Advanced Review

Systems Biology of Oxygen Homeostasis

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ABSTRACT

Metazoan species maintain oxygen homeostasis through the activity of hypoxia-inducible factors (HIFs), which are transcriptional activators that match O_2 supply and demand. Here, we review the involvement of HIFs in the molecular physiology and pathophysiology of cellular O_2 sensing, O_2 delivery, O_2 utilization, and systemic O_2 sensing.

INTRODUCTION

A constant supply of O_2 must be delivered to all cells for their continued survival. O_2 is utilized as the final electron acceptor in the process of oxidative phosphorylation. The function of the respiratory chain is optimized for physiological PO_2 levels and sustained deviations from normoxia cause increased production of reactive oxygen species (ROS) by the electron transport chain (ETC). ROS cause oxidation of lipids, proteins, and nucleic acids that can result in cellular dysfunction or death. Thus, homeostatic mechanisms exist to tightly regulate O_2 levels within cells and tissues.

Hypoxia-inducible factors (HIFs) function as master regulators of oxygen homeostasis, which is critical for the survival of all metazoan species (Semenza, 2010). HIFs are heterodimeric proteins, consisting of an O_2-regulated HIF-1α, HIF-2α, or HIF-3α subunit and a constitutively expressed HIF-1β subunit. Depending on the context, HIF-1 and HIF-2 activate transcription of target genes that mediate adaptive or maladaptive responses at the systemic level to regulate O_2 delivery and at the cellular level to regulate O_2 utilization (Prabhakar and Semenza, 2012). The role of HIF-3 is much less well studied (Duan, 2016). Both cellular and systemic O_2 sensing mechanisms are employed to ensure a proper balance between O_2 supply and demand; dysregulation of these mechanisms results in disease pathology. In this four-part review article, we will describe the involvement of HIFs in the molecular physiology and pathophysiology of cellular O_2 sensing, O_2 delivery, O_2 utilization, and systemic O_2 sensing.

CELLULAR O_2 SENSING

The activity of HIFs is regulated by O_2-dependent hydroxylation of the HIF-α subunits (Figure 1). Hydroxylation of two proline residues (Pro-402 and Pro-564 in human HIF-1α) is mediated by prolyl hydroxylase domain (PHD) proteins, principally PHD2 (Berra et al., 2003; Kaelin and Ratcliffe, 2008). Prolyl hydroxylation is required for binding of the von Hippel-Lindau (VHL) protein, which recruits an ubiquitin-protein ligase complex (consisting of Elongin B, Elongin C, Cullin 2, RBX2, and an E2 ligase) that ubiquitinates HIF-α proteins and thereby targets them for proteasomal destruction (Maxwell et al., 1999; Ivan et al., 2002). The PHDs are dioxygenases that utilize O_2 and α-ketoglutarate as substrates and contain Fe(II) in their catalytic centers, such that PHD activity may be inhibited by any of the following conditions: reduced availability of O_2 or α-ketoglutarate; oxidation of Fe(II) to Fe(III); iron chelation; or administration of an α-ketoglutarate analog (Kaelin and Ratcliffe, 2008). HIF-α subunits begin to accumulate as soon as cells are subjected to hypoxia, iron chelators, or α-ketoglutarate analogs, with HIF-1α levels peaking after 4 to 6 hours in many cell lines. Peak expression of many HIF target gene mRNAs occurs after 24 hours of continuous hypoxia. Even under conditions of mild hypoxia, increased production of ROS by the mitochondrial ETC may contribute to the inhibition of PHD activity (Waypa et al., 2016).

In addition to prolyl hydroxylation, the HIF-1α and HIF-2α subunits are subject to hydroxylation of an asparagine residue in the transactivation domain (Asn-803 in human HIF-1α), which blocks interaction with the coactivator proteins CBP and P300 (Lando et al., 2002). Asparagine hydroxylation and repression of HIF transcriptional activity is mediated by factor inhibiting HIF-1 (FIH-1), which is also an O_2- and α-ketoglutarate-dependent dioxygenase with biochemical properties that are similar to those described above for the

PHDs, although FIH-1 activity may be inhibited at higher O_2 levels than those required for inhibition of PHD activity (Mahon et al., 2001; Lando et al., 2002; Dayan et al., 2006).

O_2 DELIVERY

Erythropoiesis

A primary means of increasing O_2 delivery systemically is to increase erythropoiesis, as red blood cells are responsible for transport of O_2, bound to hemoglobin, from the lungs to all body tissues. The synthesis of hemoglobin in erythroid progenitor cells requires iron. Iron uptake/delivery and red blood cell production are dependent on the HIF-mediated expression of the following genes: *EPO*, which encodes erythropoietin, the hormone that stimulates the survival, proliferation, and differentiation of erythroid progenitor cells (Semenza and Wang, 1992); *EPOR*, which encodes the EPO receptor on erythroid progenitor cells (Yoon et al., 2006); *CYBRD1*, which encodes the duodenal cytochrome b (DCYTB; also known as ferric reductase) that reduces Fe(III) to Fe(II) for intestinal uptake (Shah et al., 2009); *SLC11A2*, which encodes the apical divalent metal transporter (DMT1; also known as NRAMP2) that allows uptake of Fe(II) from the intestinal lumen into enterocytes (Mastrogiannaki et al., 2009; Shah et al., 2009); *SLC40A1*, which encodes ferroportin (FPN1), the basolateral protein that transports iron out of enterocytes and into blood vessels for binding to transferrin and delivery to the bone marrow (Taylor et al., 2011); *TF*, which encodes transferrin, the protein that transports iron from the intestine to the bone marrow (Rolfs et al., 1997); *TFRC*, which encodes the transferrin receptor on erythroid cells that takes up iron from transferrin (Lok and Ponka, 1999; Tacchini et al., 1999); and FECH, which encodes ferrochelatase, the enzyme that catalyzes the insertion of

iron into protoporphyrin to form heme (Liu et al., 2004). Thus, HIFs act as master regulators that coordinately activate the transcription of multiple genes required for a single physiological response; in this case, HIF-2 regulates at least 8 genes encoding proteins that mediate erythropoiesis (Figure 2).

Congenital Polycythemia: A Hereditable Disorder of Oxygen Sensing

Hereditary erythrocytosis or congenital polycythemia is a hereditable disorder of erythropoiesis that is characterized by excess red blood cell production. The increase in hematocrit can lead to occlusion of cerebral vessels and stroke-like symptoms. Remarkably, mutations in *VHL*, *EGLN1*, and *EPAS1* genes, which encode VHL, PHD2, and HIF-2α, respectively, have been identified in patients with this disorder. Investigation of a form of congenital polycythemia that was endemic to a region of Russia known as Chuvashia revealed that affected individuals were homozygous for a *VHL* missense mutation in which Arg was replaced by Trp at codon 200 (Ang et al., 2002). The mutant VHL^{R200W} protein was shown to have reduced binding to hydroxylated HIF-1α. As a result, any given O_2 concentration, the expression of HIF target genes, including *EPO*, is higher in cells expressing VHL^{R200W} as compared to cells expressing wild type VHL. As a result, patients with Chuvash polycythemia have higher-than-normal EPO levels despite having significantly increased red blood cell counts. Remarkably, patients with Chuvash polycythemia are not just polycythemic, they also have an increased respiratory rate and increased pulmonary artery pressure, which are physiological responses to hypoxia (Smith et al., 2006). Thus, homozygosity for the VHL^{R200W} mutation results in a global defect in O_2 sensing.

Missense mutations in the *EGLN1* gene encoding PHD2 were found to cause polycythemia in several individuals. These missense mutations result in the substitution of evolutionarily conserved residues (P317R or R371H) near the catalytic center of the protein, suggesting that they result in decreased hydroxylase activity (Percy et al., 2007). Finally, missense mutations in that result in the substitution of evolutionarily conserved residues (M535V, M535I, G537W, G537R) in HIF-2α that are located near a proline residue that is subject to hydroxylation (Pro-532), suggesting that the substitutions reduce the efficiency with which the hydroxylases modify the mutant protein (Percy et al., 2008). Patients with *EPAS1* missense mutations exhibit increased pulmonary artery pressure, cardiac output, heart rate, and respiratory rate (Formenti et al., 2011). Thus, human genetics provides compelling evidence that the HIF-PHD-VHL pathway plays a critical role in the regulation of erythropoiesis as well as many other physiological responses to hypoxia.

Angiogenesis

A primary means of increasing local O_2 delivery is by vascular remodeling (increased luminal diameter of existing vessels) and angiogenesis (development of new blood vessels). Again, HIFs function as master regulators by activating the transcription of multiple genes encoding secreted angiogenic growth factors and cytokines that stimulate the proliferation of resident endothelial cells and the recruitment of bone marrow-derived angiogenic cells (BMDACs). The following are some of the HIF target genes that encode angiogenic growth factors and cytokines (Figure 3): *VEGFA*, which encodes vascular endothelial growth factor A, a protein that binds to VEGFR2 on endothelial cells to stimulate angiogenesis, vascular

permeability, and recruitment of VEGFR2$^+$ BMDACs; *ANGPT1* and *ANGPT2*, which encode angiopoietins 1 and 2, proteins that bind to the TIE2 receptor to modulate endothelial-pericyte interactions; *ANGPTL4*, which encodes angiopoietin-like 4, which stimulates endothelial cell proliferation and vascular permeability; *EPO*, which encodes a protein (erythropoietin) that binds to its receptor (EPOR) on endothelial cells to stimulate their survival and proliferation; *KITL*, which encodes kit ligand (also known as stem cell factor, a protein that mediates the recruitment of c-KIT$^+$ BMDACs; *PDGFB*, which encodes platelet-derived growth factor B, which binds to its receptor (PDGFR) on pericytes to modulate interactions with endothelial cells; *PGF*, which encodes placental growth factor, a protein that mediates the recruitment of VEGFR1$^+$ BMDACs; and *CXCL12*, which encodes chemokine (C-X-C motif) ligand 12 (also known as stromal-derived factor 1), a protein that mediates the recruitment of CXCR4$^+$ BMDACs (Semenza et al., 2014).

Pathological Angiogenesis in Ischemic Retinopathies

One of the major complications of diabetes is the development of proliferative diabetic retinopathy (PDR), in which excess proliferation of blood vessels in the eye leads to hemorrhage and retinal detachment that results in vision loss, which can progress to blindness (Campochiaro, 2013). Increased levels of VEGF were found in fluid taken from the eyes of diabetic patients (Aiello et al., 1994). In mouse models of retinal ischemia, increased HIF-1α production by Mueller cells was found to precede the induction of VEGF mRNA and protein expression in the retina (Ozaki et al., 1999). Clinical trials demonstrated that treatment of diabetic patients with anti-VEGF therapy slowed disease progression in some but not all patients: in one study of patients with diabetic retinopathy, the cumulative

probability of clinical progression over two years was reduced from 34% to 11% (Ip et al., 2012). Mouse models revealed that in addition to VEGF, HIF-dependent expression of multiple angiogenic factors is induced by retinal ischemia (Yoshida et al., 2010), suggesting that the limited response to anti-VEGF therapy reflected the pathogenic role of other angiogenic factors.

Interrogation of a panel of angiogenic growth factors and cytokines revealed that oxygen-induced retinopathy, which is a mouse model of retinal ischemia, was characterized by dramatically increased expression of two angiogenic factors in the eye: VEGF and ANGPTL4 (Babapoor-Farrokhran et al., 2015). Analysis of aqueous fluid from the eyes of diabetic patients revealed that whereas the mean VEGF level was increased in diabetic patients with PDR as compared to diabetic patients without PDR, there was extensive overlap between groups; in contrast, mean ANGPTL4 levels were increased in PDR and there was virtually no overlap with the non-PDR group (Babapoor-Farrokhran et al., 2015). Furthermore, aqueous fluid from PDR patients treated with anti-VEGF therapy still activated endothelial cells in vitro and addition of anti-VEGF antibody to the aqueous fluid did not prevent endothelial activation, whereas addition of anti-ANGPTL4 antibody did prevent endothelial activation (Babapoor-Farrokhran et al., 2015). As in the case of VEGFA, ANGPTL4 promotes permeability as well as proliferation of endothelial cells (Xin et al., 2013). Thus, anti-ANGPTL4 therapy (either alone or in combination with anti-VEGF therapy) may improve outcome in PDR patients (Figure 4). An alternative strategy is the use of small molecule inhibitors of HIF-1, which were originally shown to inhibit tumor growth and angiogenesis (Zhang et al., 2008; Lee et al., 2009a,b), to block the expression of

ANGPTL4, VEGF, and other angiogenic factors that are induced by retinal ischemia (Yoshida et al., 2010; Iwase et al., 2013).

O_2 UTILIZATION

All human cells require a constant supply of O_2 to carry out oxidative phosphorylation in the mitochondria for ATP generation. O_2 is utilized as the final electron acceptor in the mitochondrial ETC, resulting in the generation of water. Under hypoxic conditions, the efficiency of electron transfer is impaired and electrons react with O_2 prior to reaching complex IV, to form superoxide radicals, which can be converted to hydrogen peroxide by superoxide dismutase. Superoxide and hydrogen peroxide are ROS that oxidize cellular macromolecules and alter their biochemical or physical properties, resulting in cell dysfunction or death. HIFs play a critical role in maintaining redox homeostasis under hypoxic conditions. Many cancers contain hypoxic regions due to high rates of cell proliferation coupled with the formation of vasculature that is structurally and functionally abnormal. Primary tumors with low oxygenation (PO_2 < 10 mmHg) are associated with an increased risk of metastasis and patient mortality (Vaupel et al., 2007). In order to maintain redox homeostasis in the presence of intratumoral hypoxia, HIFs activate the transcription of target genes that serve to decrease mitochondrial oxidant production or increase mitochondrial antioxidant production.

HIF-1 Inhibits Mitochondrial Oxidant Production

Cancer cells are characterized by increased glucose and glutamine uptake. In the Embden-Meyerhof pathway, glucose is metabolized to pyruvate, which is then converted to acetyl

CoA by pyruvate dehydrogenase (PDH) for entry into the tricarboxylic acid (TCA; also known as Krebs) cycle (Figure 5). Glucose metabolites are also shunted into the pentose phosphate pathway for production of nucleic acids and NADPH or the serine synthesis pathway for production of serine, glycine, and NADPH. Glutamine is converted to glutamate and then to α-ketoglutarate for entry into the TCA cycle. In addition to glucose and glutamine, fatty acids are the other source of energy for cells. Fatty acids can be taken up from the extracellular media, generated by the metabolism of triglycerides from lipid droplets (Carracedo et al., 2013), or synthesized *de novo* from acetyl CoA. *De novo* synthesis of fatty acids is required for membrane synthesis and therefore for cell growth and proliferation. Fatty acids are catabolized by fatty acid oxidation (FAO; also known as β-oxidation). FAO generates one molecule of acetyl CoA in each oxidation cycle and two in the last cycle (Figure 5). Oxidation of acetyl CoA derived from glucose or fatty acids in the TCA cycle generates NADH and $FADH_2$, which donate their electrons to the ETC, leading to the formation of a proton gradient that drives the production of ATP. Thus, the flux of acetyl CoA metabolized by the TCA cycle determines the flux of electrons delivered to the ETC.

As in the case of angiogenesis and erythropoiesis, HIF-1 functions as a master regulator of glucose metabolism by activating the transcription of multiple target genes that inhibit the generation of acetyl CoA from glucose (Semenza, 2014), including: PDH kinase, isozyme 1 (PDK1) and PDK3; BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L); and lactose dehydrogenase A (LDHA) (Figure 5). PDKs phosphorylate and inactivate PDH, thereby inhibiting the conversion of pyruvate to acetyl CoA (Kim et al., 2006; Papandreou et al., 2006; Lu et al., 2008). LDHA competes with PDH for pyruvate and converts it to lactate, the terminal product of glycolysis (Le et al., 2010).

BNIP3 and BNIP3L trigger mitochondrial autophagy as a means to reduce oxidative metabolism of glucose, glutamine and fatty acids (Zhang et al., 2008; Bellot et al., 2009).

HIF-1 also inhibits FAO, thereby decreasing intracellular generation of acetyl CoA, by repressing the expression of the medium-chain and long-chain acyl-CoA dehydrogenase (*MCAD* and *LCAD*) genes under hypoxia (Huang et al., 2014). MCAD and LCAD are key enzymes catalyzing the first step of FAO in the mitochondria (Figure 5). Under hypoxia, HIF-1α suppresses the expression of CMYC, which otherwise would activate transcription of the gene encoding PGC-1β, which is a coactivator of *MCAD* and *LCAD* transcription. This phenomenon was observed in prostate and liver cancer cells. Consistent with the suppression of FAO, there an increase in lipid accumulation was observed in the cells under hypoxia. The decrease in FAO under hypoxia was necessary to prevent ROS accumulation and cell death under hypoxic conditions (Huang et al., 2014). Thus, regulation of FAO represents an additional mechanism by which HIF-1 regulates oxidant production as a critical metabolic adaptation to hypoxic conditions. The pharmacologic blockade of FAO has been pursued for treatment of heart diseases. Ranolazine targets carnitine palmitoyl transferase I, which catalyzes fatty acid transport into mitochondria for FAO and is approved for use in cancer patients (Currie et al., 2013). Cancer cells rely on FA as cellular building blocks for membrane formation, energy storage and production of signaling molecules. Recent preclinical studies suggest that FAO inhibitors may have therapeutic utility in hematological malignancies and triple negative breast cancer with CMYC overexpression (Samudio et al., 2010; Camarda et al., 2016). Inhibition of HIF-1 represents an alternative therapeutic strategy and has the benefit of blocking oxidative metabolism of both glucose and fatty acids.

HIFs Stimulates Mitochondrial Antioxidant Production

Reduced glutathione is the principal antioxidant in human cells and NADPH is required to maintain glutathione in a reduced form. Two different glycolytic shunt pathways generate NADPH: the pentose phosphate pathway and the combined activity of the serine synthesis pathway (SSP) and one-carbon (folate cycle) metabolism (1CM). In the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) converts the glycolytic intermediate glucose-6-phosphate and NADP to 6-phosphogluconate and NADPH in the cytosol (Figure 6). In the SSP, the glycolytic intermediate 3-phosphoglycerate is converted to serine via three reactions, which are catalyzed by phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH). Serine and $NADP^+$ are then utilized for 1CM, either in the cytosol or mitochondria, which generates glycine and NADPH. The enzymes required for mitochondrial 1CM are serine hydroxymethyltransferase 2 (SHMT2), methylene tetrahydrofolate dehydrogenase 2 (MTHFD2), and MTHFD1-like (MTHFD1L), whereas SHMT1 and MTHFD1 carry out these same reactions in the cytosol (MTHFD1 catalyzes the reactions performed by both MTHFD2 and MTHFD1L). The major role of 1CM in NADPH generation and redox regulation has only recently become appreciated (Fan et al., 2014). Here we will discuss how HIFs facilitate mitochondrial antioxidant production under hypoxic conditions.

In breast cancer cells, irrespective of the estrogen receptor (ER) status, the expression of all six genes encoding the SSP enzymes (PHGDH, PSAT1 and PSPH) and mitochondrial 1CM enzymes (SHMT2, MTHFD2 and MTHFD1L) are induced under hypoxic conditions in a HIF-dependent manner. Expression of the genes encoding cytosolic 1CM

enzymes (SHMT1 and MTHFD1) is not induced by hypoxia in most cell lines. *G6PD* gene expression was repressed by hypoxia in all breast cancer cell lines, indicating a reprogramming of glucose metabolism to increase flux through the SSP and decrease flux through the PPP under hypoxic conditions, which was confirmed by metabolomic analyses.

When PHGDH, the first enzyme of the SSP was knocked down in breast cancer cells (irrespective of ER status), mitochondrial ROS levels and apoptosis were increased in the knockdown subclones, as compared to subclones expressing a non-targeting control shRNA (NTC) under hypoxic conditions (Samanta et al., 2016). The ratio of reduced:oxidized glutathione increased dramatically under hypoxic conditions in NTC but not in PHGDH knockdown subclones, which also had significantly reduced levels of NADPH (Samanta et al., 2016). These results suggest that knockdown of PHGDH in breast cancer cell lines increased oxidant stress. Similarly, knockdown of SHMT2 in neuroblastoma cell lines increased oxidant stress and cell death under hypoxic conditions (Ye et al., 2014). SHMT2 expression promoted survival of glioblastoma cells in the hypoxic tumor microenvironment (Kim et al., 2015). SHMT2 was also induced in 6 breast cancer cell lines under hypoxic conditions (Samanta et al., 2016). These results indicate that induction of the SSP and mitochondrial 1CM by HIFs is another critical metabolic adaptation to hypoxia. Presumably knockdown of PSAT1, PSPH, MTHFD2, or MTHFD1L would have similar consequences as observed for knockdown of PHGDH or SHMT2.

PHGDH knockdown in ER^+ MCF-7 or ER^- MDA-MB-231 breast cancer cells resulted in reduced NADPH levels as well as increased mitochondrial ROS and apoptosis in the bulk cancer cell population (Samanta et al., 2016). However, the effect of PHGDH knockdown on the breast cancer stem cell (BCSC) population was particularly dramatic. Exposure of NTC

subclones to hypoxia for 3 days resulted in a greater-than-five-fold increase in the percentage of BCSCs, which was abrogated in PHGDH knockdown subclones. *In vivo*, PHGDH knockdown subclones exhibited a dramatic impairment in lung metastasis that was consistent with the observed reduction of BCSCs in the primary tumor (Samanta et al., 2016). PHGDH knockdown subclones were also much more sensitive to treatment with doxorubicin or carboplatin, which are chemotherapy drugs that act by generating ROS. Interestingly, whereas HIF-1 alone is responsible for activating transcription of genes required to decrease mitochondrial oxidant production, both HIF-1 and HIF-2 contribute to the regulation of the genes encoding SSP and mitochondrial 1CM enzymes that increase mitochondrial antioxidant capacity.

SYSTEMIC OXYGEN SENSING

The Carotid Body Activity Mediates Acute Physiological Responses to Hypoxemia

Nearly 150 years ago, Pflüger reported that hypoxia stimulates breathing (Pflüger, 1868). Whereas the cell-autonomous O_2 sensing that occurs as a result of hydroxylation-dependent modulation of HIF activity enables each cell to respond individually to hypoxia, systemic hypoxia, which occurs in response to blood loss or ascent to high altitude, is sensed as hypoxemia (reduced blood O_2 content) by the carotid bodies (CBs), which are tiny bilateral organs located at the bifurcation of the common carotid artery into the internal and external carotid arteries (De Castro, 1926; Heymans and Heymans, 1927). The response of the CB to hypoxia is notable for its speed and sensitivity: a 20% reduction in arterial PO_2 is sufficient to induce a response within seconds (Prabhakar and Semenza, 2015). Glomus cells are excitable cells in the CB that depolarize in response to hypoxemia.

Axons from glomus cells course through the carotid sinus nerve (CSN) to the nucleus tractus solitarius, from which reflex arcs project to: the diaphragm, to increase respiratory rate; the heart, to increase heart rate; and via the rostral ventro-lateral medulla and sympathetic nervous system, to the adrenal medulla (AM), to stimulate the secretion of catecholamines (epinephrine and norepinephrine) that increase blood pressure (Figure 7). These cardiovascular and respiratory adaptations increase cardiac output and ventilation, respectively, thereby increasing the capture of O_2 by red blood cells for delivery throughout the body.

Whereas O_2 sensing in non-depolarizable cells occurs via alterations in rates of HIF-α hydroxylation, O_2 sensing in the depolarizable glomus cells of the CB occurs by the generation of carbon monoxide (CO) by the enzyme heme oxygenase 2 (HO2), which utilizes O_2 as a substrate (Figure 8). CO binds to the heme moiety of soluble guanylate cyclase, which activates its catalytic activity, converting GTP to cyclic GMP (cGMP), which binds to and activates cGMP-dependent protein kinase (protein kinase G [PKG]). Phosphorylation of cystathionine-γ-lyase (CSE) by PKG inhibits CSE catalytic activity, which mediates the generation of hydrogen sulfide (H_2S). Thus, O_2 sensing/signal transduction in the CB involves two additional gas messengers, CO and H_2S: under normoxic conditions, CO levels are high, H_2S levels are low, and CSN activity is low; in contrast, hypoxemia leads to decreased CO production (due to decreased substrate availability) and increased H_2S production, which induces glomus cell depolarization and increased CSN activity (Prabhakar and Semenza, 2015; Yuan et al., 2015).

When CBs from wild type mice are perfused with a hypoxic gas mixture, there is a rapid increase in carotid sinus nerve activity. However, in *Cse*$^{-/-}$ mice, which lack CSE

activity, basal H_2S levels are reduced by half and do not increase in response to hypoxia, whereas CB responses to hypercarbia were intact (Peng et al., 2010). Similarly, CBs from *Hif1a*$^{+/-}$ mice, which are heterozygous for a null (knockout) allele at the *Hif1a* locus encoding HIF-1α, exhibit no increase in neural activity in response to hypoxia, despite normal histological appearance and normal responses to hypercarbia or cyanide, indicating a specific defect in O_2 sensing (Kline et al., 2002).

The Carotid Body Activity Mediates Pathological Responses to Intermittent Hypoxia

The classic homeostatic responses of the CB that are described in the preceding section are subverted in obstructive sleep apnea (OSA). In this condition, the upper airway becomes occluded by relaxation of the tongue, tonsils, and pharyngeal soft tissue during sleep, resulting in apnea (cessation of breathing) for 15-30 sec, which results in hypoxemia that is sensed by the carotid body. The patient awakens, clears the airway, resulting in reoxygenation, and goes back to sleep. This cycle of hypoxemia and reoxygenation is repeated dozens of times each night. The major pathological consequence of OSA is the development of hypertension, which places the patient at risk for heart failure, myocardial infarction, and stroke. Among adult Americans, 1 in 5 males and 1 in 15 females are affected by OSA. It is the major cause of treatment-resistant hypertension. Patients are treated with continuous positive airway pressure to prevent airway occlusion, which lowers blood pressure in some but not all patients.

Although OSA results in chronic intermittent hypoxemia as well as chronic intermittent hypercarbia, pioneering studies revealed that exposure of rodents to repeated short cycles of ambient hypoxia and reoxygenation, i.e. chronic intermittent hypoxia (CIH),

was sufficient to cause hypertension, which was associated by increased plasma levels of norepinephrine. Furthermore, transection of the carotid sinus nerve blocked the increase in catecholamine levels and blood pressure in rodents subjected to CIH, demonstrating involvement of the CB. Remarkably *Hif1a*$^{+/-}$ mice were completely protected from increased catecholamine levels and blood pressure in response to CIH (Peng et al., 2006).

CIH generates increased ROS in the CB and AM and treatment of rodents with the radical scavenger manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) blocked the development of hypertension in rodents subjected to CIH. Exposure of wild type mice to CIH induced HIF-1α expression in the central nervous system, which was also blocked by MnTMPyP administration, indicating that HIF-1α was downstream of ROS (Peng et al., 2006). However, CIH induced increased levels of oxidants in the brains of wild type, but not *Hif1a*$^{+/-}$ mice, indicating that HIF-1α was upstream of ROS (Peng et al., 2006). This paradox was resolved by the discovery that expression of NADPH oxidase 2 (NOX2), an enzyme that generates superoxide, was induced by CIH in wild type but not in *Hif1a*$^{+/-}$ mice (Yuan et al., 2011). CIH causes an initial ROS signal, which induces HIF-1α expression. HIF-1 then induces NOX2 activity, which leads to further ROS generation. Thus, a feed-forward loop is established creating a vicious cycle of increased ROS and HIF-1α levels. Further mechanistic studies revealed that increased ROS result in an increase in intracellular calcium that activates protein kinase C and mTOR, which mediate increased HIF-1α synthesis and stability (Yuan et al., 2008).

Remarkably, whereas basal levels of HIF-1α protein in the CB are low and increase after CIH, basal HIF-2α levels are high and decrease after CIH (Nanduri et al., 2009). Increased intracellular calcium levels induced increased activity of calpains, which are

calcium-dependent proteases, that degraded HIF-2α. CIH repressed expression in the CB and AM of *Sod2*, which is a known HIF-2 target gene that encodes the mitochondrial superoxide dismutase. Treatment of rodents with the calpain inhibitor ALLM blocked CIH-induced increases in ROS, plasma catecholamines, and blood pressure (Nanduri et al., 2009). Thus, CIH increases the expression of HIF-1α and the pro-oxidant enzyme NOX2 but decreases the expression of HIF-2α and the anti-oxidant enzyme SOD2, all of which are required for the development of hypertension (Figure 9).

Epas1$^{+/-}$ mice, which are heterozygous for a knockout allele at the locus encoding HIF-2α, display a phenotype that is in striking contrast to *Hif1a*$^{+/-}$ mice: under room air conditions, the mice have elevated blood pressure and instability of breathing, and their CB and AM show augmented responses to hypoxia, all of which are observed in wild type mice that have been subjected to CIH (Peng et al., 2011). Furthermore, SOD2 expression was decreased and ROS were increased in the CB and AM of *Epas1*$^{+/-}$ mice under room air conditions. Remarkably, treatment of *Epas1*$^{+/-}$ mice with MnTMPyP for 15 days normalized blood pressure, plasma norepinephrine levels and the CB response to hypoxia (Peng et al., 2011). Furthermore, HIF-1α protein expression was increased in the CB of *Epas1*$^{+/-}$ mice and treatment with digoxin, a drug that inhibited the accumulation of HIF-1α protein in the CB, also normalized CB sensitivity and corrected the ROS imbalance (overly oxidized state), plasma norepinephrine levels, breathing, and blood pressure (Yuan et al., 2013). Conversely, treatment of *Hif1a*$^{+/-}$ mice with 2-methoxyestradiol, a drug that inhibited the accumulation of HIF-2α protein in the CB, corrected the ROS imbalance (overly reduced state) and completely restored responsiveness of the CB and AM to hypoxia (Yuan et al., 2013).

The response of *Epas1*$^{+/-}$ and *Hif1a*$^{+/-}$ mice to pharmacological inhibitors of HIF-1α and HIF-2α, respectively, suggested that proper functioning of the CB and AM required proper redox balance, which in turn was dependent on a proper balance between HIF-1α and HIF-2α rather than the absolute levels of these proteins. To test this hypothesis, *Epas1*$^{+/-}$;*Hif1a*$^{+/-}$ doubly-heterozygous mice were analyzed (Yuan et al., 2013). Remarkably, despite reduced expression of both HIF-1α and HIF-2α, these mice exhibited normal redox state and sensitivity of the CB and AM to hypoxia, normal blood pressure, and normal breathing -- a dramatic confirmation of the model.

Does the dysregulation of redox homeostasis that is induced by CIH impact on the acute carotid body O_2 sensing mechanism described in the preceding section? Recent studies have revealed that ROS inhibits HO2 activity (Figure 8), thereby increasing the generation of H_2S by CSE (Yuan et al., 2016). Pharmacologic or genetic inhibition of CSE activity blocked carotid body-mediated activation of the sympathetic nervous system and the development of hypertension in rodents subjected to CIH (Yuan et al., 2016). Thus, CIH dysregulates redox balance, HIF-1α:HIF-2α transcriptional balance, and gas messenger signaling in the carotid body to induce sympathetic activation and hypertension.

The remarkable homeostatic mechanism described above can be interpreted in an evolutionary, as well as a physiological, context. The evolution of vertebrates with greatly increased body mass required the elaboration of cardiovascular, respiratory, and nervous systems that ensured the delivery of O_2 to every cell of the organism. Whereas HIF-1α is found in all metazoan species with differentiated cell types, HIF-2α is only found in vertebrates. In keeping with the importance of redox homeostasis described earlier in this review in the context of cellular metabolism, mutual antagonism between pro-oxidant

activity of HIF-1α and anti-oxidant activity of HIF-2α generate a redox balance that determines the set point of the CB, sympathetic nervous system, and AM, which in turn establish set points for breathing, heart rate, and blood pressure.

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FIGURE LEGENDS

FIGURE 1. O_2-dependent hydroxylation of HIF-α subunits. Hydroxylation of specific proline (Pro) and asparagine (Asn) residues promote VHL binding and block P300/CBP binding, respectively. VHL recruits an E3-ubiquitin protein ligase (BCCRE2) consisting of Elongin B, Elongin C, Cullin 2, RBX1, and an E2 ubiquitin ligase that ubiquitinates HIF-α, thereby targeting the protein for proteasomal degradation.

FIGURE 2. HIF-2 is a master regulator of erythropoiesis. 8 genes activated by HIF-2 encode protein products that are required for iron absorption, transport, and incorporation into hemoglobin for red blood cell production. *CYBRD1, SLC11A2*, and *SLC40A1* are expressed in duodenal enterocytes; *TF* in expressed in hepatocytes; *EPO* is expressed in renal interstitial cells; *EPOR, FECH*, and *TFRC* are expressed in erythroid progenitors.

FIGURE 3. HIF-1 and HIF-2 are master regulators of tissue vascularization. 8 genes activated by HIF-1 and HIF-2 encode secreted proteins that bind to cognate receptors on vascular cells to stimulate angiogenesis and vascular remodeling in response to hypoxia/ischemia.

FIGURE 4. The HIF target genes *VEGFA* and *ANGPTL4* encode secreted proteins that mediate angiogenesis and vascular permeability that leads to proliferative retinopathy and macular edema, respectively. VEGFA binds to VEGFR2 on retinal vascular endothelial cells (VECs); the receptor for ANGPTL4 on retinal VECs has not been identified. Drugs inhibiting VEGFA binding to VEGFR2 on VECs are used to treat ocular diseases; inhibition of HIF

activity or ANGPTL4 binding to its receptor on retinal VECs may also have therapeutic utility.

FIGURE 5. Oxygen-dependent regulation of oxidant production by HIF-1. In hypoxic cells, HIF-1 suppresses glucose oxidation, by activating the transcription of *LDHA* and *PDK1* (blue), and fatty acid oxidation, by repressing the expression of *MCAD* and *LCAD* (green). In addition, under hypoxic conditions, HIF-1 activates the transcription of *BNIP3* (blue) to induce mitochondrial-selective autophagy and thereby suppress both glucose and fatty acid oxidation. BNIP3, BCL2/adenovirus E1B 19-kDa protein-interacting protein 3; LCAD, long-chain acyl-CoA dehydrogenase; LDHA, lactate dehydrogenase A; MCAD, medium-chain acyl-CoA dehydrogenase; and PDK1, pyruvate dehydrogenase kinase 1.

FIGURE 6. Oxygen-dependent regulation of antioxidant production by HIF1. Under hypoxic conditions, HIFs activate the transcription of *PHGDH, PSAT1*, and *PSPH* (red) to increase conversion of glucose to serine (serine synthesis pathway); and *SHMT2, MTHFD2*, and *MTHFD1L* (blue) to increase generation of mitochondrial NADPH (mitochondrial one-carbon metabolism), which is required to convert glutathione from oxidized (GSSG) to reduced (GSH) form to protect against increased ROS generated by the ETC. Genes encoding proteins that generate cytosolic NADPH are either not consistently induced [*SHMT1, MTHFD1* (purple)] or actively repressed [*G6PD* (green)] under hypoxic conditions. G6PD, glucose-6-phosphate dehydrogenase; MTHFD, methylene tetrahydrofolate dehydrogenase; MTHFD1L, MTHFD1-like; PHGDH, phosphoglycerate dehydrogenase;

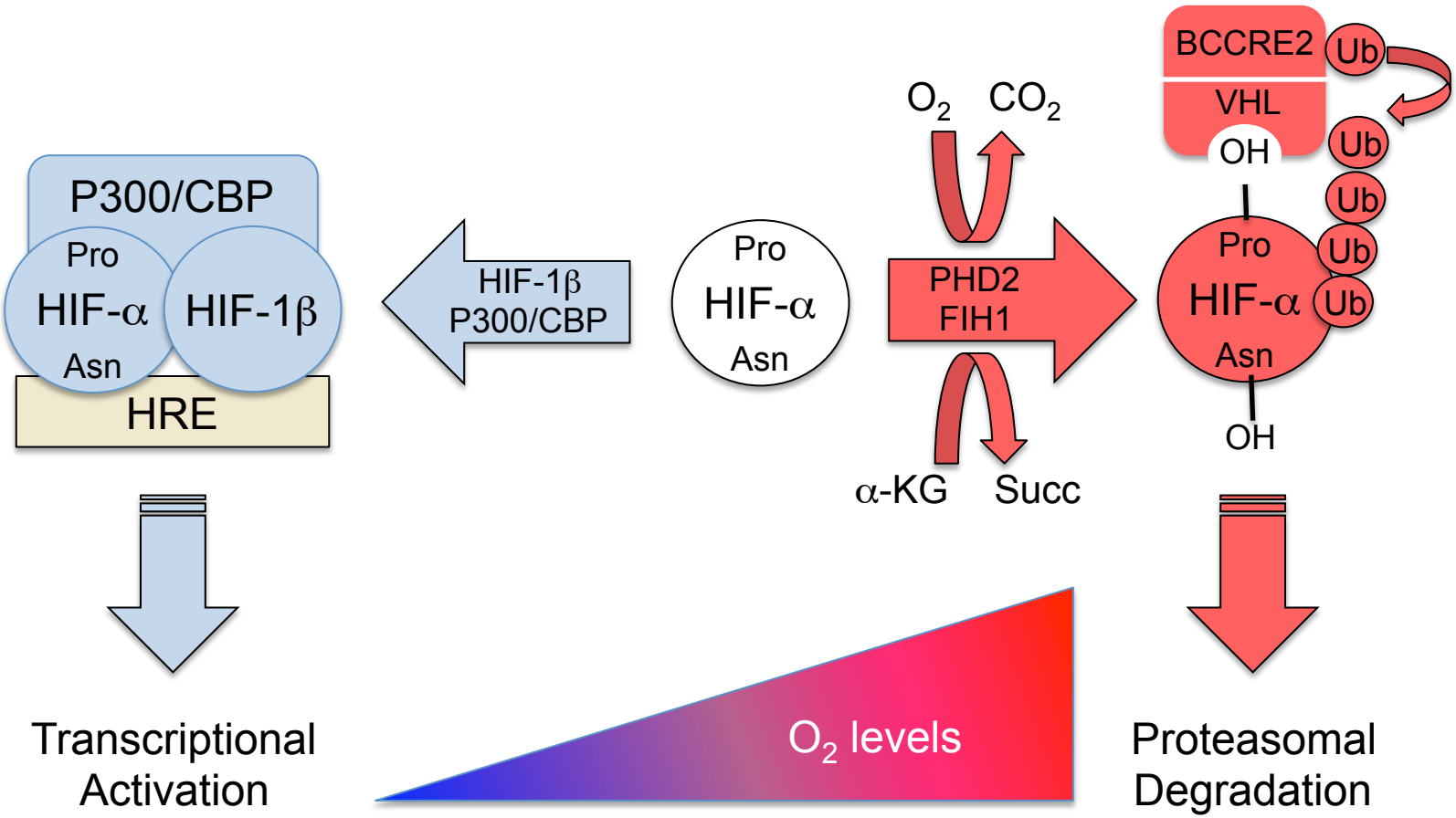
PSAT, phosphoserine aminotransferase; PSPH, phosphoserine phosphatase; SHMT, serine hydroxymethyltransferase.

FIGURE 7. Neural transmission of reflex arcs that result in changes in heart rate (HR), respiratory rate (RR), and blood pressure (BP) in response to depolarization of glomus cells in the carotid body (CB). AM, adrenal medulla; ASN, adrenal sympathetic nerve; CSN, carotid sinus nerve; CST, corticospinal tract; Epi, epinephrine; NE, norepinephrine; nTS, nucleus tractus solitarius; RVLM, rostral ventro-lateral medulla; SG, sympathetic ganglion.

FIGURE 8. O_2 sensing and signaling by gas messengers in the carotid body. cGMP, cyclic GMP; CIH, chronic intermittent hypoxia; CSE, cystathionine–γ-lyase; CSN, carotid sinus nerve; Cys-265, cysteine residue 265; HO2, heme oxygenase 2; Homocys, homocysteine; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase. O_2 stimulates CO production, which inhibits H_2S production, thereby inhibiting glomus cell depolarization. ROS generated by CIH inhibit HO2 activity, probably by oxidation of Cys-265, thereby increasing H_2S production and CSN activity.

FIGURE 9. Chronic intermittent hypoxia disrupts redox and HIF balance, leading to sympathetic activation and hypertension. HIF, hypoxia-inducible factor; mTOR, mammalian target of rapamycin; NOX, NADPH oxidase; PKC, Ca^{2+}-dependent protein kinase; SOD, superoxide dismutase.

Figure 1


P300/CBP
Pro
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HIF-1β
Asn
HRE
HIF-1β
P300/CBP
Pro
HIF-α
Asn
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PHD2
FIH1
α-KG
Succ
BCCRE2
VHL
OH
Ub
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Ub
Pro
HIF-α
Asn
OH
Transcriptional
Activation
O2 levels
Proteasomal
Degradation

Figure 2

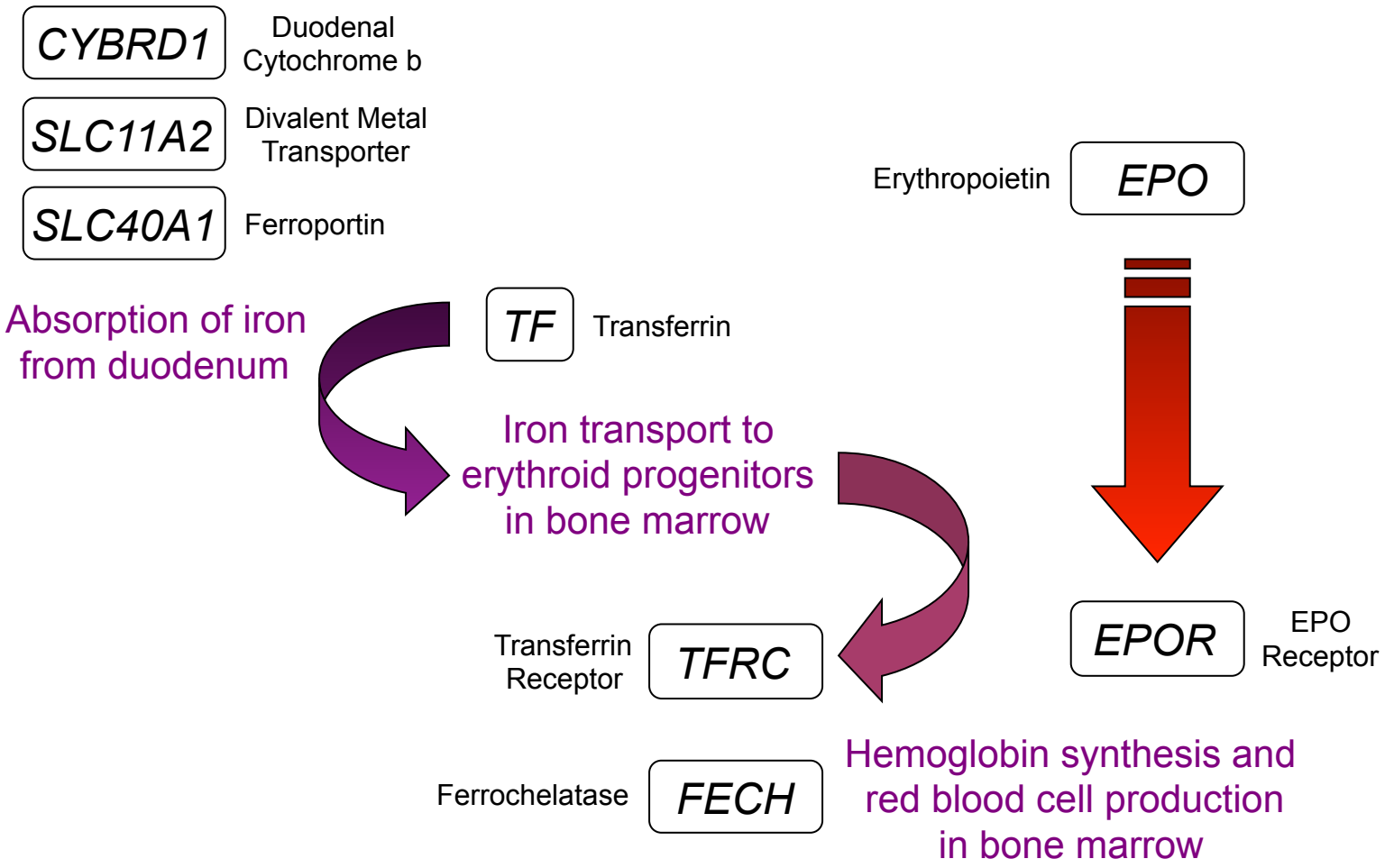

CYBRD1
Duodenal Cytochrome b
SLC11A2
Divalent Metal Transporter
SLC40A1
Ferroportin
Absorption of iron from duodenum
TF
Transferrin
Iron transport to erythroid progenitors in bone marrow
Transferrin Receptor
TFRC
Ferrochelatase
FECH
Hemoglobin synthesis and red blood cell production in bone marrow
Erythropoietin
EPO
EPOR
EPO Receptor

Figure 3

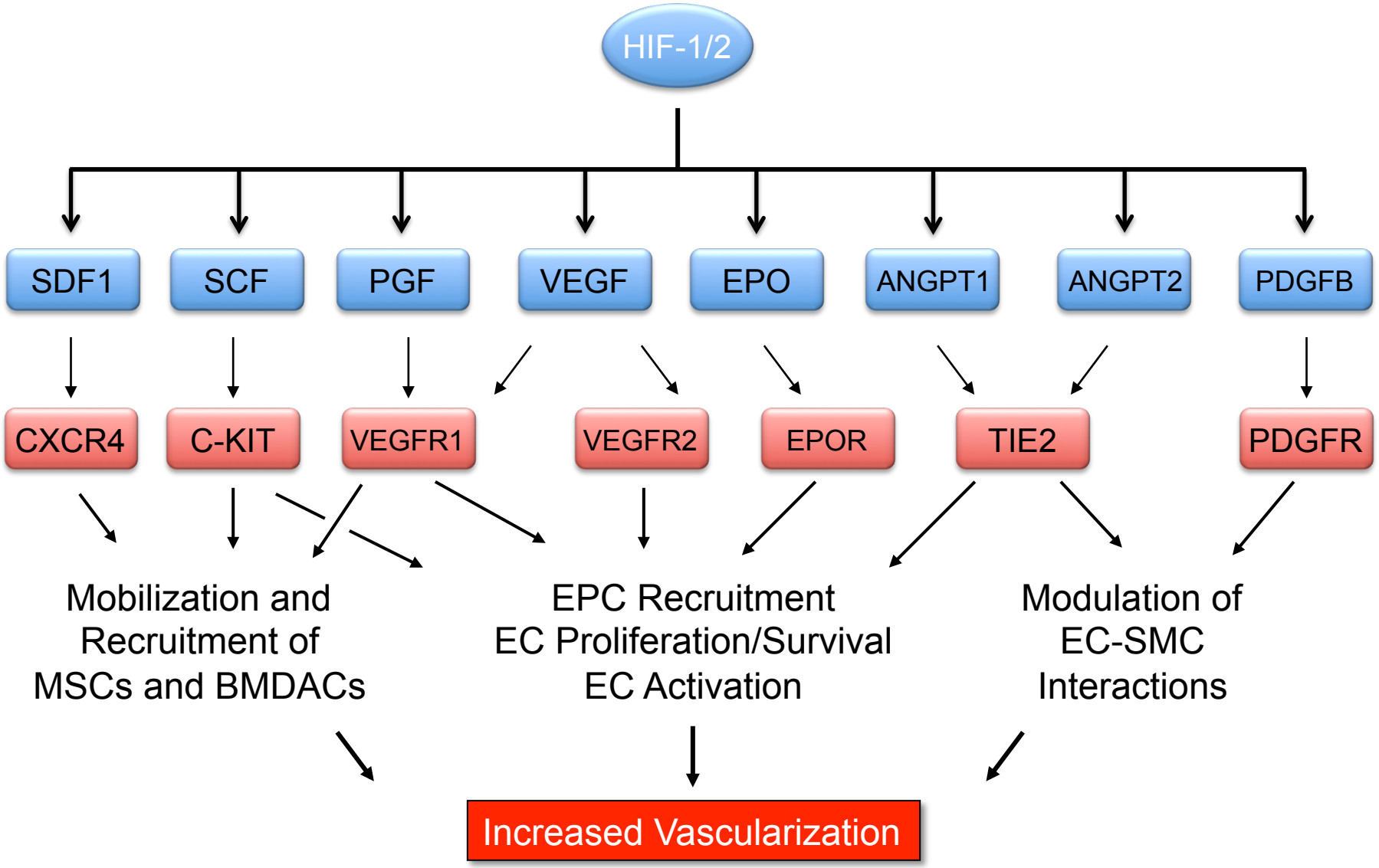

HIF-1/2
SDF1
SCF
PGF
VEGF
EPO
ANGPT1
ANGPT2
PDGFB
CXCR4
C-KIT
VEGFR1
VEGFR2
EPOR
TIE2
PDGFR
Mobilization and Recruitment of MSCs and BMDACs
EPC Recruitment
EC Proliferation/Survival
EC Activation
Modulation of EC-SMC Interactions
Increased Vascularization

Figure 4

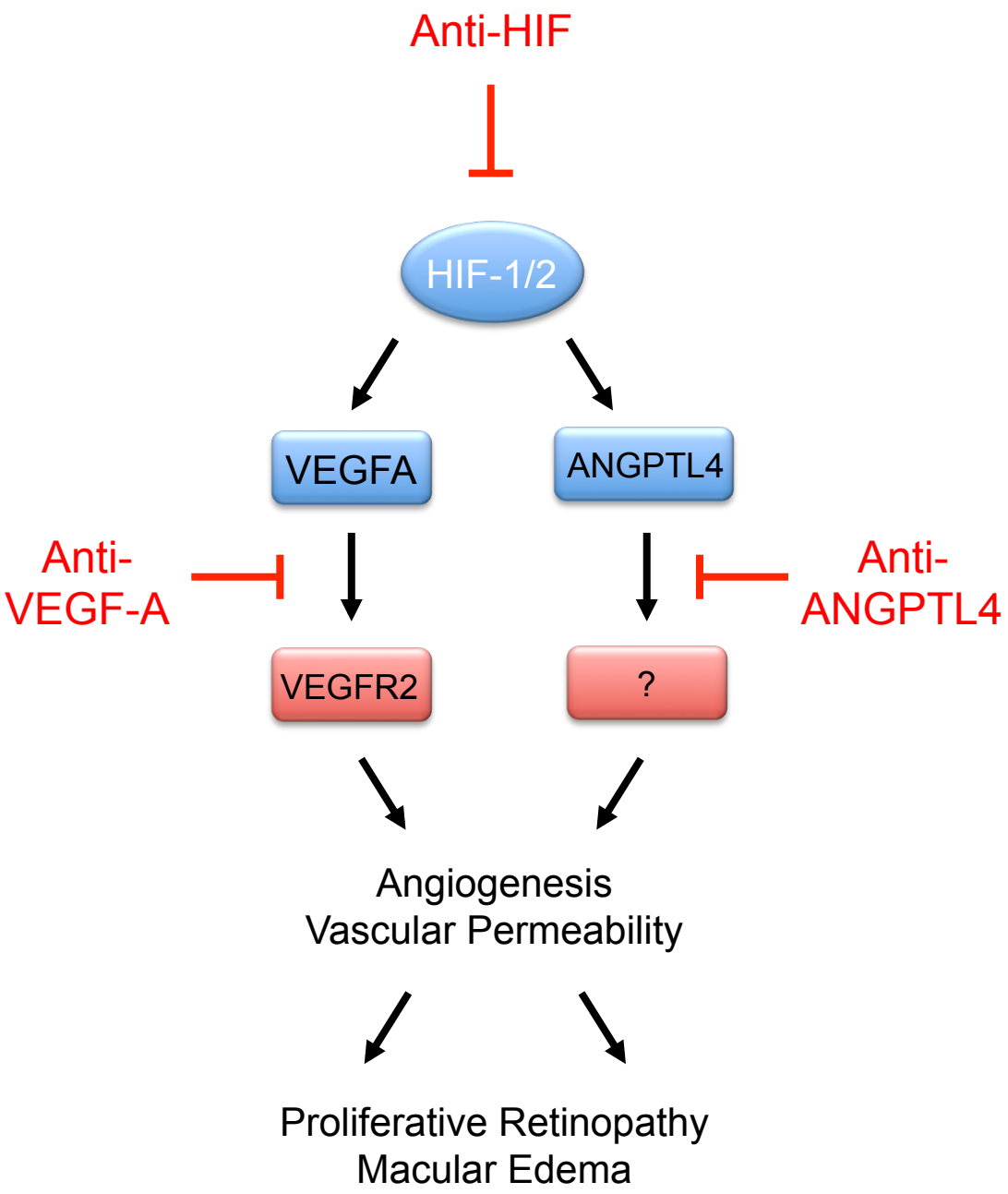

Anti-HIF
HIF-1/2
VEGFA
ANGPTL4
Anti-
VEGF-A
Anti-
ANGPTL4
VEGFR2
?
Angiogenesis
Vascular Permeability
Proliferative Retinopathy
Macular Edema

Figure 5

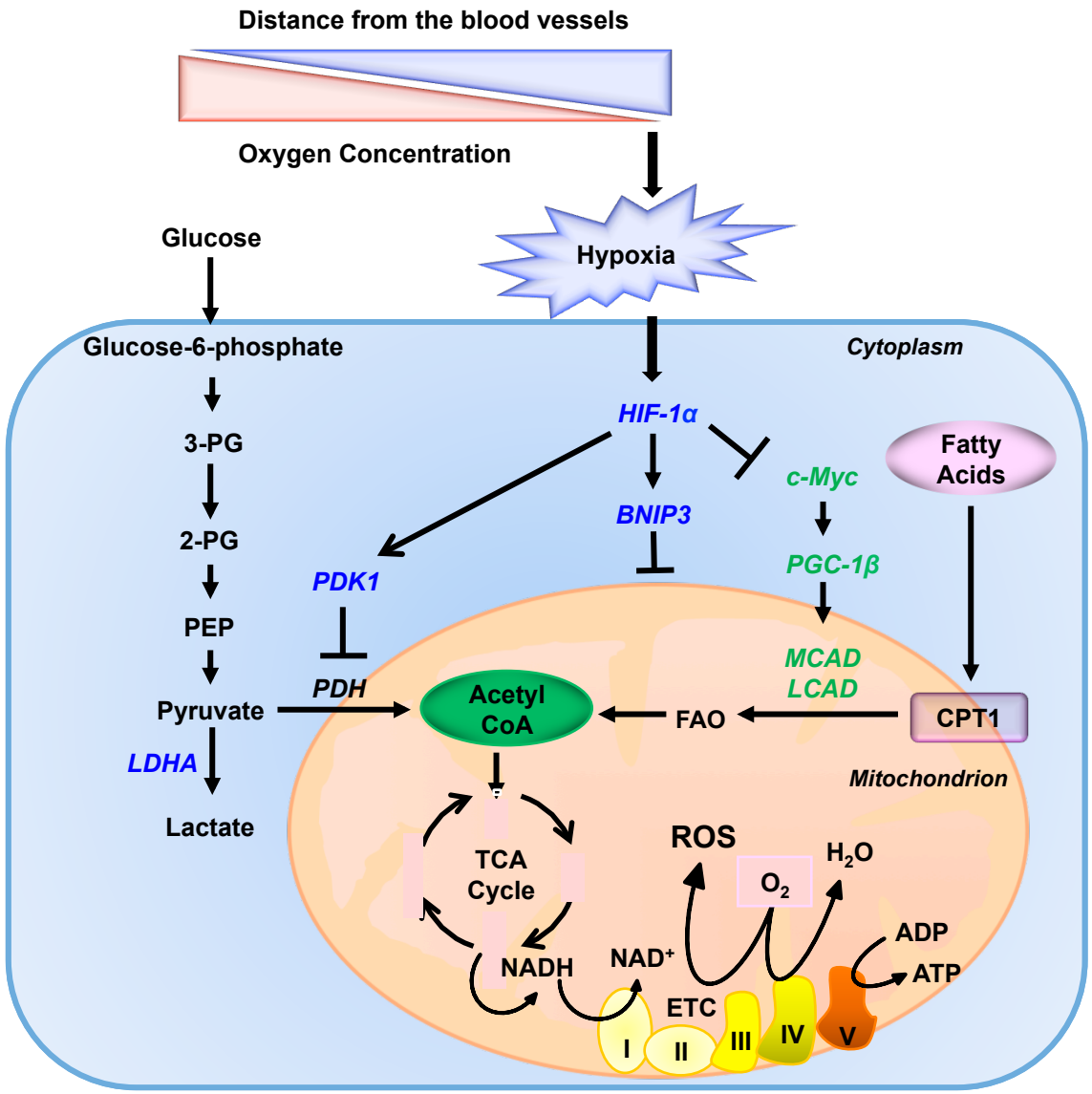

Distance from the blood vessels
Oxygen Concentration
Glucose
Hypoxia
Glucose-6-phosphate
Cytoplasm
HIF-1α
3-PG
c-Myc
Fatty Acids
BNIP3
2-PG
PDK1
PGC-1β
PEP
MCAD
LCAD
PDH
Pyruvate
Acetyl CoA
FAO
CPT1
LDHA
Mitochondrion
Lactate
ROS
TCA Cycle
H2O
O2
ADP
NADH
NAD+
ATP
ETC
I
II
III
IV
V

Figure 6

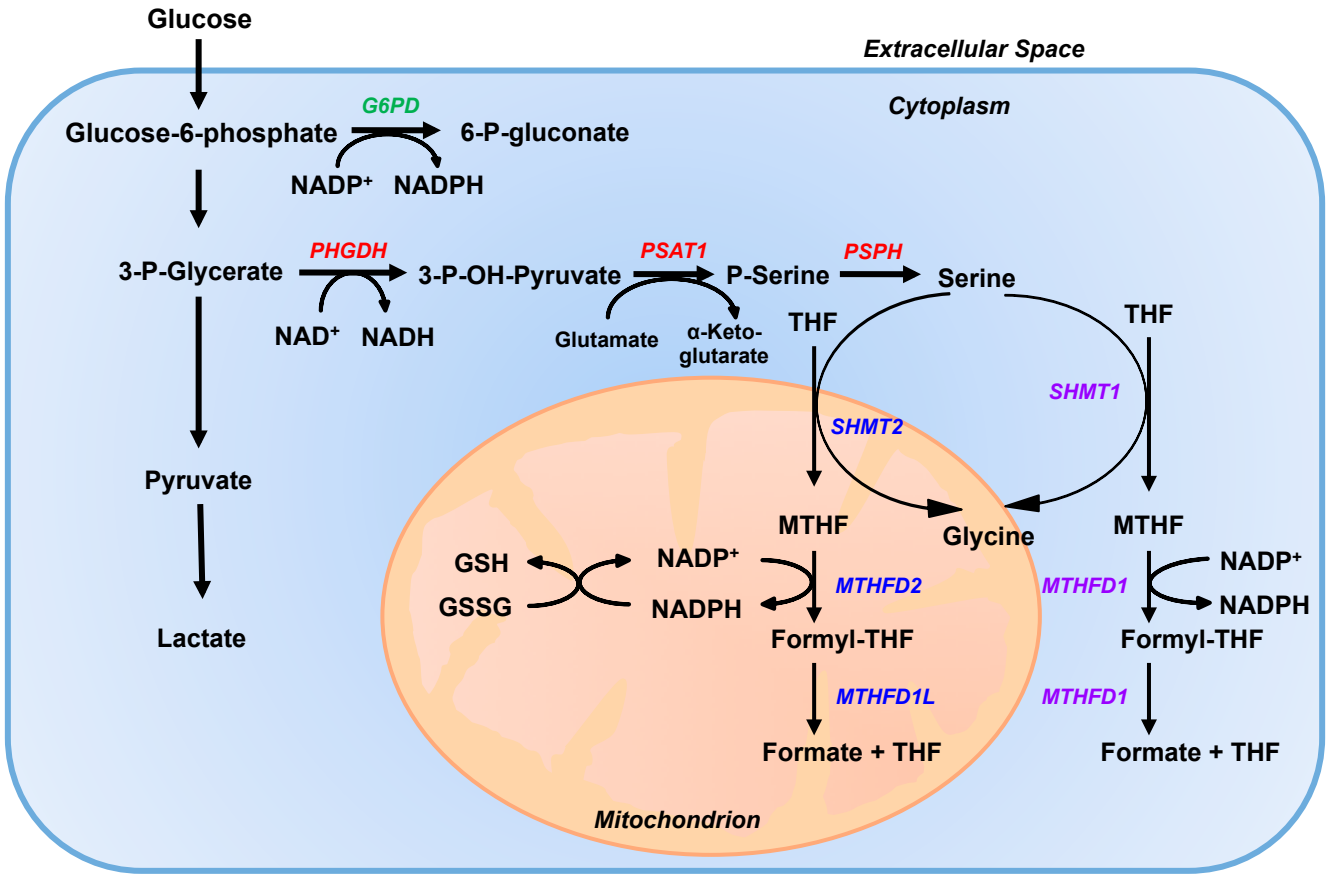

Glucose
Extracellular Space
Cytoplasm
G6PD
Glucose-6-phosphate
6-P-gluconate
NADP+
NADPH
PHGDH
3-P-Glycerate
3-P-OH-Pyruvate
NAD+
NADH
PSAT1
Glutamate
α-Keto-
glutarate
PSPH
P-Serine
Serine
THF
THF
SHMT1
SHMT2
Pyruvate
Lactate
MTHF
Glycine
MTHF
GSH
GSSG
NADP+
NADPH
MTHFD2
MTHFD1
NADP+
NADPH
Formyl-THF
Formyl-THF
MTHFD1L
MTHFD1
Formate + THF
Formate + THF
Mitochondrion

Figure 7

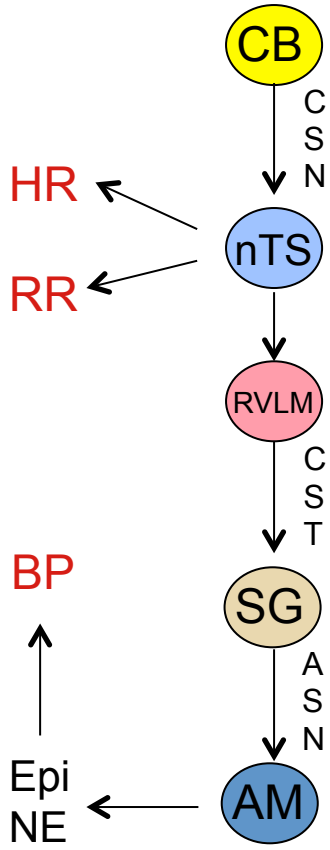


Figure 8

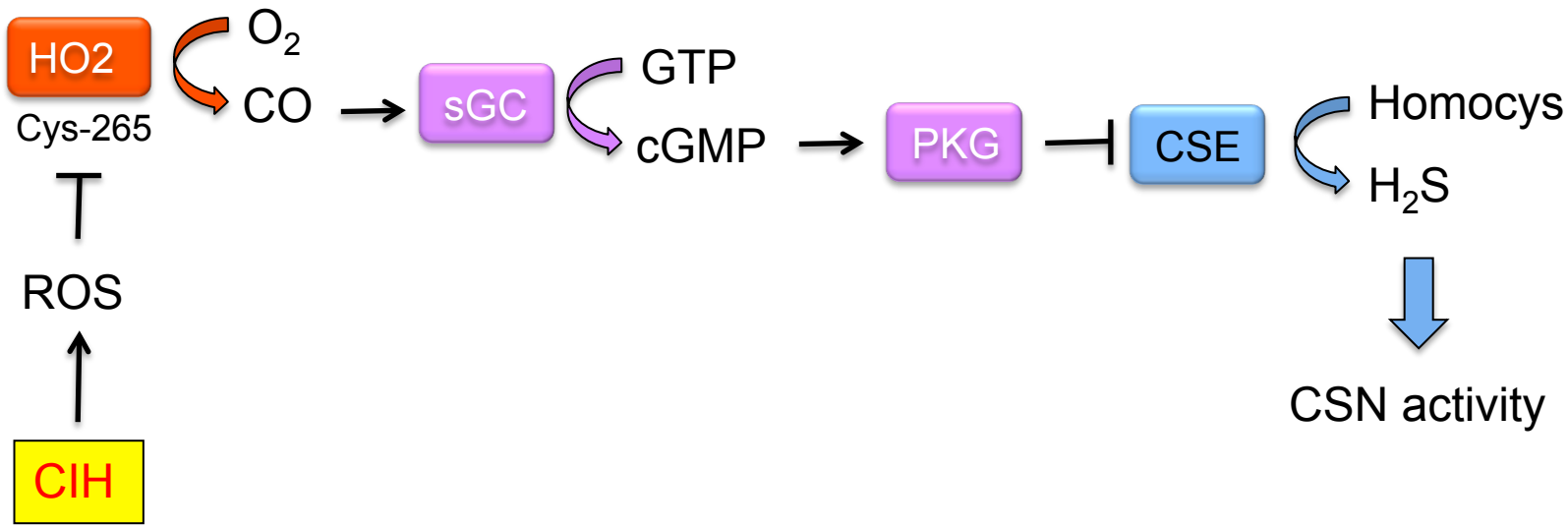
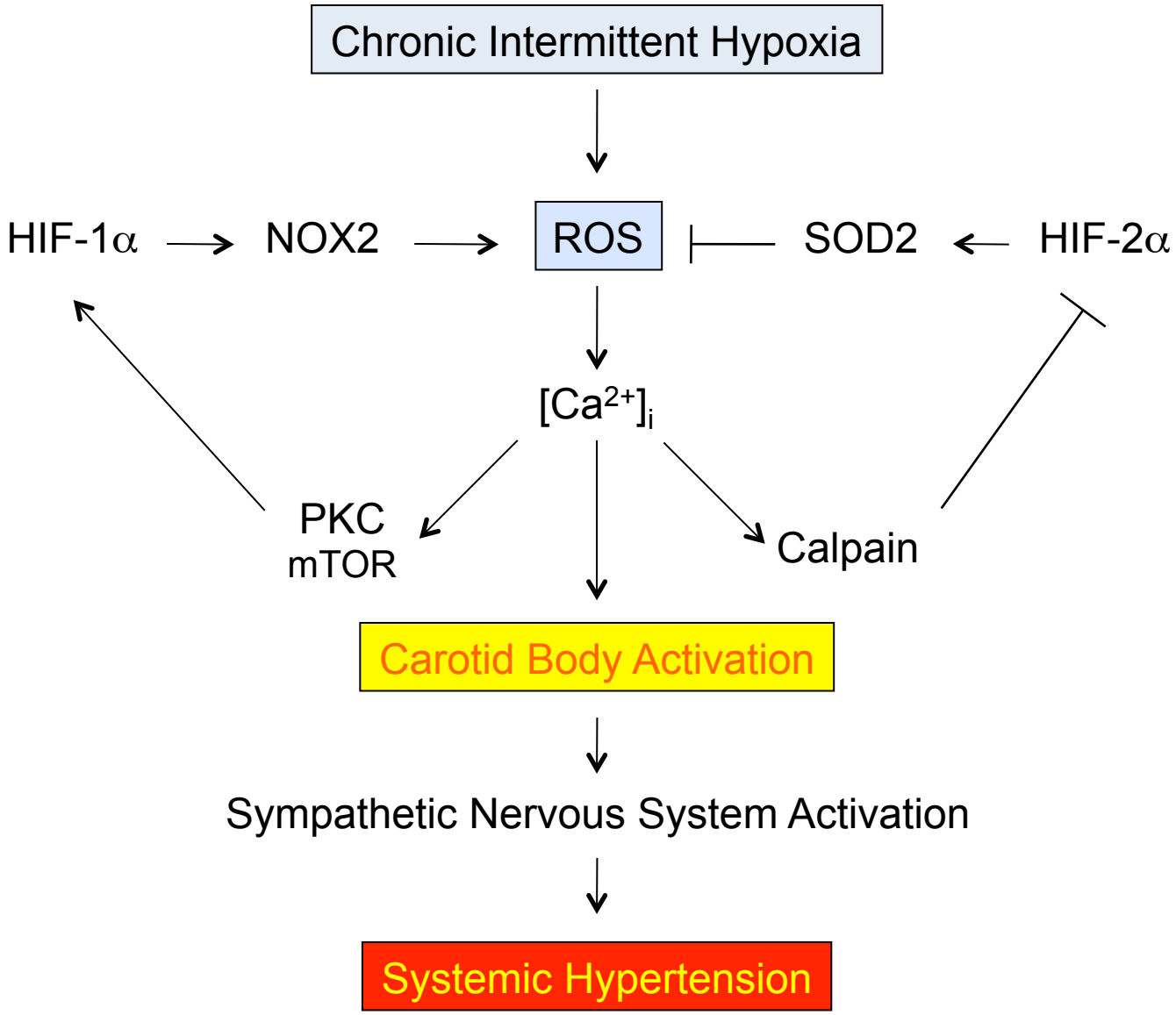

HO2
Cys-265
O_2
CO
sGC
GTP
cGMP
PKG
CSE
Homocys
H_2S
CSN activity
ROS
CIH

Figure 9


Chronic Intermittent Hypoxia
HIF-1α
NOX2
ROS
SOD2
HIF-2α
[Ca2+]i
PKC
mTOR
Calpain
Carotid Body Activation
Sympathetic Nervous System Activation
Systemic Hypertension