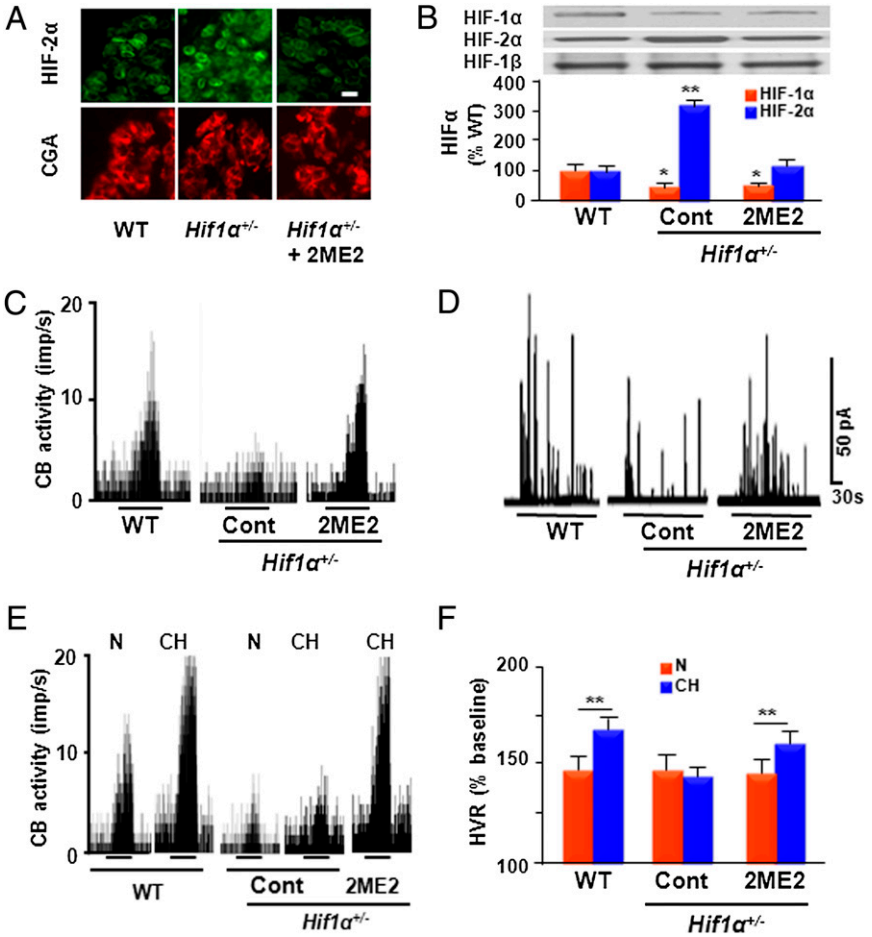


**Fig. 2.** Analysis of *Hif1α*$^{+/-}$ mice. Three groups were studied: WT mice (*Hif1α*$^{+/+}$), control (Cont) *Hif1α*$^{+/-}$ mice, and *Hif1α*$^{+/-}$ mice treated with 2ME2. (*A*) The expression of HIF-2α and CGA in glomus cells of the CB was analyzed by immunofluorescence. (Scale bar, 20 μm.) (*B*) Representative immunoblots (*Upper*) and densitometric analysis (*Lower*) of HIF-1α and HIF-2α levels in the AM relative to untreated WT mice are shown. (*C*) CB responses to hypoxia ($PO_2$ ~40 mmHg for 3 min; black bar). (*D*) Catecholamine (CA) secretion from AM chromaffin cells in response to hypoxia (black bar). (*E*) CB Responses to hypoxia from mice maintained under normoxic conditions (N) or exposed to chronic hypoxia (CH; 72 h at 0.4 atmospheres). (*F*) Hypoxic ventilatory response (HVR) after 72 h of CH was analyzed as ratio of minute ventilation ($V_E$) to $O_2$ consumption ($V_{O2}$) and presented as percent of baseline (i.e., before CH). Data in bar graphs are presented as mean ± SEM, $n$ = 7–8 mice; *$P < 0.05$, **$P < 0.01$.

acute hypoxic challenge, whereas the effect of chronic hypobaric hypoxia on ventilation was absent in *Hif1α*$^{+/-}$ mice (15). Normalization of HIF-2α levels by 2ME2 treatment restored the CB sensory response (Fig. 2*E*; quantified in Fig. S2*F*), as well as the hypoxic ventilatory response (Fig. 2*F*), in *Hif1α*$^{+/-}$ mice exposed to chronic hypoxia.

**Physiological Responses to HIF-α Isoform Inhibition in WT Mice.** Because digoxin and 2ME2 attenuated HIF-1α and HIF-2α protein levels in *Hif2α*$^{+/-}$ and *Hif1α*$^{+/-}$ mice, respectively, we investigated whether treatment with these drugs also affected HIF-α isoform expression in WT mice. Compared with vehicle-treated controls, WT mice treated with digoxin (1 mg per kg per d i.p. for 3 d) exhibited decreased HIF-1α and increased HIF-2α protein levels in the AM (Fig. S3*A*), an impaired CB response to hypoxia (Fig. S3 *B* and *C*), and decreased plasma catecholamine levels (Fig. S3*D*), which was similar to the phenotype of *Hif1α*$^{+/-}$ mice. However, digoxin treatment had no effect on blood pressure or breathing in WT mice (Fig. S3 *E*–*H*).

Compared with vehicle-treated littermates, WT mice treated with 2ME2 (200 mg per kg per d i.p. for 3 d) exhibited increased HIF-1α and decreased HIF-2α protein levels in the AM (Fig. S4*A*), an augmented CB response to hypoxia, irregular breathing, an increased incidence of postsigh apnea, an increase in mean blood pressure of 8 ± 1.2 mmHg, and markedly increased plasma catecholamine levels (Fig. S4 *B*–*J*), which was similar to the phenotype of *Hif2α*$^{+/-}$ mice.

**Hypoxic Sensitivity and Cardio-Respiratory Function in Double-Heterozygous Mice.** We hypothesized that the phenotypes of *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice were not because of reductions in the absolute levels of HIF-1α and HIF-2α, respectively, but rather because of the imbalanced levels of HIF-1α and HIF-2α. To test this hypothesis, we generated *Hif1α*$^{+/-}$;*Hif2α*$^{+/-}$ double-heterozygous mice. Both HIF-1α and HIF-2α protein levels were significantly but equally reduced in the AM from double heterozygous mice (Fig. 3*A*). Remarkably, the CB and AM responses to hypoxia, blood pressure, plasma catecholamine levels, breathing pattern, as well as the CB and ventilatory responses to chronic hypoxia in double-heterozygous mice were all indistinguishable from WT littermates (Fig. 3 *B*–*H* and Fig. S5 *A*–*F*).

**Altered Redox State in the AM of *Hif2α*$^{+/-}$ and *Hif1α*$^{+/-}$ Mice.** A previous study reported that mice with HIF-2α deficiency exhibited oxidative stress and that antioxidant treatment corrected cardio-respiratory abnormalities in *Hif2α*$^{+/-}$ mice (16). The oxidative stress in *Hif2α*$^{+/-}$ mice was attributed to impaired transcription of genes encoding antioxidant enzymes, including *Sod2*, which encodes

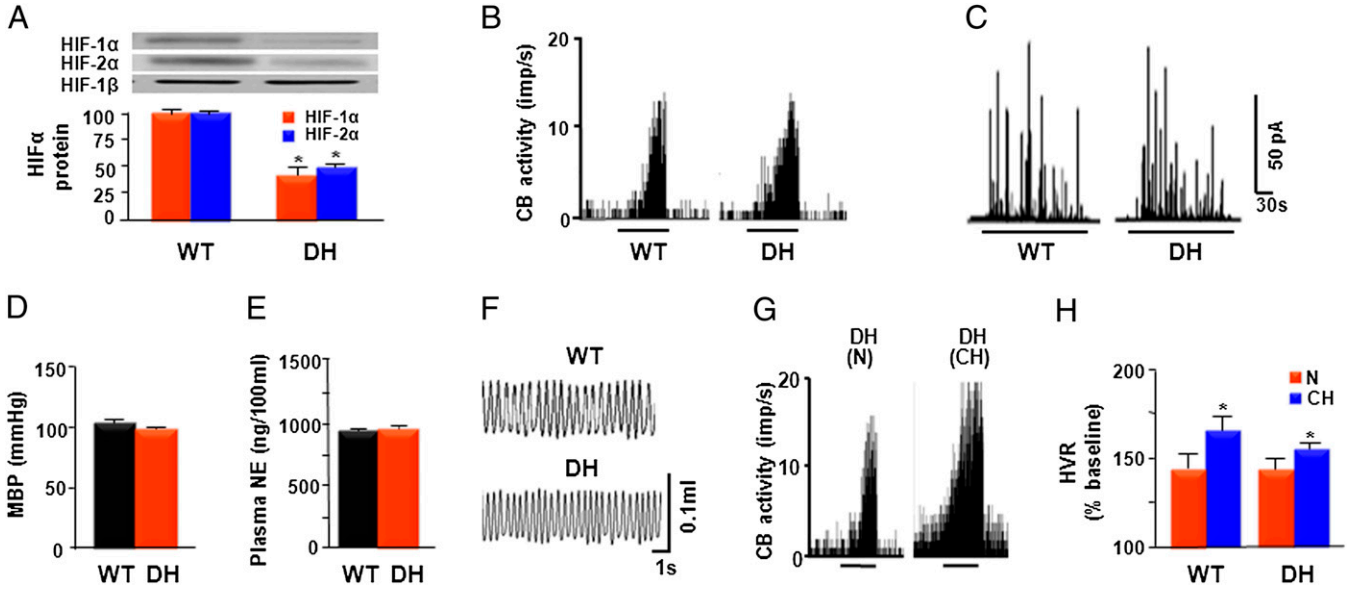


**Fig. 3.** Analysis of double-heterozygous mice. Two experimental groups were studied: WT mice (*Hif1α*$^{+/+}$;*Hif2α*$^{+/+}$), and double-heterozygous (DH) mice (*Hif1α*$^{+/-}$;*Hif2α*$^{+/-}$). (*A*) Representative immunoblots (*Upper*) and densitometric analysis (*Lower*) (mean ± SEM, *n* = 6 mice each) of HIF-1α and HIF-2α levels in the AM from DH relative to WT mice are shown. (*B*) CB responses to hypoxia ($PO_2$ ~40 mm Hg for 3 min; black bar) are shown. (*C*) AM chromaffin cell CA secretory responses to hypoxia (black bar). (*D* and *E*) MBP (*D*) and plasma NE levels (*E*) are shown. (*F*) Breathing patterns of WT and DH mice. (*G*) Responses to hypoxia in CBs from mice maintained under normoxic conditions (N) and mice exposed to chronic hypoxia (CH). (*H*) HVR after 72 h of CH was analyzed as ratio of $V_E$ to $V_{O2}$ and percent of baseline (i.e., before CH) is presented as mean ± SEM from six mice. *$P < 0.05$ (bar graph in *A* and *H*).

superoxide dismutase 2 (11, 16, 18). In contrast, HIF-1α regulates expression of the *Nox2* gene, which encodes the pro-oxidant enzyme Nox2 (17). We hypothesized that increased HIF-1α levels contribute to oxidative stress in *Hif2α*$^{+/-}$ mice by activation of Nox2 expression. Nox2 and Sod2 mRNA levels and enzyme activities were determined in the AM. In addition, because aconitase is inactivated by oxidation (22), aconitase enzyme activity in cytosolic and mitochondrial fractions was determined as a measure of oxidative stress. These analyses were performed on the AM because of limited availability of the CB tissue, which weighs ~25 μg.

Nox2 mRNA and enzyme activity were significantly increased in *Hif2α*$^{+/-}$ mice compared with WT littermates, and these abnormalities were corrected by digoxin treatment (Fig. 4*A*). In contrast, digoxin treatment had no effect on the decreased Sod2 mRNA levels and enzyme activity caused by partial HIF-2α deficiency (Fig. 4*B*). Untreated *Hif2α*$^{+/-}$ mice exhibited oxidative stress, as evidenced by significantly decreased cytosolic and mitochondrial aconitase activities, which were corrected by digoxin treatment (Fig. 4*C*). WT mice treated with digoxin exhibited increased Sod2 and decreased Nox2 levels in the AM compared with vehicle-treated controls (Fig. S4*K*).

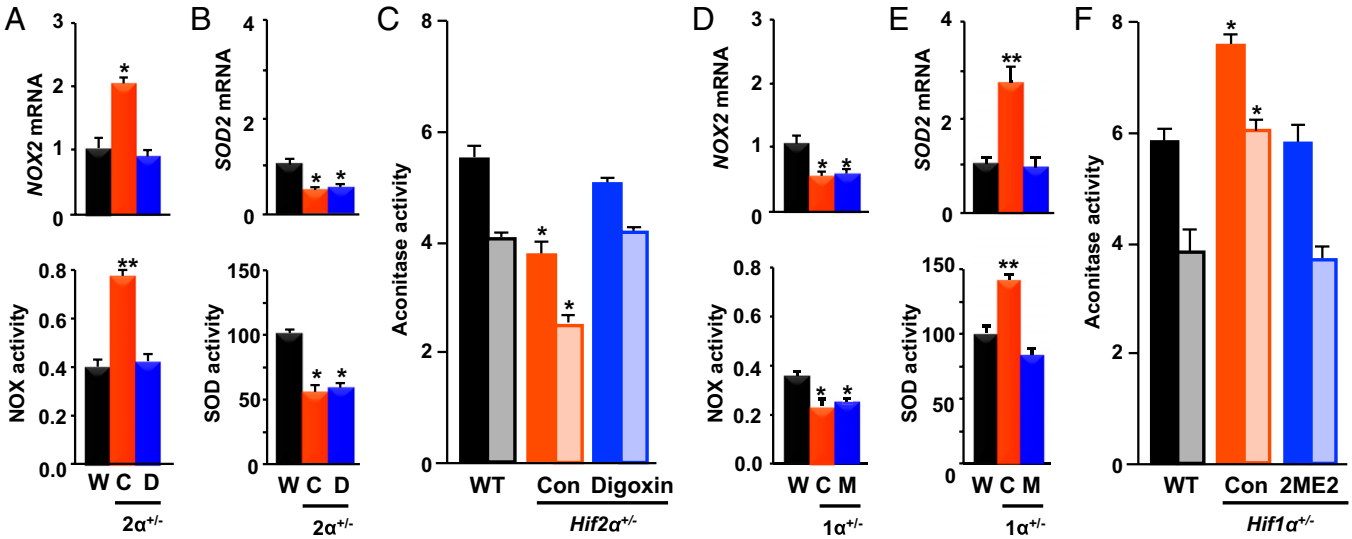


**Fig. 4.** Analysis of intracellular redox state in *Hif2α*$^{+/-}$ and *Hif1α*$^{+/-}$ mice. Nox2, Sod2 mRNA and enzyme activity, and cytosolic (solid bars) and mitochondrial (shaded bars) aconitase activities were determined in AM from the following groups of mice: wild-type mice (W or WT); control (C or Con) *Hif1α*$^{+/-}$ or *Hif2α*$^{+/-}$ mice; *Hif2α*$^{+/-}$ mice treated with digoxin (D); and *Hif-1α*$^{+/-}$ mice treated with 2ME2 (M). (*A*–*C*) Data from WT, *Hif2α*$^{+/-}$ and *Hif2α*$^{+/-}$ + Digoxin groups. (*D*–*F*) Data from WT, *Hif1α*$^{+/-}$ and *Hif1α*$^{+/-}$ + 2ME2 groups. Data in bar graphs are presented as mean ± SEM, *n* = 6–8 mice per group; *$P < 0.05$, **$P < 0.01$.

Analysis of the AM in *Hif1α*$^{+/-}$ mice revealed decreased Nox2 mRNA levels and enzyme activity (Fig. 4*D*), and increased Sod2 mRNA levels and enzyme activity (Fig. 4*E*). Cytosolic and mitochondrial aconitase activities were significantly increased in the AM from *Hif1α*$^{+/-}$ mice compared with WT littermates (Fig. 4*F*). These findings indicate that partial HIF-1α deficiency leads to a reduced intracellular redox state. Treatment of *Hif1α*$^{+/-}$ mice with 2ME2 normalized HIF-2α protein levels (Fig. 2*B*), corrected Sod2 expression/activity (Fig. 4*E*) and normalized aconitase activity in cytosolic and mitochondrial fractions of the AM (Fig. 4*F*). However, 2ME2 had no effect on Nox2 mRNA levels or enzyme activity (Fig. 4*D*). Treatment of WT mice with 2ME2 resulted in increased Nox2 and decreased Sod2 protein levels in the AM (Fig. S4*K*).

In contrast to the altered redox states in the AM of *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice, analysis of AM from double heterozygous mice revealed that Nox2 and Sod2 mRNA levels and enzyme activities, as well as cytosolic and mitochondrial aconitase activities, were comparable to WT littermates (Fig. S5 *G–I*).

**Altered Mammalian Target of Rapamycin and Prolyl Hydroxylase Activity in HIF-2α–Deficient Cells.** We next investigated the mechanisms by which HIF-2α deficiency leads to increased HIF-1α protein levels. These studies used PC12 cells because they involved silencing or forced expression of HIF-2α, which are technically challenging in primary cultures of CB glomus cells or AM chromaffin cells. PC12 cells were chosen because they are derived from the AM and respond to hypoxia with increased catecholamine secretion (23) similar to CB glomus cells (reviewed in ref. 19).

Transfection of PC12 cells with a small interfering RNA (siRNA) targeted against HIF-2α (siHIF-2α), but not a nontargeting scrambled siRNA, led to decreased HIF-2α and Sod2 levels, increased HIF-1α and Nox2 protein levels, and decreased aconitase activity compared with nontransfected control cells (Fig. 5 *A* and *B*), recapitulating the phenotype seen in the AM of *Hif-2α*$^{+/-}$ mice. Forced expression of Sod2 in siHIF-2α–transfected cells corrected the oxidative stress and blocked the increase in HIF-1α protein levels (Fig. 5*B*), suggesting that oxidative stress triggers increased HIF-1α protein levels in HIF-2α-deficient PC12 cells.

We next investigated how oxidative stress increases HIF-1α protein levels in HIF-2α–deficient PC12 cells. We previously reported that oxidative stress induced by intermittent hypoxia increases HIF-1α protein levels via increased protein synthesis, as evidenced by studies with cycloheximide, and that this effect was mediated by activation of mammalian target of rapamycin (mTOR), a serine/threonine kinase that stimulates HIF-1α protein synthesis (24). Compared with cells transfected with scrambled siRNA, phosphorylated (active) mTOR levels were increased in siHIF-2α–transfected cells, whereas the levels of phosphorylated mTOR and HIF-1α were normalized by correcting the oxidative stress either by treatment with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a membrane permeable antioxidant, or by forced expression of Sod2 (Fig. 5*C*). Treatment of siHIF-2α–trans-

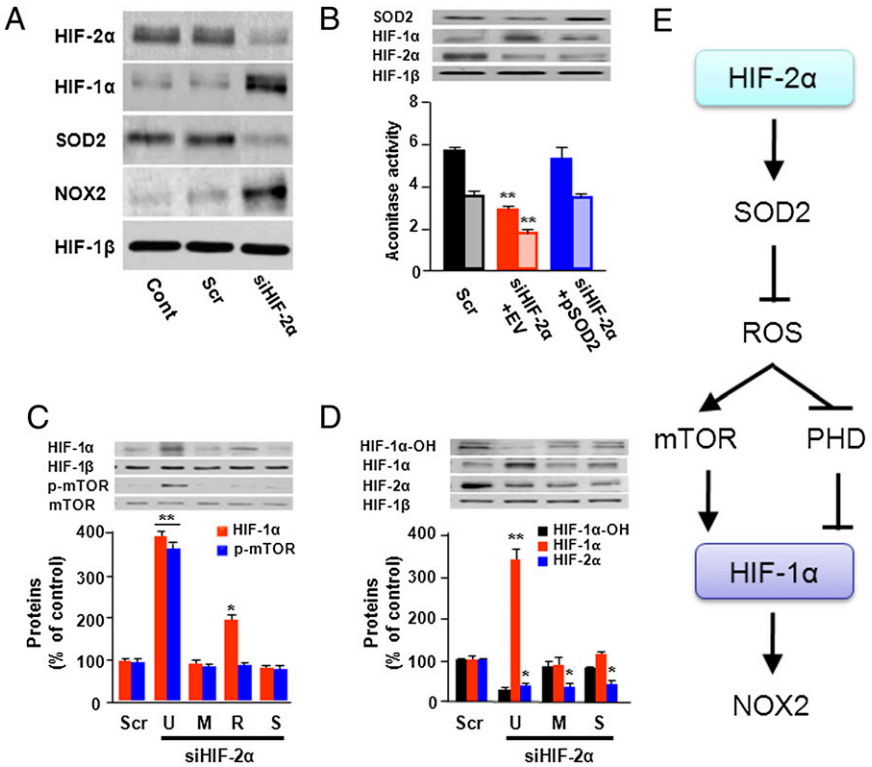


**Fig. 5.** Analysis of mTOR signaling and HIF-1α prolyl hydroxylation in HIF-2α–deficient PC12 cells. (*A*) Immunoblot assays were performed to analyze the expression of HIF-α isoforms, Sod2, Nox2, and HIF-1β in nontransfected control cells and in cells transfected with scrambled siRNA (Scr) or HIF-2α siRNA (siHIF-2α). (*B*) Cells were transfected with scrambled siRNA (Scr) or siHIF-2α and either empty vector (EV) or Sod2 expression vector (pSod2), followed by immunoblot assays (*Upper*) and measurement of aconitase activities (*Lower*) in cytosolic (solid bars) and mitochondrial (shaded bars) fractions of the same samples. (*C* and *D*) Cells were transfected with Scr or siHIF-2α and either left untreated (U) or treated with MnTMPyP (M; 50 μM) or rapamycin (R; 100 nM) or transfected with Sod2 expression vector (S). Immunoblot assays were performed for total mTOR (mTOR), phosphorylated mTOR (p-mTOR), HIF-1α, and HIF-1β (*C*); and prolyl hydroxylated HIF-1α (HIF-1α-OH) and total levels of HIF-α isoforms (*D*). Representative immunoblots (*Upper*) and densitometric analysis (*Lower*) are shown. Data in bar graphs are presented as mean ± SEM, $n = 4–5$ independent experiments; $^*P < 0.05$, $^{**}P < 0.01$. (*E*) The mechanisms and consequences of increased HIF-1α levels in HIF-2α–deficient cells are shown.

fected cells with rapamycin, a selective mTOR inhibitor, blocked mTOR phosphorylation and reduced HIF-1α protein levels by ~60% (Fig. 5*C*), indicating that mTOR activation contributes to the increased HIF-1α levels in HIF-2α–deficient cells.

$O_2$-dependent hydroxylation by prolyl hydroxylases triggers degradation of HIF-1α, whereas oxidation of Fe (II) in the catalytic center of prolyl hydroxylases renders them inactive (25). Silencing of HIF-2α led to a significant decrease in the hydroxylated form of HIF-1α and a concomitant increase in total HIF-1α protein levels, and these effects were abolished by treatment with MnTMPyP or forced expression of Sod2 (Fig. 5*D*). Thus, both reactive oxygen species (ROS)-dependent inhibition of prolyl hydroxylation and ROS-dependent activation of mTOR contribute to increased HIF-1α protein levels in HIF-2α–deficient cells (Fig. 5*E*).

**Altered $Ca^{2+}$ Levels and Calpain Activity in HIF-1α–Deficient Cells.** We next investigated the mechanisms underlying increased HIF-2α protein levels in HIF-1α–deficient cells. Transfection of PC12 cells with siHIF-1α led to decreased HIF-1α and Nox2 levels, increased HIF-2α and Sod2 levels, and a reduced intracellular redox state as evidenced by increased aconitase activity (Fig. 6 *A* and *B*). Forced expression of Nox2 in siHIF-1α–transfected cells normalized aconitase activity and HIF-2α protein levels (Fig. 6*B*).

We previously reported that in PC12 cells exposed to intermittent hypoxia, ROS-dependent elevation of $[Ca^{2+}]_i$ leads to the degradation of HIF-2α by calpains, which are $Ca^{2+}$-dependent proteases, rather than by the proteasome (18). Because HIF-1α–deficient cells showed a reduced intracellular redox state, we hypothesized that HIF-2α levels were increased as a result of reduced calpain activity. To test this possibility, $[Ca^{2+}]_i$ levels were analyzed in PC12 cells cotransfected with an expression vector encoding yellow fluorescent protein (YFP) and a vector encoding HIF-1α siRNA. YFP allowed identification of cells that were transfected with HIF-1α siRNA for analysis (Fig. S6*A*). Cells transfected with siHIF-1α, but not with scrambled siRNA, manifested decreased $[Ca^{2+}]_i$ and calpain activity, and these effects were reversed by correcting the redox state by forced expression of Nox2 (Fig. 6*C*). Treating siHIF-1α–transfected cells with ionomycin or thapsigargin to increase $[Ca^{2+}]_i$ levels led to increased calpain activity and restored HIF-2α to control levels (Fig. 6*D*). Conversely, HIF-1α–overexpressing cells exhibited increased $[Ca^{2+}]_i$ and calpain activity, and decreased HIF-2α levels, whereas N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), a calpain inhibitor, restored HIF-2α levels in HIF-1α–overexpressing PC12 cells (Fig. S6 *B* and *C*). Thus, loss of Nox2 expression in HIF-1α–deficient cells impairs the ROS- and $Ca^{2+}$-dependent negative regulation of HIF-2α (Fig. 6*E*).

## Discussion

Our results have delineated a hitherto uncharacterized functional antagonism between HIF-1α and HIF-2α, which regulates hypoxic sensing and is essential for cardiovascular and respiratory

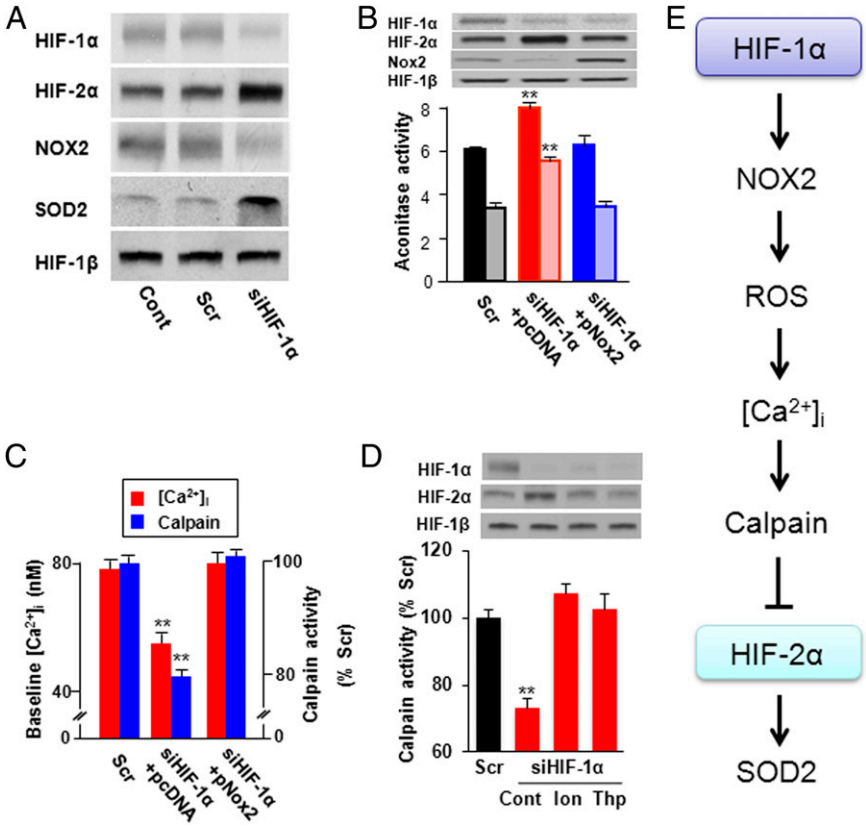


**Fig. 6.** Analysis of $Ca^{2+}$-dependent calpain activity in HIF-1α-deficient PC12 cells. (*A*) Immunoblot assays were performed to analyze the expression of HIF-α isoforms, Nox2, Sod2, and HIF-1β in control cells and cells transfected with either scrambled siRNA (Scr) or HIF-1α siRNA (siHIF-1α). (*B*) Cells were transfected with Scr or siHIF-2α and either empty vector (pcDNA) or Nox2 expression vector (pNox2), followed by immunoblot assays (*Upper*) and measurements of aconitase activity (*Lower*) in cytosolic (solid bars) and mitochondrial (shaded bars) fractions. (*C*) $[Ca^{2+}]_i$ (red bars) and calpain activity (blue bars) were determined in cells transfected with Scr or siHIF-2α and either empty vector (pcDNA) or Nox2 vector (pNox2). $[Ca^{2+}]_i$ data (mean ± SEM) were obtained from 122 cells. (*D*) HIF-α isoform expression (*Upper*) and calpain activity (*Lower*) were analyzed in PC12 cells transfected with Scr or transfected with siHIF-2α and left untreated (Cont) or treated with either 3 μM ionomycin (Ion) or 1.5 μM thapsigargin (Thp). Data in bar graphs are presented as mean ± SEM, $n$ = 5–6 independent experiments; $^{**}P < 0.01$. (*E*) The mechanisms and consequences of increased HIF-2α levels in HIF-1α-deficient cells are shown.

homeostasis. CB responses to hypoxia were impaired in *Hif1α*$^{+/-}$ mice and were augmented in *Hif2α*$^{+/-}$ mice, a finding that is consistent with previous reports (15, 16). We further demonstrate that partial deficiency of either HIF-1α or HIF-2α also alters hypoxic sensing by AM chromaffin cells. Further investigation is required to determine whether HIF-α subunits contribute to the responses of other paraganglionic cells that are sensitive to hypoxia (26). Altered hypoxic sensing by the CB and AM was associated with disrupted cardio-respiratory homeostasis manifested by impaired ventilatory responses to chronic hypoxia in *Hif1α*$^{+/-}$ mice, and by hypertension and respiratory arrhythmias in *Hif2α*$^{+/-}$ mice. HIF-1α is ubiquitously expressed in cells of all metazoan species, whereas HIF-2α is expressed only in certain cell types of vertebrate species (9). The finding that partial HIF-2α deficiency disrupts cardio-respiratory homeostasis in adult mice under normoxic conditions provides a physiological basis for the co-evolution of HIF-2α with the complex circulatory and respiratory control systems of vertebrate organisms.

A major finding of the present study is that the systemic effects of partial loss of either HIF-1α or HIF-2α were associated with increased expression of the other isoform in the CB and AM. Reducing HIF-1α levels in *Hif2α*$^{+/-}$ mice by digoxin treatment, and HIF-2α levels in *Hif1α*$^{+/-}$ mice by 2ME2 treatment, normalized CB and AM responses to hypoxia and restored cardio-respiratory homeostasis. Remarkably, treatment of WT mice with digoxin selectively inhibited HIF-1α and led to increased HIF-2α levels, whereas 2ME2 treatment selectively inhibited HIF-2α and led to increased HIF-1α protein levels, with profound effects on the CB response to hypoxia and cardio-respiratory function, recapitulating the phenotype seen in *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice, respectively. Furthermore, double-heterozygous mice, which were equally deficient in HIF-1α and HIF-2α, exhibited hypoxic responses and cardio-respiratory function that were indistinguishable from WT littermates. Taken together, these findings demonstrate the existence of functional antagonism between HIF-1α and HIF-2α, and indicate that a fine balance between HIF-α subunits, rather than their absolute levels, is critical for appropriate $O_2$ sensing by the CB and AM and for proper regulation of cardio-respiratory function.

Although HIF-1 and HIF-2 regulate the same genes in many tissues (27), recent studies revealed that in the CB and AM they activate expression of gene products with opposing functions that regulate the redox state (11, 17, 18). Our results demonstrated that the balance between HIF-α subunits profoundly affects the intracellular redox state in the CB and AM. *Hif2α*$^{+/-}$ mice exhibited an oxidized, whereas *Hif1α*$^{+/-}$ mice displayed a reduced, intracellular redox state. HIF-1α–dependent expression of the pro-oxidant enzyme Nox2 contributed to an oxidized redox state in *Hif-2α*$^{+/-}$ mice, whereas HIF-2α–dependent expression of the antioxidant enzyme Sod2 contributed to the reduced redox state in *Hif1α*$^{+/-}$ mice. Remarkably, correcting the redox state alone was sufficient to normalize the CB and AM responses to hypoxia in *Hif1α*$^{+/-}$ and in *Hif2α*$^{+/-}$ mice. Thus, complex feed-forward and feed-back relationships between ROS, HIF-α isoforms, and redox regulatory enzymes establish the redox state, which in turn determines the set point for $O_2$ sensing in the CB and AM (Fig. 7). A variety of signaling mechanisms including $K^+$ channels, $Ca^{2+}$ signaling, mitochondria, biogenic amines, and gas messengers have been implicated in responses of the CB and AM to hypoxia (reviewed in refs. 19 and 28). Whether the HIF-dependent redox regulation described in this study affects these signaling mechanisms is a question that requires further study.

Our results demonstrate that complex redox-dependent mechanisms contribute to the reciprocal regulation of HIF-α isoforms. ROS-dependent activation of mTOR signaling and inhibition of prolyl hydroxylation, which have been shown to increase HIF-1α protein synthesis (24) and stability (25), respectively, contributed

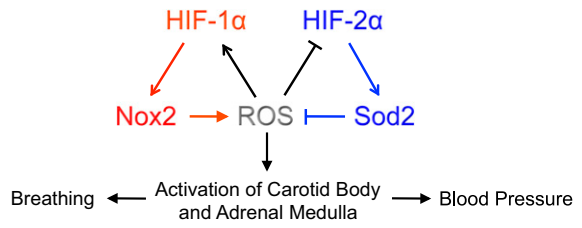


**Fig. 7.** Mutual antagonism between HIF-1α and HIF-2α controls redox status in the CB and AM and regulates breathing and blood pressure. Note the reciprocal positive relationship between HIF-1α and ROS and reciprocal negative relationship between HIF-2α and ROS.

to the increased HIF-1α levels in HIF-2α–deficient cells. On the other hand, loss of ROS-dependent $Ca^{2+}$-activated calpain activity contributed to the increased HIF-2α levels in HIF-1α–deficient cells. Although for technical reasons these studies used PC12 cell cultures, similar mechanisms are likely operative in the CB and AM of *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice. Further studies are needed to assess the mechanisms by which HIF-1α deficiency leads to decreased $[Ca^{2+}]_i$ and to identify the mechanisms by which calpains degrade HIF-2α protein. Nonetheless, these findings demonstrate that partial loss of either HIF-1α or HIF-2α increased the expression of the other HIF-α isoform, resulting in a disruption of redox homeostasis that was both caused by, and required for, the imbalance in HIF-α subunits.

Our data indicate that mutual antagonism between HIF-1α and HIF-2α establishes the set point for $O_2$ sensing in the CB and AM, which in turn is important for maintaining cardio-respiratory homeostasis. Emerging evidence suggests that hypersensitivity of the CB and AM to hypoxia has an adverse impact on physiological systems. For example, patients with obstructive sleep apnea exhibit an exaggerated CB chemosensory reflex, increased plasma catecholamine levels, hypertension, and breathing abnormalities (reviewed in ref. 29). Simulating recurrent apnea by exposing rodents to intermittent hypoxia produces an imbalance in HIF-α isoforms manifested by increased HIF-1α (30) and decreased HIF-2α (18) protein levels in the CB and AM. Intermittent hypoxia-exposed rodents exhibit oxidative stress, increased Nox2 and decreased Sod2 levels, augmented hypoxic responses by the CB and AM, hypertension, and breathing abnormalities (30–32), which recapitulate the phenotype of *Hif-2α*$^{+/-}$ mice. Remarkably, either blockade of changes in HIF-α isoforms or normalization of the intracellular redox state by antioxidants reverses the effects of intermittent hypoxia in rodents (18, 29–33). Thus, correcting the changes in intracellular redox state by selective modulation of either HIF-1α or HIF-2α expression may represent a unique therapeutic approach for the treatment of cardio-respiratory abnormalities caused by sleep disordered breathing with apnea. Additional studies are required to investigate whether imbalanced expression of HIF-α isoforms and altered redox state in the CB, AM, or other neuroendocrine cells are involved in other cardio-respiratory diseases. Finally, the development of HIF-2α–selective inhibitors for cancer therapy has been proposed (34), but our present results suggest that this strategy might lead to an imbalance between HIF-1α and HIF-2α in the CB and AM, resulting in hypertension and respiratory abnormalities.

## Materials and Methods

**Mice.** Experiments were approved by the University of Chicago Institutional Animal Care and Use Committee and performed by individuals blinded to genotype using male, age-matched *Hif1α*$^{+/-}$ and *Hif2α*$^{+/-}$ mice and their WT littermates (10, 11).

**Measurements of Breathing and Blood Pressure.** Ventilation and $O_2$ consumption ($V_{O2}$) were monitored by whole-body plethysmography, and blood

pressure was measured by tail cuff, in conscious mice (16, 30). *Hif2α*$^{+/-}$ mice were anesthetized (70 mg/kg ketamine and 7 mg/kg xylazine, intraperitoneally), the carotid sinus nerves were transected bilaterally, and mice were allowed to recover from surgery for 1 wk. Breathing and blood pressure were determined before/after sectioning of sinus nerves. Tidal volume ($V_T$) and minute ventilation ($V_E$) were normalized to body weight. Breathing variability was analyzed by Poincarè plots. Breath-to-breath intervals were analyzed as previously described (16).

**Exposure to Hypobaric Hypoxia.** The protocols for exposing conscious mice to hypobaric hypoxia were described previously (15). Briefly, after baseline measurements of breathing, mice were placed in a hypobaric chamber (0.4 atmospheres) for 72 h, and then respiratory responses to acute hypoxia were determined in conscious mice by whole-body plethysmography. Subsequently, mice were anesthetized, CBs were harvested and CB responses to hypoxia were determined in vitro as described below.

**CB Sensory Activity.** Sensory activity from the CB was recorded ex vivo as described previously (16, 30). Clearly identifiable action potentials (two to three active units) were recorded from one of the nerve bundles of the sinus nerve with a suction electrode. "Single" units were selected based on the height and duration of the individual action potentials using a spike discrimination program (Spike Histogram Program, Power Laboratory; AD Instruments). The $PO_2$ and $PCO_2$ of the superfusion medium were determined by a blood gas analyzer (ABL 5, Radiometer). CB sensory activity from "single" units was averaged during the 3 min of baseline and during the 3 min of gas challenge and expressed as impulses per second.

**Measurements of Plasma Norepinephrine Levels.** Blood was collected from anesthetized mice by cardiac puncture and plasma norepinephrine was determined by HPLC combined with electrochemical detection using dihydroxybenzylamine as an internal standard (16, 30).

**Measurements of Catecholamine Secretion from AM Chromaffin Cells.** AM chromaffin cells were plated on coverslips coated with collagen (Sigma) in F-12K medium with 1% (vol/vol) FBS, insulin-transferrin-selenium, and penicillin/streptomycin/glutamine under 7% (vol/vol) $CO_2$/20% $O_2$ for 24 h at 37 °C. Catecholamine secretion from individual AM chromaffin cells was monitored by carbon fiber amperometry as previously described (21). The number of secretory events and the amount of catecholamine secreted per event were analyzed and the data were expressed as total catecholamine molecules secreted.

**Fura-2 Imaging.** $[Ca^{2+}]_i$ was monitored in PC12 cells using Fura-2 AM. Cells that were transfected with HIF-1α vector or HIF-1α siRNA were identified by cotransfection with a vector encoding YFP. Background fluorescence was subtracted from signals. Image intensity at 340 nm was divided by 380-nm image intensity to obtain the ratiometric image. Ratios were converted to free $[Ca^{2+}]_i$ using calibration curves constructed in vitro by adding Fura-2 (50 μM, free acid) to solutions containing known concentrations of $Ca^{2+}$ (0–2,000 nM). The recording chamber was continually superfused with solution from gravity-fed reservoirs.

**Reverse-Transcriptase Quantitative PCR.** HIF-1α, HIF-2α, Nox2, and Sod2 mRNA levels were analyzed by quantitative real-time RT-PCR using SYBR Green (18) normalized to the 18S rRNA signal. The following primers were used: HIF-1α (rat/mouse): 5′-CCA CAG GAC AGT ACA GGA TG-3′ and 5′-TCA AGT CGT GCT GAA TAA TAC C-3′. HIF-2α (mouse): 5′-TAC GAA GTG GTC TGT GGG CAA TCA-3′ and 5′-TCA GCT TGT TGG ACA GGG CTA TCA-3′; Nox2 (rat): 5′-GTG GAG TGG TGT GTG AAT GC-3′ and 5′-TTT GGT GGA GGA TGT GAT GA-3′; Nox2 (mouse): 5′-AGC TAT GAG GTG GTG ATG TTA GTG G-3′ and 5′-CAC AAT ATT TGT ACC AGA CAG ACT TGA G-3′; Sod2 (rat/mouse): 5′-GGC CAA GGG AGA TGT TAC AGC-3′ and 5′-GGC CTG TGG TTC CTT GCA G-3′; 18S rRNA (rat/mouse): 5′-CGC CGC TAG AGG TGA AAT TC-3′ and 5′-CGA ACC TCC GAC TTT CGT TCT-3′. HIF-1α, HIF-2α, Nox2, Sod2, and scrambled siRNAs were obtained from Santa Cruz.

**Cell Culture.** PC12 cells were cultured in DMEM supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL), under 90% air and 10% $CO_2$ at 37 °C. Before all experiments, the cells were placed in antibiotic free medium and serum starved for 16 h to avoid confounding effects of serum on HIF-1α or HIF-2α protein expression. Cells were pretreated for 30 min with either drug or vehicle. PC12 cells were transfected with DNA using Lipofectamine (Gibco).

**Immunoblot Assays.** Immunoblot assays of HIF-1α, HIF-2α, HIF-1β, Nox2, and Sod2 in the AM and PC12 lysates were performed as described (17, 18, 24). The following primary antibodies were used: HIF-1α, HIF-2α, HIF-1β, and prolyl-hydroxylated HIF-1α (Novus Biologicals); Nox2 (Santa Cruz); Sod2 (Millipore); phosphorylated and total mTOR (Cell Signaling); and α-tubulin (Sigma).

**Enzyme Assays.** Aconitase, Nox2, and calpain activities were determined as described (17, 18). Sod2 activity was measured using an assay kit (Dojondo Molecular Technologies). Protein levels were determined by Bradford assay kit (Bio-Rad).

**Data Analysis and Statistics.** Depending on the experiment, data were analyzed with either one-way ANOVA or two-way ANOVA with repeated measures followed by Tukey's test. Values are given as means ± SEM and $P$ values of $< 0.05$ were considered significant. Unless otherwise stated, $n$ refers to independent experiments and individual biochemical assays were performed in triplicate.

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1. Lane N, Martin W (2010) The energetics of genome complexity. *Nature* 467(7318): 929–934.
2. Heymans J, Heymans C (1927) Sur les modifications directes et sur la regulation reflexe de l'activitie du centre respiratoire de la tete isolee du chien. *Arch Int Pharmacodyn Ther* 33:273–372.
3. Comline RS, Silver M (1961) The release of adrenaline and noradrenaline from the adrenal glands of the foetal sheep. *J Physiol* 156:424–444.
4. Seidler FJ, Slotkin TA (1986) Ontogeny of adrenomedullary responses to hypoxia and hypoglycemia: Role of splanchnic innervation. *Brain Res Bull* 16(1):11–14.
5. Jones CT, Roebuck MM, Walker DW, Lagercrantz H, Johnston BM (1987) Cardiovascular, metabolic and endocrine effects of chemical sympathectomy and of adrenal demedullation in fetal sheep. *J Dev Physiol* 9(4):347–367.
6. Thompson RJ, Jackson A, Nurse CA (1997) Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *J Physiol* 498(Pt 2):503–510.
7. García-Fernández M, Mejías R, López-Barneo J (2007) Developmental changes of chromaffin cell secretory response to hypoxia studied in thin adrenal slices. *Pflugers Arch* 454(1):93–100.
8. Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. *Cell* 148(3): 399–408.
9. Loenarz C, et al. (2011) The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Rep* 12(1):63–70.
10. Iyer NV, et al. (1998) Cellular and developmental control of $O_2$ homeostasis by hypoxia-inducible factor 1 α. *Genes Dev* 12(2):149–162.
11. Scortegagna M, et al. (2003) Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1$^{-/-}$ mice. *Nat Genet* 35(4): 331–340.
12. Roux JC, Brismar H, Aperia A, Lagercrantz H (2005) Developmental changes in HIF transcription factor in carotid body: Relevance for $O_2$ sensing by chemoreceptors. *Pediatr Res* 58(1):53–57.
13. Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL (1998) The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev* 12(21): 3320–3324.
14. Tian H, McKnight SL, Russell DW (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 11(1):72–82.
15. Kline DD, Peng YJ, Manalo DJ, Semenza GL, Prabhakar NR (2002) Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 α. *Proc Natl Acad Sci USA* 99(2):821–826.
16. Peng YJ, et al. (2011) Hypoxia-inducible factor 2α (HIF-2α) heterozygous-null mice exhibit exaggerated carotid body sensitivity to hypoxia, breathing instability, and hypertension. *Proc Natl Acad Sci USA* 108(7):3065–3070.
17. Yuan G, et al. (2011) Hypoxia-inducible factor 1 mediates increased expression of NADPH oxidase-2 in response to intermittent hypoxia. *J Cell Physiol* 226(11):2925–2933.
18. Nanduri J, et al. (2009) Intermittent hypoxia degrades HIF-2α via calpains resulting in oxidative stress: Implications for recurrent apnea-induced morbidities. *Proc Natl Acad Sci USA* 106(4):1199–1204.
19. Kumar P, Prabhakar NR (2012) Peripheral chemoreceptors: Function and plasticity of the carotid body. *Compr Physiol* 2(1):141–219.
20. Zhang H, et al. (2008) Digoxin and other cardiac glycosides inhibit HIF-1α synthesis and block tumor growth. *Proc Natl Acad Sci USA* 105(50):19579–19586.

21. Souvannakitti D, et al. (2010) NADPH oxidase-dependent regulation of T-type $Ca^{2+}$ channels and ryanodine receptors mediate the augmented exocytosis of catecholamines from intermittent hypoxia-treated neonatal rat chromaffin cells. *J Neurosci* 30(32): 10763–10772.
22. Gardner PR (2002) Aconitase: Sensitive target and measure of superoxide. *Methods Enzymol* 349:9–23.
23. Kumar GK, et al. (1998) Release of dopamine and norepinephrine by hypoxia from PC-12 cells. *Am J Physiol* 274(6 Pt 1):C1592–C1600.
24. Yuan G, Nanduri J, Khan S, Semenza GL, Prabhakar NR (2008) Induction of HIF-1α expression by intermittent hypoxia: Involvement of NADPH oxidase, $Ca^{2+}$ signaling, prolyl hydroxylases, and mTOR. *J Cell Physiol* 217(3):674–685.
25. Kaelin WG, Jr., Ratcliffe PJ (2008) Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Mol Cell* 30(4):393–402.
26. Fried G, Wikström M, Lagercrantz H (1988) Postnatal development of catecholamines and response to hypoxia in adrenals and paraganglia of rabbits. *J Auton Nerv Syst* 24(1-2):65–70.
27. Prabhakar NR, Semenza GL (2012) Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiol Rev* 92(3):967–1003.
28. Prabhakar NR, Semenza GL (2012) Gaseous messengers in oxygen sensing. *J Mol Med (Berl)* 90(3):265–272.
29. Prabhakar NR, Kumar GK, Nanduri J, Semenza GL (2007) ROS signaling in systemic and cellular responses to chronic intermittent hypoxia. *Antioxid Redox Signal* 9(9):1397–1403.
30. Peng YJ, et al. (2006) Heterozygous HIF-1α deficiency impairs carotid body-mediated systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia. *J Physiol* 577(Pt 2):705–716.
31. Kumar GK, et al. (2006) Chronic intermittent hypoxia induces hypoxia-evoked catecholamine efflux in adult rat adrenal medulla via oxidative stress. *J Physiol* 575(Pt 1):229–239.
32. Kuri BA, Khan SA, Chan SA, Prabhakar NR, Smith CB (2007) Increased secretory capacity of mouse adrenal chromaffin cells by chronic intermittent hypoxia: involvement of protein kinase C. *J Physiol* 584(Pt 1):313–319.
33. Zhan G, et al. (2005) NADPH oxidase mediates hypersomnolence and brain oxidative injury in a murine model of sleep apnea. *Am J Respir Crit Care Med* 172(7): 921–929.
34. Keith B, Johnson RS, Simon MC (2012) HIF-1α and HIF-2α: Sibling rivalry in hypoxic tumor growth and progression. *Nat Rev Cancer* 12(1):9–22.