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 Hif1α−/− 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.

Vertebrate organisms have evolved complex respiratory and cardiovascular systems that are designed to ensure optimal O2 delivery to each cell. O2 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 O2 availability also affect cellular redox homeostasis. Thus, O2, energy, and redox homeostasis are inextricably linked to cardio-respiratory function. The ability of mammals to maintain homeostasis in response to changes in O2 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 O2 levels and stimulates breathing in response to hypoxemia, thereby ensuring adequate O2 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 O2 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 O2-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),
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 O2 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 O2 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 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 hypervactive 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 PO2, 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
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. 2E; quantified in Fig. S2F), as well as the hypoxic ventilatory response (Fig. 2F), 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. S3A), an impaired CB response to hypoxia (Fig. S3B and C), and decreased plasma catecholamine levels (Fig. S3D), 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. S4A), an augmented CB response to hypoxia, irregular breathing, an increased incidence of posthypoxic 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 Cardiorespiratory 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. 3A). 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 cardiorespiratory 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 (PO2 ~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 Vt to Vt0, 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). In contrast, digoxin treatment had no effect on the decreased Sod2 mRNA levels and enzyme activity caused by partial HIF-2α deficiency (Fig. 4B). Untreated Hif2α+/− mice exhibited oxidative stress, as evidenced by significantly decreased cytosolic and mitochondrial aconitase activities, which were corrected by digoxin treatment (Fig. 4C). WT mice treated with digoxin exhibited increased Sod2 and decreased Nox2 levels in the AM compared with vehicle-treated controls (Fig. S4K).

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 Hif1α+/− 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.

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Analysis of the AM in Hif1α+/− mice revealed decreased Nox2 mRNA levels and enzyme activity (Fig. 4D), and increased Sod2 mRNA levels and enzyme activity (Fig. 4E). Cytosolic and mitochondrial aconitase activities were significantly increased in the AM from Hif1α+/− mice compared with WT littermates (Fig. 4F). 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. 2B), corrected Sod2 expression/activity (Fig. 4E) and normalized aconitase activity in cytosolic and mitochondrial fractions of the AM (Fig. 4F). However, 2ME2 had no effect on Nox2 mRNA levels or enzyme activity (Fig. 4D). Treatment of WT mice with 2ME2 resulted in increased Nox2 and decreased Sod2 protein levels in the AM (Fig. S4K).

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 non-targeting 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. 5A and B), recapitulating the phenotype seen in the AM of Hif2α+/− mice. Forced expression of Sod2 in siHIF-2α–transfected cells corrected the oxidative stress and blocked the increase in HIF-1α protein levels (Fig. 5B), 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. 5C). Treatment of siHIF-2α–trans-
fected cells with rapamycin, a selective mTOR inhibitor, blocked mTOR phosphorylation and reduced HIF-1α protein levels by ~60% (Fig. 5C), 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. 5D). 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. 5E).

Altered Ca²⁺ 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. 6B).

We previously reported that in PC12 cells exposed to intermittent hypoxia, ROS-dependent elevation of [Ca²⁺], leads to the degradation of HIF-2α by calpains, which are Ca²⁺-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, [Ca²⁺], 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. S6A). Cells transfected with siHIF-1α, but not with scrambled siRNA, manifested decreased [Ca²⁺], and calpain activity, and these effects were reversed by correcting the redox state by forced expression of Nox2 (Fig. 6C). Treating siHIF-1α–transfected cells with ionomycin or thapsigargin to increase [Ca²⁺], levels led to increased calpain activity and restored HIF-2α to control levels (Fig. 6D). Conversely, HIF-1α–overexpressing cells exhibited increased [Ca²⁺], 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 Ca²⁺-dependent negative regulation of HIF-2α (Fig. 6E).

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 Ca²⁺-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 (pDNA) 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) [Ca²⁺] (red bars) and calpain activity (blue bars) were determined in cells transfected with Scr or siHIF-2α and either empty vector (pDNA) or Nox2 vector (pNox2). [Ca²⁺], 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 hypoxia 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 hypoxia 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 arhythmias 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 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 Hif1α+/− 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 O2 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 act in opposition. 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 Hif2α−/+ 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 O2 sensing in the CB and AM (Fig. 7). A variety of signaling mechanisms including K+ channels, Ca2+ 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 to the increased HIF-1α levels in HIF-2α-deficient cells. On the other hand, loss of ROS-dependent Ca2+−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 [Ca2+]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 O2 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 Hif2α−/+ 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 O2 consumption (V(O2)) were monitored by whole-body plethysmography, and blood
pressure was measured by tail cuff, in conscious mice (16, 30). HiF-α+/− 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 and after sectioning of sinus nerves. Tidal volume (VT) and minute ventilation (Ve) were normalized to body weight. Breathing variability was analyzed by Poincaré plots. Breath-to-breath intervals were determined during the 3 min of baseline and during the 3 min of gas challenge and expressed as impulses per second.

**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 PO2 and PCO2 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 95% air and 5% CO2 at 37 °C. The recording chamber was continually superfused with solution from gravity-fed reservoirs.


Fig. S1. Quantitative analysis of hypoxia-inducible factor-2 (Hif2α)−/− mice. The following experimental groups were studied: WT mice (Hif2α+/+), Hif2α−/− mice, and Hif2α+/− mice treated with digoxin (Dig). (A and B) HIF-1α mRNA expression in the carotid body (CB) and adrenal medulla (AM) was analyzed. (C) CB response to hypoxia [hypoxia − baseline = Δ impulses/sec (Δ imp/s)]; n = 12–15 fibers from each group. (D) Catecholamine (CA) secretion from AM chromaffin cells presented as total CA molecules secreted; n = 18–22 cells in each group. (E and F) Quantitative analysis of breathing was performed. Mean SD 1 (SD1; datapoints from the ascending 45° line; E) and mean SD2 (data points from the line orthogonal to SD1; F) derived from Poincaré plot analysis of breath-to-breath intervals shown in Fig. 1H. (G) Apnea index for data derived from Fig. 1I. (H) Breathing pattern in Hif-2α−/− mice with intact CB and after CB denervation (CBD). (I–L) Quantitative analysis of breathing was performed using Poincaré plots (I and J) to derive SDs (K and L). Data in bar graphs represent mean ± SEM, 7–8 mice per group; *P < 0.05, **P < 0.01.
Fig. S2. Quantitative analysis of *Hif1α*<sup>−/−</sup> mice. The following experimental groups were studied: WT mice (*Hif1α*<sup>+/+</sup>), *Hif1α*<sup>−/−</sup> mice, and *Hif1α*<sup>−/−</sup> mice treated with 2-methoxyestradiol (2ME2). (A and B) HIF-2α mRNA expression in the CB (A) and AM (B) was analyzed by reverse-transcription quantitative real-time PCR. (C) Representative immunoblot (from four independent experiments) showing HIF-2α protein levels in PC12 cells treated with 2ME2 (10 μM) compared with vehicle-treated control cells (Con). (D) CB response to hypoxia (*n* = 11–16 fibers per group). (E) CA secretion from AM chromaffin cells (*n* = 18–22 cells in each group). (F) CB responses to hypoxia in mice exposed to normoxia (N) or chronic hypoxia (CH); *n* = 15–18 fibers per group. Data in bar graphs represent mean ± SEM from 6 to 8 mice in each group; **P < 0.01.
Fig. S3. Effect of digoxin in WT mice. (A) Representative immunoblots (Upper) and densitometric analysis (Lower) of HIF-1α, HIF-2α, and HIF-1β levels in the AM of digoxin-treated WT mice relative to untreated WT mice are shown. (B) CB responses to hypoxia (PO$_2$ ∼40 mmHg for 3 min; black bar under tracing) are shown. Integrated sensory activity is presented as impulses per second [CB activity (imp/s)]. (C) CB responses to hypoxia (n = 11–14 fibers in each group). (D and E) Plasma norepinephrine levels (NE; D) and mean blood pressure (MBP; E) are shown. (F–H) Analysis of breathing, including breathing pattern (F), Poincaré plots of breath-to-breath interval (G), and derived SDs (H). Data in bar graphs are presented as mean ± SEM, n = 6 mice per group; **P < 0.01.
Fig. S4. Effect of 2ME2 in WT mice. (A) Representative immunoblots (Upper) and densitometric analysis (Lower) of HIF-1α, HIF-2α, and HIF-1β levels in the AM of 2ME2-treated WT mice relative to untreated WT mice are shown. (B and C) Analysis of CB responses to hypoxia, including representative tracings (B) and mean data (C; n = 14–17 fibers per group). (D–H) Analysis of breathing, including breathing pattern (D), Poincaré plots (E), and derived SDs, representative postsigh apneas (G), and apnea index (H). (I and J) Cardiovascular analysis, including MBP (I) and plasma NE levels (J). (K) Representative immunoblots (Upper) and densitometric analysis (Lower) of NADPH oxidase 2 (Nox2), superoxide dismutase 2 (Sod2), and tubulin protein levels in WT mice and WT mice treated with digoxin (Dig) or 2ME2. Data in bar graphs are presented as mean ± SEM, n = 6 mice per group; *P < 0.05, **P < 0.01.
Fig. 55. Quantitative analysis of double-heterozygous mice. Two experimental groups were studied: WT mice (Hif1α+/−;Hif2α+/−); and double-heterozygous (DH) mice (Hif1α+/−;Hif2α−/−). (A) CB sensory response to hypoxia (n = 10–14 fibers in each group). (B) CA secretion from AM chromaffin cells (n = 20–24 cells per group). (C and D) SDs derived from Poincaré plots. (E) Apnea index. (F) CB responses to hypoxia in mice exposed to normoxia (N) or chronic hypoxia (CH); n = 10–12 fibers per group. (G–I) Nox2 and Sod2 mRNA levels and enzyme activities (G and H) and cytosolic (black bars) and mitochondrial (green bars) aconitase activities (I) in AM from WT and DH mice. Data in bar graphs represent mean ± SEM; n = 6–7 mice in each group; **P < 0.01.

Fig. 56. Analysis of changes in [Ca2+]i and calpain activity with forced expression of HIF-1α in PC12 rat pheochromocytoma cells. (A) PC12 cells were transfected with an expression vector encoding yellow fluorescent protein (YFP, Upper) and either empty vector (pCEP4) or HIF-1α expression vector (pHIF-1α). [Ca2+]i was measured by Fura-2 imaging (Middle) and YFP and Fura-2 images were merged (Lower). (B) [Ca2+]i was measured in control (nontransfected) cells or cells transfected with either empty vector (pCEP4) or HIF-1α vector (pHIF-1α); n = 96–115 cells per group. (C) Calpain activity and HIF-α levels were determined in control cells and cells transfected with either empty vector (pCEP4) or HIF-1α vector (pHIF-1α) in the absence or presence of calpain inhibitor (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal; 10 μM). Data in bar graphs is presented as mean ± SEM, n = 4 independent experiments; **P < 0.01.