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The Expanding Universe of Hypoxia

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Introduction

Oxygen is the substrate of multicellular life. Reduced oxygen availability (hypoxia) is sensed and triggers homeostatic responses, which impact on virtually all areas of biology and medicine. Since the purification and molecular cloning of hypoxia-inducible factor 1 (HIF-1) in 1995, the scientific community has witnessed an explosive expansion of this field. From January 15 to 20, 2008, the beautiful Canadian coastal city of Vancouver hosted over 400 scientists for the Keystone Symposium on Molecular, Cellular, Physiological, and Pathogenic Responses to Hypoxia. The meeting, which is one of the largest Keystone symposia of the year, was focused on: the molecular mechanisms underlying adaptive responses to hypoxia; the role of hypoxia as a stimulus for normal developmental and physiological processes; the role of hypoxia in the pathogenesis of cardiovascular disease, cancer, and other common human diseases; and novel therapeutic strategies that target hypoxia response pathways. In this report, we will attempt to summarize the major developments in the field that were presented during the course of the meeting.

Oxygen Sensing

Sten Lindahl (Karolinska Institutet) provided a historical perspective to the discovery of oxygen and a biochemical timeline that highlighted the evolution of cellular metabolism and the various substrates employed as electron donors, such as H_2 , S_2 , H_2S , and O_2 . He noted that in order to sense systemic changes in O_2 tension, the human body has developed specialized tissues, such as the carotid body, for which Heymans received the Nobel Prize in 1938. In addition, all cells must have a sensing mechanism for O_2 and he highlighted the discovery of HIF-1 (1).

Peter Ratcliffe (University of Oxford) reviewed the O_2 -, Fe(II)- and 2-oxoglutarate-dependent hydroxylation of prolyl and asparaginyl residues in HIF-1 α . Hydroxylation of Pro-402 and Pro-564 by prolyl hydroxylase domain (PHD) proteins leads to binding of the von Hippel-Lindau protein (VHL) followed by ubiquitination and degradation of HIF-1 α , whereas hydroxylation of Asn-803 by factor inhibiting HIF-1 (FIH-1) blocks binding of the coactivators p300 and CBP. Novel substrates for FIH-1 include Notch, tankyrase, and I κ B α (2,3), which contain an ankyrin repeat domain (ARD) and can be hydroxylated at one or more sites within the ARD. The Notch1 ARD binds to FIH-1 with greater affinity than does HIF-1 α and ARD overexpression leads to reduced HIF-1 α hydroxylation, suggesting that ARD proteins may serve as a sink for hydroxylation.

Paul Schumacker (Northwestern University) summarized data showing that production of reactive oxygen species (ROS) by mitochondrial electron transport complex III is required for inhibition of prolyl hydroxylases and stabilization of HIF-1 α under hypoxic conditions (4).

SUMO, like ubiquitin, is a small protein that is covalently ligated to proteins as a post-translational modification. Addition and removal of SUMO are mediated by SUMOylases and deSUMOylases, respectively. **Edward Yeh** (University of Texas-M.D. Anderson Cancer Center) reported that knockout (KO) mice lacking expression of the deSUMOylase SENP1 die by embryonic day 15 with decreased erythropoietin mRNA and protein expression and decreased HIF-1 α protein stability. SENP1 deficiency increases HIF-1 α SUMOylation, which promotes the binding of VHL to HIF-1 α protein in a hydroxylation-independent manner (5).

In severely hypoxic cells translation is inhibited through the unfolded protein response leading to activation of endoplasmic reticulum (ER) kinase PERK and phosphorylation of eukaryotic translation initiation factor eIF2 α (6). Using the influenza virus hemagglutinin as a model protein, **Bradly Wouters** (Ontario Cancer Institute) reported that hypoxia reversibly inhibits disulfide bond formation in the ER.

Walter Boron (Case Western Reserve University) and **Jose Lopez-Barneo** (Hospital Universitario Virgen del Rocío) presented evidence that the water channel aquaporin 1 (AQP1) also functions as a gas channel. Cellular responses to hypoxia require rapid transmembrane gas equilibration, but gas permeability through the lipid bilayer is limited. 60% of CO₂ passes through red blood cells via AQP1, whereas only 5% of CO₂ is permeable through the lipid bilayer (7). AQP1 deficient mice were deficient in endurance exercise, which could be partially corrected by increasing the ambient O₂ concentration. AQP1 overexpression in PC12 cells was associated with increased expression of HIF-2 α and target genes under hypoxic conditions and more rapid loss of HIF-2 α upon reoxygenation, consistent with more rapid equilibration of intracellular and extracellular O₂ concentrations. (8).

Metabolism

Chi Dang (Johns Hopkins University School of Medicine) proposed that the functional interaction of c-Myc and HIF-1 is different under physiological versus tumorigenic conditions. Under non-tumorigenic conditions, HIF-1 α inhibits normal c-Myc expression and function at multiple levels to diminish mitochondrial function and increase glycolysis (22). In contrast, many tumors manifest deregulated expression of c-Myc, which collaborates with HIF-1 to enhance the adaptation of tumor cells to the hypoxic microenvironment by cooperatively activating the transcription of genes such as *HK2*, *PDK1*, and *VEGF* (9). Deregulated c-Myc expression is associated with increased ROS production and anti-oxidants such as N-acetylcysteine or vitamin C inhibit c-Myc-mediated tumorigenesis by reactivating PHD2 and diminishing HIF-1 α levels (10). Hence, HIF-1 α is essential for c-Myc-mediated tumorigenesis, and the cooperation of these two factors enhances the tumor metabolic phenotype characterized by enhanced glucose uptake and lactate production.

Kathleen O'Hagan (University College Dublin) discussed the regulation of mitochondrial biogenesis by the coactivator PGC-1 α , which is induced in skeletal muscle in response to exercise, in brown fat in response to cold exposure, and in the developing heart. She reported that PGC-1 α overexpression enhances mitochondrial biogenesis and O₂ consumption, leading to intracellular hypoxia, which decreases PHD activity and stabilizes HIF-1 α .

Julian Aragonés (Flanders Interuniversity Institute for Biotechnology) reported that KO mice lacking expression of PHD1 in skeletal muscle have decreased exercise tolerance and O₂ consumption, but have increased ischemic tolerance and that these phenotypes were mediated by HIF-2 α and PPAR- α (11).

Robert Abraham (Wyeth Pharmaceuticals) discussed the interplay between mTOR signaling and autophagy during metabolic stress in human cancer cells. He showed that mTOR inhibitors repress tumor growth and angiogenesis (12).

Keith Laderoute (SRI International) presented an overview of 5'-AMP-activated protein kinase (AMPK). Activation of AMPK is involved in the metabolic stress response and energy homeostasis and may promote the growth of some solid tumors (13).

ROS and Redox

Salvador Moncada (University College London) described interactions between the nitric oxide (NO) and hypoxia pathways. The NO pathway is involved in the neuronal, cardiovascular, and immune systems. Under hypoxic conditions, NO functions as an activator of soluble guanylate cyclase as well as an inhibitor of cytochrome *c* oxidase. NO promotes ROS production by reducing cytochrome *c*. Formation of ROS in the presence of NO regulates mitochondria redox states and cell respiration (14). NO inhibits the stabilization of HIF-1 α during hypoxia (15), which is respiration-dependent. As the mitochondrion is an important signaling organelle and HIF-1 α is a critical factor functioning in adaptation to hypoxia response, the relative levels of O₂ and NO may determine the signaling consequences of hypoxia.

Nanduri Prabhakar (University of Chicago) reported that sleep apnea, which affects ~4% of postmenopausal females and males over 45 years of age, causes chronic intermittent hypoxia (CIH), leading to an increased risk of hypertension, myocardial infarction, and stroke. In a mouse model, 10 days of CIH resulted in increased brain ROS and HIF-1 α levels, increased systemic blood pressure and plasma catecholamines, and ventilatory abnormalities, which could be blocked by concurrent anti-oxidant therapy (16). In mice heterozygous for a KO allele at the locus encoding HIF-1 α , all of the pathogenic responses to CIH were absent.

Stephen Archer (University of Chicago) reviewed the mechanism of hypoxic pulmonary vasoconstriction (HPV). Exposure of pulmonary artery smooth muscle cells (PASMCs) to hypoxia leads to inhibition of voltage-gate potassium (K_v) channels, increased membrane potential, activation of L-type Ca²⁺ channels, Ca²⁺ influx, and cell depolarization. HPV is lost in K_v1.5 KO mice. Chronic hypoxia results in loss of K_v1.5 expression, loss of HPV, and development of pulmonary arterial hypertension. (17,18). The expression of pyruvate dehydrogenase kinase (PDK) is increased in PAH and treatment of rats with the PDK inhibitor, dichloroacetate (DCA), reverses PAH.

Kay Macleod (University of Chicago) discussed the involvement of BNIP3 and NIX (BNIP3L) in hypoxia-induced autophagy (19). **Patrick van Uden** (Dundee University) reported that NF- κ B binds to *HIF1A* promoter and activates gene transcription.

Masayuki Yamamoto (Tohoku University) presented elegant work delineating the mechanism by which the transcription factor Nrf2 is activated by oxidant stress (20). Keap1 binds to Cul3 and forms an E3 ubiquitin ligase complex, which degrades Nrf2 under non-stress conditions. Oxidative stress inactivates Keap1 (by oxidation of cysteine residues 273 and 288), which leads to stabilization of Nrf2 followed by nuclear translocation and target

gene expression. Thus, the Keap1/Cul3/Nrf2 oxidative stress pathway is analogous to the VHL/Cul2/HIF-1 α hypoxia response pathway.

Adaptive Responses to Hypoxia

Gregg Semenza (Johns Hopkins University School of Medicine) described three recently-identified adaptive metabolic responses to hypoxia. First, HIF-1 coordinates a switch in the composition of cytochrome *c* oxidase (mitochondrial electron-transport chain complex IV) from COX4-1 to COX4-2 subunit utilization, which increases the efficiency of cytochrome *c* oxidase under hypoxic conditions (21). Second, HIF-1 activates transcription of the *PDK1* gene encoding a kinase that phosphorylates and inactivates pyruvate dehydrogenase, thereby shunting pyruvate away from the mitochondria by preventing its conversion to acetyl CoA (22). Third, HIF-1 represses mitochondrial biogenesis and respiration by inhibiting Myc activity (23). Each of those three responses is mediated by HIF-1 and inhibition of these responses increases ROS production and apoptosis under hypoxic conditions.

Joseph Garcia (University of Texas Southwestern) described the selective regulation of HIF-2 α by Sirt1, which is the most well characterized member of the mammalian sirtuin family. Sirt1 selectively augments the activity of HIF-2 α , but not HIF-1 α . Sirt1 augmentation requires its enzymatic activity as a histone deacetylase, and is mediated through interaction with the carboxyl terminal transactivation domain of HIF-2 α .

John Wood (University of Kansas) described the involvement of mast cells in hypoxia-induced microvascular inflammation in the mesenteric microcirculation (24). Hypoxia resulted in rapid mast cell degranulation and blockade of mast cell degranulation with cromolyn prevented or attenuated hypoxia-induced increases in ROS, leukocyte adherence, and vascular permeability. Antioxidant lipoic acid and NO/iNOS prevent mast cell degranulation during hypoxia. These results suggest that mast cells play a key role in hypoxia-induced inflammation and that alterations in the ROS-NO balance may be involved in mast cell activation during hypoxia.

MicroRNAs (miRs) are involved in a wide variety of normal and pathological cellular processes. **Mircea Ivan** (Tufts-New England Medical Center) reported that microarray-based expression profiles identified a specific spectrum of miRs that is induced in response to hypoxia, and some of these appeared to be regulated by HIF-1 (25). The vast majority of hypoxia-induced miRs are also overexpressed in human tumors (26). In hypoxic cells, HIF-1 α and HIF-2 α regulate the expression of miR-210, which functions as a prosurvival factor under stress conditions by inhibiting hypoxia-induced caspase activation and apoptosis.

Cynthia Beall (Case Western Reserve University) presented data regarding the distinct adaptations of Andean, Tibetan and East African indigenous high-altitude populations (27). Andean people have higher hemoglobin concentrations than Tibetans, who have higher hemoglobin concentrations than East Africans. Arterial O₂ content is also remarkably different among these groups. Tibetans have higher NO levels, higher pulmonary blood flow, and very high plasma nitrite and nitrate concentrations, which are unprecedented for healthy people (100 times higher than lowlanders).

Hypoxia and Development/Differentiation

Randall Johnson (University of California, San Diego) reported that hepatic erythropoietin (EPO) production was dramatically increased in a HIF-1 α /HIF-2 α -dependent manner in mice with VHL KO in the skin due to reduced hepatic blood flow. Treatment with the NO synthase inhibitor L-NAME prevented increased EPO production.

The prolyl hydroxylase domain proteins PHD1, PHD2, and PHD3 mediate O₂-dependent degradation of HIF- α subunits. **Guo-Hua Fong** (University of Connecticut Health Center) presented data on PHD KO mice (28,29). *Phd1*^{-/-} and *Phd3*^{-/-} mice were viable and did not manifest vascular defects. *Phd2*^{-/-} mice died at embryonic day 12.5 with cardiac malformations. Postnatal tamoxifen-inducible conditional KO of PHD2 led to increased tissue vascularization and increased vascular endothelial growth factor (VEGF) expression. Endothelial cell-specific PHD2 KO led to increased vessel size in the liver.

Erinn Rankin (University of Pennsylvania) used Cre-Lox mediated recombination to inactivate HIF-1 α or HIF-2 α and found that HIF-2 α is required for development of liver hemangiomas and for the development of hepatic steatosis in VHL deficient mice, whereas HIF-1 α regulates glycolysis (30).

Kidney disease affects over 20 million people in the U.S., and the glomerular filtration barrier is often the target of injury. **Susan Quaggin** (Samuel Lunenfeld Research Institute) reported that VEGF conditional KO in renal podocytes revealed that VEGF is critical for establishment and maintenance of the glomerular filtration barrier (31). Mice with VHL KO in podocytes developed rapidly progressive glomerulonephritis that was associated with increased CXCR4 expression and could be treated by CXCR4 inhibitors (32). Overexpression of CXCR4 in podocytes was sufficient to cause a mild glomerular disease. Collectively, these results suggest novel therapeutic targets for glomerulonephritis.

Ellen van Rooijen (Hubrecht Institute) showed that VHL deficiency in zebrafish embryos leads to severe polycythemia, increased vascularization, increased numbers of CD31⁺ hematopoietic stem cells, and increased heart rate, cardiac output, and ventilatory rate. Unlike mice, *vh1*^{-/-} zebrafish can complete embryogenesis without VHL. Death on day 8 (larval stage) may be due to heart failure.

Lorenz Poellinger (Karolinska Institutet) discussed O₂-dependent regulation of cellular differentiation. Exposure of human neuroblastoma cells to hypoxia decreased the expression of neuronal/neuroendocrine marker genes and induced expression of neural crest marker genes, including ID2, Notch, ID1, Hes1, and Hey1 (33). Hypoxia promotes the undifferentiated cell state in several stem/precursor cell populations in a Notchdependent manner (34). Hypoxia activates Notch-responsive promoters and increases expression of Notch target genes. The Notch intracellular domain (ICD) interacts with HIF-1 α , and HIF-1 α is recruited to Notchresponsive promoters upon Notch activation under hypoxic conditions.

Celeste Simon (University of Pennsylvania) provided new insight into the regulation of stem and progenitor cell differentiation by hypoxia. Oct-4, c-Myc, SOX2, β -catenin, and Notch are expressed in stem cells and are each regulated by HIF-1 α and/or HIF-2 α (35).

Endothelial Progenitors, Ischemia and Cardiovascular Disease

VEGF plays a critical role in hypoxia-driven angiogenesis. **Eli Keshet** (Hebrew University) reported that reversible VEGF blockade in the heart, by tissue-specific expression of soluble VEGFR1, results in a reversible reduction in myocardial vascularization and the induction of hibernation, in which regions of heart muscle cease contraction in an effort to conserve energy (36). He also reported a new role for VEGF in neurogenesis in the dentate gyrus of adult mice.

Hematopoietic stem cells (HSCs)/endothelial progenitor cells (EPCs) home to sites of VEGF and SDF-1 expression to repair injured retinal and choroidal vasculature by participating in the formation of new blood vessels. **Maria Grant** (University of Florida) discussed the role

of IGF binding protein-3 (IGFBP3) in HSC/EPC regulation. *IGFBP3* is a HIF-1 target gene, and IGFBP3 induces the migration of CD34⁺ EPCs to the ischemic retina, their differentiation, and incorporation into nascent capillaries (37). She noted that the products of three HIF-1 target genes, *VEGF*, *SDF1*, and *IGFBP3*, all have pro-angiogenic effects on EPCs and HSCs.

Vivek Mittal (Cold Spring Harbor Laboratory) presented evidence that bone marrow (BM)-derived EPCs contribute to blood vessel formation in breast tumors and that the CXCR4-SDF1 axis regulates EPC recruitment to tumors in mice. EPCs also participated in the vascularization of metastases. Tumors have high ID1 protein expression in EPCs and *Id1* promoter activity marks BM-derived EPCs and ECs. Expression of ID1 short hairpin RNA (shRNA) in BM leads to EPC loss-of-function, blocks metastasis formation and increases mouse survival (38). E4G10 is a monoclonal antibody that specifically recognizes unengaged VE-Cadherin on EPCs, but not on ECs. Injection into tumor-bearing mice of E4G10 conjugated to ²²⁵Actinium resulted in a reduction in EPCs.

Progenitor cells derived from bone marrow, from circulating blood or tissue-resident stem cells may contribute to the functional recovery and repair of infarcted myocardium by promoting neovascularization in the ischemic area and cardiac regeneration (39). **Stefanie Dimmeler** (University of Frankfurt) provided new insights regarding bone marrow progenitor cell therapy for myocardial infarction. She reported that circulating EPCs isolated from patients during cardiopulmonary bypass expressed mesenchymal and endothelial markers (suggestive of a “mesoangioblast” phenotype). The cells expressed the stem cell markers OCT3/4, KLF4, and c-Myc as well as Islet-1, which is expressed by cardiac progenitor cells.

Cancer

William Kaelin (Dana Farber Cancer Institute) discussed the von Hippel-Lindau syndrome, which is characterized by cerebellar hemangioblastoma, clear cell renal carcinoma, and pheochromocytoma, and is caused by inactivating mutations of the *VHL* tumor suppressor gene. Pheochromocytomas arise from sympathetic neuronal precursor cells, which are normally programmed to undergo apoptosis in response to NGF withdrawal (40), a process that requires c-Jun, PHD3, and the kinesin family member KIF-1B β . The mechanism by which PHD3 induces KIF-1B β activity is unknown. Missense mutations in the gene encoding KIF-1B β that impair the pro-apoptotic function of the protein have been identified in medulloblastoma, neuroblastoma, and pheochromocytoma.

HACE1 has tumor suppressor activity and its loss-of-function occurs in > 75% of Wilms tumor cases (41). **Poul Sorensen** (British Columbia Cancer Research Center) presented data showing that HACE1 is a novel E3 ubiquitin ligase that promotes the degradation of HIF-1 α and HIF-2 α under hypoxic conditions in a VHL-independent manner.

Jacques Pouyssegur (University of Nice) discussed two functions of HIF-1 in hypoxic tumor cells: autophagy, which is mediated by BNIP3/BNIP3L; and extracellular acidification, which is mediated by the sodium hydrogen exchanger NHE1, monocarboxylate (lactate) transporters MCT1 and MCT4, and carbonic anhydrases CA9 and CA12 (42).

Amato Giaccia (Stanford University) reported that expression of shRNA against PHD2 enhanced tumor xenograft growth, in a HIF-1 α /HIF-2 α -independent manner, and increased angiogenin and IL-8 expression, an effect that was independent of PHD2 hydroxylase activity, leading to increased tumor angiogenesis.

Erwin van Meir (Emory University) reported that the low molecular weight compound KCN1 blocks HIF-1 transcriptional activity and inhibits tumor growth by reducing nuclear levels of heat shock protein 90 (HSP90). KCN1 binds to Plectin-1 directly and Plectin-1 is required for nuclear exclusion of HSP90 by KCN1.

Mark W. Dewhirst (Duke University) reviewed studies demonstrating that radiotherapy or chemotherapy increased HIF-1 α and VEGF expression in tumor cells, which protected the tumor microvasculature from therapy-induced apoptosis (43).

Annamaria Rapisarda (SAIC-Frederick, Inc.) presented data on the increased anti-tumor effect of combining the anti-angiogenic agent bevacizumab with the HIF-1 α inhibitor Topotecan. The results suggested that blocking hypoxia-dependent survival pathways may improve the efficacy of anti-angiogenic therapy.

Respiratory Disease

Peter Robbins (University of Oxford) described enhanced ventilatory and pulmonary vascular responses to hypoxia in patients with Chuvash polycythemia, who are homozygous for a missense mutation in the *VHL* gene. He also reported that infusion of the iron chelator desferrioxamine results in enhanced pulmonary vascular responses to hypoxia in normal volunteers (44).

Larrisa Shimoda (Johns Hopkins University School of Medicine) reported that hypoxia induces HIF-1-dependent decreased expression of K_v1.5 and K_v2.1 channels, increased expression of TRPC1 and TRPC6 calcium channels, and increased expression of NHE1 sodium-hydrogen exchanger in PSMCs, leading to alterations in intracellular K⁺ and Ca²⁺ levels, membrane potential, and pH that contribute to vasoconstriction and vessel remodeling, which result in PAH in animals subjected to chronic hypoxia (45,46).

Michael Maitland (University of Chicago) described a Phase I trial of sorafenib in PAH patients. Sorafenib is an inhibitor of multiple receptor tyrosine kinases, including VEGFR2, and is approved for the treatment of renal cell carcinoma (47).

Agnes Görlach (German Heart Center Munich) presented data regarding stress-responsive serum- and glucocorticoid-inducible kinase SGK1, the expression and activity of which is induced by hypoxia. SGK1 overexpression up-regulates HIF-1 α mRNA and protein levels and increases HIF-1 target gene transcription. *Sgk1*^{-/-} cells have reduced HIF-1 α expression and *Sgk1*^{-/-} mice have reduced pulmonary vascular remodeling in response to hypoxia. It is not known whether SGK1 directly regulates HIF-1 α or acts through other pathways, such as PI3K/AKT or NF- κ B (48).

Michail Sitkovsky (Northeastern University) reported that hypoxia increases extracellular adenosine levels, which inhibits T cell killing activity, thus contributing to the Hellstrom paradox (49): T cells kill tumor cells *ex vivo* but not *in vivo*. Inhibitors of the A2a adrenergic receptor may be useful in promoting the activity of cytotoxic T cells as a cancer therapy. Hypoxia-induced adenosine production is also important in limiting collateral damage during the immune response to pathogens as in the case of pneumonia. Administration of O₂ at high concentrations blocks hypoxic activation of the adenosinergic pathway and exacerbates the inflammatory lung injury.

Conclusion

While it has been billions of years since O₂ became established as the substrate of life and hundreds of years since the scientific discovery of O₂, our understanding of biological

responses to hypoxia and their medical significance has greatly increased only within the last several decades. The rapid progress being made on multiple fronts is both remarkable and exciting, especially with regard to the prospects for translation of basic science advances into novel treatments for cancer, cardiovascular disease, and other common causes of mortality.

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