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Review

Physiological and Pathological Responses to Hypoxia

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As the average age in many countries steadily rises, heart infarction, stroke, and cancer become the most common causes of death in the 21st century. The causes of these disorders are many and varied and include genetic predisposition and environmental influences, but they all share a common feature in that limitation of oxygen availability participates in the development of these pathological conditions. However, cells and organisms are able to trigger an adaptive response to hypoxic conditions that is aimed to help them to cope with these threatening conditions. This review provides a description of several systems able to sense oxygen concentration and of the responses they initiate both in the acute and also in long-term hypoxia adaptation. The role of hypoxia in three pathological conditions, myocardial and cerebral ischemia as well as tumorigenesis, is briefly discussed. ***(Am J Pathol 2004, 164:1875–1882)***

The ability to maintain oxygen homeostasis is essential to the survival of all vertebrate species. Physiological systems have evolved to ensure the optimal oxygenation of all cells in each organism. This occurred through the evolution of a complex physiological infrastructure for O_2 delivery that includes an entry (lungs), transport vehicle (erythrocytes), a highway and secondary road system (vasculature), and a propulsion device (heart). The precise establishment of these systems during development and their regulation in organisms provide the basis for oxygen homeostasis.

Sensing of increased (hyperoxia) or decreased (hypoxia) O_2 level occurs through specialized chemoreceptor cells that regulate cardiovascular and ventilatory rates. In addition, all nucleated cells sense O_2 concentration and respond to reduced O_2 availability acutely (within minutes) through the activation of pre-existing proteins and chronically (within hours) through the regulation of gene transcription. The first part of this review will provide a description of these systems. Not only is O_2 homeostasis essential for survival, but hypoxia plays also an important role in the pathogenesis of frequent and severe pathologies including myocardial and cerebral ischemia and cancer. In the second part of the review, the role of hypoxia in these pathological conditions will be described.

Physiological Responses to Hypoxia

Systemic Responses

During episodes of compromised O_2 availability, several chemosensory systems, acting in concert, rapidly modulate pulmonary ventilation and perfusion as well as blood circulation to optimize the supply of O_2 to metabolizing tissues. These responses rely both on specialized chemoreceptor cells such as the carotid bodies in the arterial circulation and neuroepithelial bodies present in the airway and on the direct response of vascular smooth muscle cells to hypoxia.

Vascular Smooth Muscle Cells

Peripheral vessels dilate in response to low oxygen, whereas the vessels of the pulmonary vasculature constrict to shunt blood away from the poorly ventilated region, thereby matching ventilation to perfusion.[1] Hypoxic pulmonary vasoconstriction is a fast response that occurs in pulmonary arteries and veins but is greatest in small resistance arteries. It is intrinsic to pulmonary vasculature smooth muscle cells and is initiated by inhibition of one or several K^+ channels which set the membrane potential.[2] The resulting depolarization activates voltage-gated Ca^{2+} channels, which raises the cytosolic calcium

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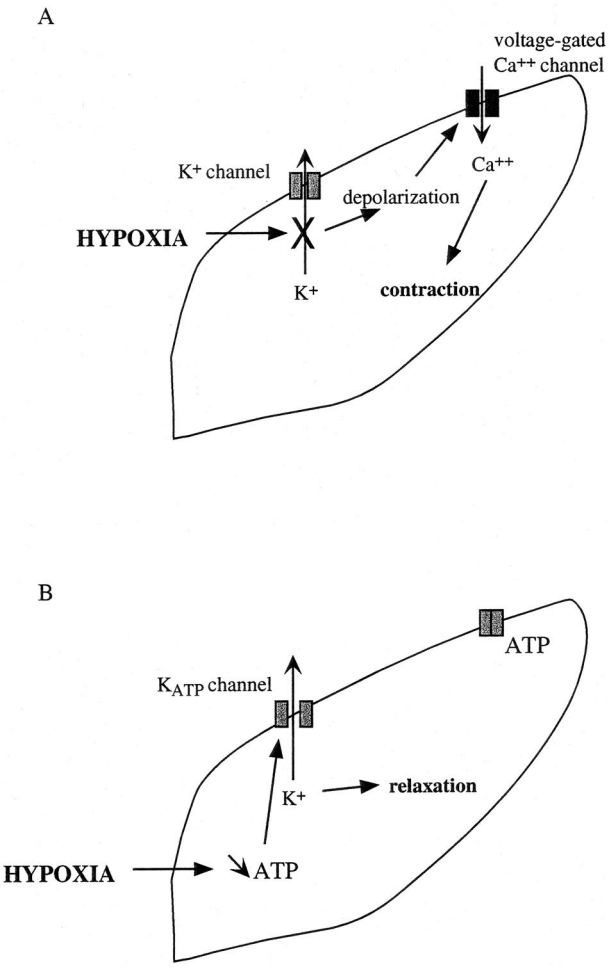

Figure 1. Schematic representation of the response of vascular smooth muscle cells to hypoxia. **A:** Pulmonary smooth muscle cells. **B:** Peripheral smooth muscle cells.

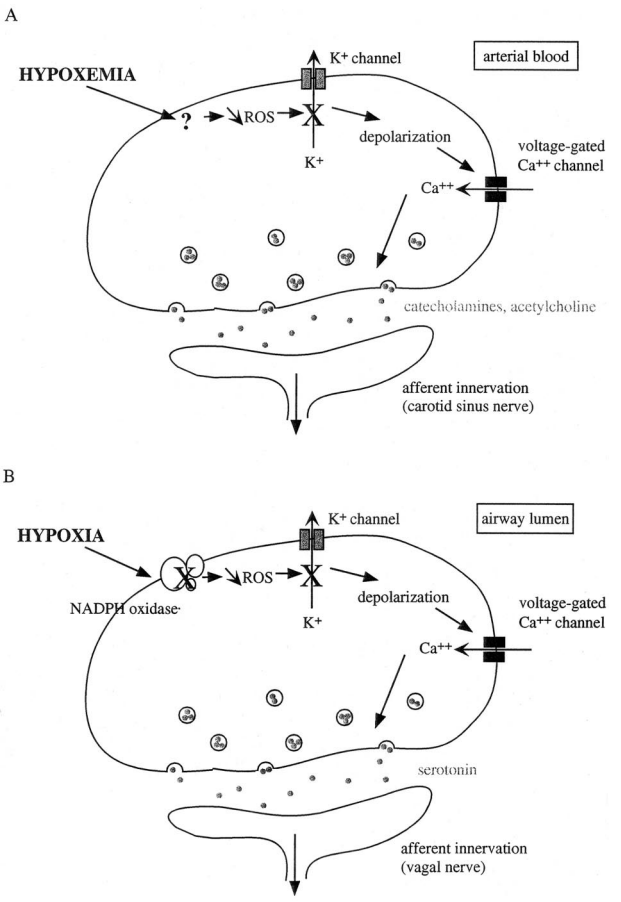

Figure 2. Schematic representation of the response of carotid and neuroepithelial bodies to hypoxia. **A:** Carotid bodies. **B:** Neuroepithelial bodies.

level and causes myocyte constriction (Figure 1A). While K^+ channels are the effectors of hypoxic pulmonary vasoconstriction, it is not clear whether they are intrinsically O_2-sensitive or under the control of a "real" O_2 sensor. Recent work from Archer et al[3] proposes that mitochondria, through the production of oxygen reactive species, could modulate the activity of the channel. Indeed, certain K_v channels are rich in cysteines and respond to the local redox environment, tending to open when oxidized and close when reduced.

Hypoxic vasodilation is another fast response that increases perfusion of blood to the O_2-deprived tissues. This is particularly well evoked in coronary and cerebral vessels. Hypoxic vasodilation is at least in part mediated by the K_{ATP} channels of vascular smooth muscle cells which open in response to hypoxia-induced decrease in ATP (Figure 1B).[4] However, there are other O_2-sensitive ionic mechanisms also involved, most probably by regulating Ca^{2+} influx.

Carotid and Neuroepithelial Bodies

Airway neuroepithelial bodies sense changes in inspired oxygen, whereas arterial oxygen levels are monitored by the carotid bodies. Both respond to decreased O_2 supply by initiating activity in efferent chemosensory fibers to produce cardiorespiratory adjustments on exposure to environmental low pO_2.[5,6]

Carotid bodies are highly vascularized organs, located at the bifurcations of the common carotid arteries. The sensory elements of the carotid bodies are type I (glomus) cells, which contain neurotransmitters, mainly catecholamines and acetylcholine. They lie in synaptic contract with efferent sensory fibers of the carotid sinus nerve. Neuroepithelial bodies are located at airway bifurcations. They are clusters of neuron-derived cells that synapse with branches of both afferent and efferent (vagal nerve) neurons, initiating information to the respiratory centers by the release of neuromediators, particularly 5-hydroxytryptamine (serotonin).

Excitation of chemoreceptor cells by hypoxia/hypoxemia depends on the presence of membrane K^+ channels whose activity is inhibited by low pO_2. Although the type of O_2-sensitive K^+ channels may differ from type I cells to neuroepithelial body cells, decreased O_2 level leads to closure of these K^+ channels and hence to membrane depolarization and Ca^{2+} influx through voltage-gated Ca^{2+} channels. The resulting increased cytosolic calcium concentration provokes neurotransmitter release and activation of efferent sensory fibers (Figure 2).

The actual O_2 sensor in glomus and neuroepithelial body cells is thought to be a heme-containing protein which is closely associated with the O_2-sensitive K^+ channel. In neuroepithelial bodies, a number of lines of evidence point toward a significant involvement of NADPH oxidase. The model suggests that under normoxic conditions, the oxidase generates reactive oxygen species which are believed to promote channel activity. Under hypoxic conditions, the level of reactive oxygen species drops, thereby inducing K^+ channel inhibition.[7,8] The observation that neuroepithelial body cell K^+ currents recorded in neuroepithelial bodies derived from gp^{91phox} (one of the subunits of NADPH oxidase) knockout mice were insensitive to hypoxia reinforce this hypothesis.[9]

In contrast, the role of NADPH oxidase as well as of mitochondria in the oxygen-dependent K^+ channel inhibition in carotid bodies has been largely discounted. The identification of the putative heme-containing O_2 sensor in these cells is still under investigation: NO synthase and heme oxygenase are possible candidates.[10] O_2 sensing in neuroepithelial and carotid bodies therefore exhibits diverse (ie, the upstream O_2 sensor and the nature of the neurotransmitters released are different) yet convergent mechanistic features, allowing the organism to face a potentially deleterious O_2 supply.

Regulation of Cellular Metabolism

One of the most fundamental parameters that healthy cells must maintain is a high content of ATP. Indeed, almost all energy-requiring processes in cells are driven, either directly or indirectly, by hydrolysis of one or two acid bouds in ATP. Maintenance of a homeostatic intracellular environment through the use of ATP-dependent ion pumping systems such as the Na^+/K^+ ATPase consumes 20 to 80% of the cell's resting metabolic rate.

Cell death occurs when ATP production fails to meet the energy maintenance demands of ionic and osmotic equilibrium. When the ATP level declines, a failure of ion-motive ATPase occurs, leading to membrane depolarization, uncontrolled Ca^{2+} influx through voltage-gated Ca^{2+} channels, and subsequent activation of calcium-dependent phospholipases and proteases. These events result in uncontrolled cell swelling, hydrolysis of main cellular components, and eventually to cell necrosis (Figure 3).[11]

Effect of Hypoxia on Mitochondria

Oxygen limitation is generally considered as an impairment of mitochondrial respiration under hypoxia or ischemia. Indeed, mitochondria are the main source of high energy phosphate bond molecules in normal cells.

Electron transport from the oxidation of NADH and $FADH_2$ to O_2 is tightly coupled to the synthesis of ATP. The electron transport occurs through protein-bound redox centers, from complex I (NADH-coenzyme Q reductase) or II (succinate-coenzyme Q reductase) to III (Coenzyme Q-cytochrome *c* reductase) and then to IV

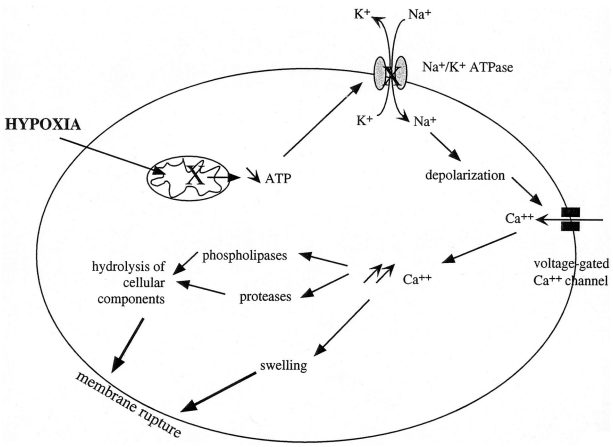

Figure 3. Schematic representation of the cascade leading to cell death when cells are exposed to severe hypoxia.

(cytochrome *c* oxidase). The free energy released by this transport is conserved by pumping out protons to create an electrochemical H^+ gradient across the inner mitochondrial membrane. The electrochemical potential of this gradient is then harnessed in the synthesis of ATP by complex V (ATP synthase): this process is known as oxidative phosphorylation.

Studies on isolated mitochondria have shown that the main effects of decreased oxygen availability on mitochondrial respiration is an inhibition of the respiratory chain and an increase of the proton leak, while phosphorylation is less affected.[12,13] The inhibition of the respiratory chain occurs at pO_2 levels high above the K_m of cytochrome *c* oxidase, indicating that a specific inhibiting mechanism still unknown is switched on well before oxygen concentration by itself would limit the activity of this enzyme.

Adaptation to Hypoxia

At the cellular level, adaptation to hypoxia is brought about on one hand by increasing the efficiency of energy-producing pathways, mainly through increased anaerobic glycolysis activity, and on the other hand by decreasing energy-consuming processes.[14] Both effects are described below.

Ion-motive ATPases and protein synthesis are the dominant energy-consuming processes of cells at standard metabolic rate, making up to more than 90% of the ATP consumption in rat skeletal muscle and as much as 66% in rat thymocytes.[15] Reallocation of cellular energy between essential and nonessential ATP demand processes as ATP supply becomes limiting is thus the key for cells to survive decreased O_2 levels. Studies on hepatocytes have shown that protein synthesis is largely inhibited in response to hypoxia.[16] Buttgereit and Brand[17] demonstrated that, in fact, the ATP consuming processes are arranged in a hierarchy, with protein synthesis and RNA/DNA synthesis being the first to be inhibited as energy becomes limiting and with Na^+/K^+ pumping and Ca^{2+} cycling taking the greatest priority. This phenomenon, known as oxygen conformance, involves precise

regulatory mechanisms notably at the level of translation initiation.[18]

Differential sensitivity to hypoxia-induced cell death in different cell types seems at least in part to be due to the extent of their electric activity, ie, to the relative importance of the ATPase ionic pumps versus other ATP-consuming processes. This ion pumping represents as much as 80% of ATP consumption in neurons while only 20% in skeletal muscle cells. In case of severe O_2 limitation, most excitable cells cannot continue to meet the energy demands of ion transporting systems, leading to cell death.[19] On the other hand, the metabolic suppression response is particularly well characterized in intact heart, where decreases in myocardial oxygen delivery result in decreased contractile activity and O_2 consumption in a phenomenon called "hibernating myocardium."[20,21]

In addition to the energy-balanced metabolic suppression, cells turn to glycolysis to meet their energetic demands in hypoxia. The switch between the two pathways of ATP regeneration from the oxygen-dependent mitochondrial respiration to the oxygen-independent glycolysis was first noted by Pasteur in the late 19th century, hence its name "Pasteur effect." Although glycolysis is less efficient than oxidative phosphorylation in the generation of ATP, in the presence of sufficient glucose, glycolysis can sustain ATP production due to increases in the activity of the glycolytic enzymes. This is manifested within minutes in the allosteric regulation of phosphofructokinase activity and chronically, in the HIF-1-dependent overexpression of many glycolytic enzymes (see below).

Phosphofructokinase is considered to be the major regulator controlling carbon flux through glycolysis. It is allosterically activated by ADP and AMP and inhibited by ATP, hence setting the glycolytic rate according to the energy demand. However, the most potent allosteric activator is fructose-2,6-biphosphate.[22] The synthesis and degradation of fructose-2,6-biphosphate depends on a single enzyme, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK-2). This enzyme is regulated within minutes by phosphorylation through the AMP-activated protein kinase (AMPK),[23] but its expression is also enhanced through transcriptional activation via HIF-1.[24] AMPK phosphorylates PFK-2 at a single site, resulting in the increase of the V_{max} of the kinase activity, thus in an increased production of fructose-2,6-biphosphate and allosteric activation of phosphofructokinase.

As well as phosphofructokinase, other metabolic enzymes are also regulated by AMP, ADP, and ATP and in the 1960s, Atkinson[25] proposed that most of the branch points between anabolism and catabolism would be controlled by these nucleotides. This was termed the "adenylate control." In 1987, Hardie's laboratory[26] identified AMPK as the primary sensor of cellular energy charge, and most of the effects of imbalance between ATP and AMP are now thought to be mediated through this kinase pathway rather than through direct effects of adenine nucleotides on enzymes. AMPK is a heterotrimeric protein comprising α, β, and γ subunits. The α subunit contains the kinase domain and an autoinhibitory region that inhibits the kinase in the absence of AMP. The β subunit appears to be the scaffold and the γ subunit might be the AMP-binding subunit. AMP binds at the interface between α and γ subunits, preventing association of the autoinhibitory region and the kinase domain, hence activation the enzyme.[27]

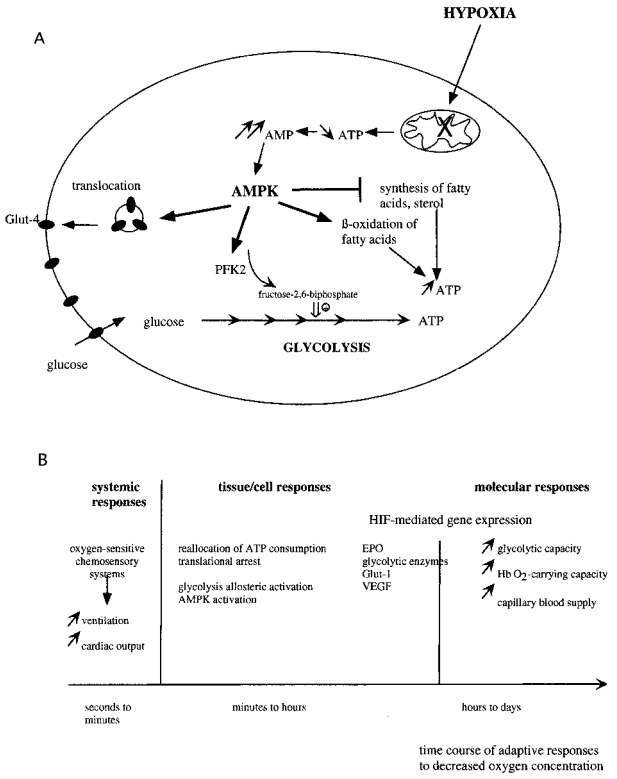

Figure 4. Schematic representation of the adaptive responses of cells to hypoxia. **A:** Schematic representation of the role of AMPK in the acute adaptation of cell metabolism to hypoxia. PFK2, 6-phosphofructose-2-kinase. **B:** Schematic representation showing the time course of metabolic adaptive responses to hypoxia. HIF, hypoxia-inducible factor; EPO, erythropoietin; VEGF, vascular endothelial growth factor; Hb, hemoglobin.

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The activated kinase switches on catabolic pathways that generate ATP and switches off anabolic pathways that consume ATP (Figure 4A).[28,29] It is achieved both acutely via direct phosphorylation and chronically via effects of gene expression. The phosphorylation of PFK-2 is one such example. AMPK activation has also been reported to stimulate translocation of the glucose transporter Glut-4 to plasma membrane and consequent glucose uptake. In the longer term, it increases the expression of Glut-4, hexokinase, and mitochondrial enzymes involved in the tricarboxylic acid cycle and respiratory chain. On the other hand, AMPK directly inhibits fatty acid, triglyceride, and sterol synthesis and the expression of enzymes of fatty acid synthesis and gluconeogenesis.[27]

Regulation of Gene Expression

Several responses are developed by cells and tissues faced with a hypoxic challenge: 1) increased ventilation and cardiac output, 2) a switch from aerobic to anaerobic metabolism, 3) promotion of improved vascularization, and 4) enhancement of the O_2 carrying capacity of the blood. Most of these processes take place very early at

the onset of hypoxia and occur through the activation of already present proteins; but in the longer term, all of them are also mediated by up-regulation of genes encoding key actors of these responses (Figure 4B). For example, such genes are: 1) tyrosine hydroxylase, which is involved in dopamine synthesis in carotid body type I cells; 2) the glycolytic enzymes phosphoglycerate kinase 1, pyruvate kinase m, phosphofructokinase, aldolase A, glyceraldehyde 3-phosphate dehydrogenase, enolase 1, and glucose transporters Glut-1 and Glut-4; 3) VEGF, PDGF to induce angiogenesis, and inducible NO synthase that increases vasodilation; and 4) erythropoietin and transferrin receptors that favor erythrocyte production.[30] This transcriptional response is mediated in large part by the action of hypoxia-inducible factor-1 (HIF-1).

HIF-1 is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator). Both subunits belong to the Per-ARNT/Ahr-Sim family of the bHLH (basic helix-loop-helix) transcription factors. HLH and PAS motifs are involved in the dimerization, while the basic helix is the DNA binding domain. HIF-1α contains two transactivation domains in the C-terminus end of the protein. ARNT is constitutively expressed and is located in the nucleus. On the other hand, while the HIF-1α mRNA levels are constant in normoxia and in hypoxia, the protein is rapidly degraded in normoxia, whereas it accumulates in hypoxia. In normoxia, HIF-1α is polyubiquitinylated and targeted to proteasome degradation. Of note, besides the fact that overall protein synthesis is diminished in hypoxia, ARNT and HIF-1α proteins are efficiently translated in normoxia and in hypoxia due to the presence of an internal ribosome entry site in their corresponding mRNA.[31]

HIF-1α contains an oxygen-dependent degradation domain within which there is a highly conserved binding domain for the tumor suppressor von Hippel-Lindau protein (pVHL). pVHL organizes the assembly of a complex that activates the E3 ubiquitin ligase which then ubiquitinylates HIF-1α, targeting its degradation. Inactivation of pVHL is associated with the von Hippel-Lindau cancer syndrome. pVHL mutations prevent its binding to HIF-1α, leading to constitutive expression of this transcription factor and its target genes. Such mutations increase the potential for angiogenesis, probably through continuous synthesis of VEGF. The interaction between HIF-1α and pVHL is regulated through hydroxylation of two proline residues of HIF-1α by a prolyl hydroxylase enzyme. In the absence of oxygen, this enzyme is no longer active: the unmodified prolyl-HIF-1α does no longer interact with pVHL and accumulates.[32,33] The absolute requirement for oxygen of this prolyl hydroxylase indicates that this enzyme may function as a direct oxygen sensor.

Other data show that stabilization and/or synthesis of HIF-1α under hypoxia is also dependent on the PI-3 kinase/Akt pathway. Indeed, the use of PI-3K inhibitors inhibits the accumulation of HIF-1α in these conditions.[34] Moreover, dominant negative mutants for PI-3K or for Akt decrease the hypoxia-induced overexpression of VEGF. Conversely, disruption of PTEN, a phosphatidylinositol triphosphate phosphatase that inactivates Akt, leads to increased VEGF expression in normoxia. Finally, growth factor- or cytokine-induced activation of HIF-1 in normoxia results from an increased synthesis of HIF-1α which is also dependent on the PI3K/Akt pathway.[35] However, it is currently unclear how the PI-3K/Akt pathway would interact with the prolyl hydroxylase-pVHL system to regulate HIF-1α protein level.

Stabilization of HIF-1α is only the first step of HIF-1 activation: adequate redox conditions, dissociation from the chaperone hsp90, association with co-activators like CBP/p300 or SRC-1 as well as phosphorylation[36] are also required for full transcriptional activity.[37] Hypoxia directly regulates the association of HIF-1α with the coactivator CBP/p300. Similarly to prolyl hydroxylase, an asparagyl hydroxylase, whose activity strictly depends on the presence of oxygen, hydroxylates HIF-1α carboxyl-terminal transactivation domain on Asn 803. This modification prevents its association with CBP/p300 in normoxia.[38]

Not only is HIF-1 required for a variety of physiological responses to chronic hypoxia, but it is also essential for embryonic survival and cardiac and vascular development. Hif1a$-/-$ mice are not viable: development of Hif1a$-/-$ embryos arrests by day E9.0 and mice die by E10.5.[39,40] There is a marked regression of blood vessels in the cephalic region and replacement by a smaller number of enlarged vascular structures. Loss of pericyte support of the endothelium leading to vascular regression is probably responsible for these defects. Concomitant with the disruption of the vascular development, massive cell death is observed in the cephalic mesenchyme. Cardiac development is also abnormal in Hif1a$-/-$ embryos. Embryonic lethality was also observed in ARNT$-/-$ mice probably due to failure of the embryonic component of the placenta to vascularize.[41] Similar vascular defects are observed in HIF-1α- and VEGF-deficient embryos indicating that hypoxia-induced VEGF overexpression is needed for harmonious development of the vascular system.

Pathological Responses to Hypoxia

Hypoxia, due to impaired blood flow, has hazardous effect on organ structure and function. This is especially the case in stroke (cerebral ischemia) and heart infarction (myocardial ischemia). Hypoxia plays also a crucial role in regulating tumor growth and metastasis. We shall briefly describe the role of hypoxia in these three pathological conditions.

Cerebral Ischemia

The high energy requirements compared to the low energy reserves render the brain particularly vulnerable to hypoxic conditions. Although it constitutes only a small fraction of total body weight (2%), it accounts for a disproportionately large percentage of O_2 consumption (about 20%). Under physiological conditions, enhanced demand for O_2 is rapidly and adequately matched by an increase in cerebral blood flow. However, in children who suffer asphyxial events or in adults undergoing stroke, hypoxemia and ischemia respectively result in brain in-

jury. The longer the duration of hypoxia/ischemia, the larger and more diffuse the areas of the brain that are affected. The most vulnerable areas seem to be the brainstem, hippocampus and cerebral cortex. Injury progresses and eventually becomes irreversible except if oxygenation is restored. Acute cell death occurs mainly through necrosis but hypoxia also causes delayed apoptosis. In addition to the deleterious processes described previously, massive glutamate release from presynaptic neurons further enhances Ca^{2+} influx and catastrophic collapse in postsynaptic cells. It has to be noted that, even if it is the only way to save the tissue, reperfusion also induces cell death, mainly through reactive oxygen species production and inflammatory cell infiltration. If the decrease in pO_2 is not too severe, cells suppress some of their functions, ie, protein synthesis and spontaneous electrical activity, in a process called "penumbra" which is characterized by reversibility, provided that O_2 supply is resumed.[42,43]

Myocardial Ischemia

Acute coronary syndromes resulting from occlusion of one of the coronary expose the heart to ischemic conditions. Brief periods of ischemia (<20 minutes) are reversible if followed by reperfusion. They are not associated with development of necrosis but result in the phenomenon of stunning. If duration of coronary occlusion is prolonged beyond this point, a wavefront of necrosis propagates from subendocardium to subepicardium. Reperfusion beyond a few hours does not reduce myocardial infarct size.

Within seconds of cessation of blood flow, energy metabolism shifts from mitochondrial respiration to anaerobic glycolysis. Simultaneously, effective contractions diminish and then cease. Lactate and protons accumulate in cardiomyocytes, inducing acidosis and osmotic load and then cell edema. In addition, intracellular Ca^{2+} rises probably due to the combined action of the Na^+/H^+ and Na^+/Ca^{2+} exchangers activated by cellular acidosis. If prolonged, this will eventually lead to cell necrosis.[44]

Restoration of arterial flow is necessary to restore aerobic metabolism and save the ischemic myocytes. It, however, induces by itself further damage: this process has been termed "reperfusion injury." A form of reperfusion injury is myocardial stunning, which was described by Braunwald and Kloner[45] as "prolonged, postischemic dysfunction of viable tissue salvaged by reperfusion," ie, myocardium exhibits temporary contractile failure even though it is alive and aerobic. A burst of reactive oxygen species liberated during the first few minutes of reperfusion is probably the cause of this contractile failure. Alterations in Ca^{2+} homeostasis rather than alteration of the contractile apparatus are probably the consequence of reactive oxygen species generation and the origin of this dysfunction.[46]

Tumor Angiogenesis

Oncogene activation and tumor suppressor inactivation result in deregulated cellular proliferation. However, most tumors grown larger than 1 mm^3 contain regions of low oxygen tension (hypoxia) due to an imbalance between oxygen supply and consumption. Formation of new blood vessels or "neoangiogenesis" is thus essential for further tumor growth. It is also important to allow tumor cell dissemination at distant sites, ie, metastasis.

The construction of this new vascular network requires different sequential steps including the release of proteases from "activated" endothelial cells with subsequent degradation of the basement membrane, migration of endothelial cells into the interstitial space, endothelial cell proliferation and differentiation into mature blood vessels. These processes are mediated by a wide array of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules. This angiogenesis process requires many interactions that must be tightly regulated in a spatial and temporal manner.[47]

The onset of neovascularization in primary tumor has been described as the angiogenic switch. Various signals that trigger this switch have been identified, including immune/inflammatory response and genetic mutations, but metabolic stress (hypoxia) is probably the most important one.[48,49] Tumor cells survive fluctuations in oxygen tension through activation of hypoxia-inducible factor-1.

Several studies using HIF-1 mutant cells have shown that HIF-1 has a profound effect on tumor biology. For example, tumors grown from HIF-1α-defective embryonic stem cells display abnormal vascularity and reduced growth rate.[40] Moreover, HIF-1 is up-regulated in a broad range of cancers, and there is a significant correlation between tumor grade, vascularization and HIF-1α overexpression.[50,51] This pattern of expression suggests that the tumor cells are responding to hypoxia by HIF-1-mediated expression of angiogenic proteins. Among them, VEGF is probably the most potent one, and its expression is regulated by HIF-1. In addition to promote VEGF release, HIF-1 is also essential for tumor cell adaptation to hypoxia, notably via increasing glycolysis capacity.[52]

Detection of Hypoxia

Tumor hypoxia is a strong prognostic factor for outcome in various cancers. Reduced drug delivery to hypoxic cells for chemotherapy as well as intrinsic radioresistance of hypoxic cells are contributing factors, which in addition to apoptosis resistance and enhanced metastasis capacity, all contribute to this poor outcome. Methods for determination of tumor oxygenation are thus of considerable clinical relevance. In addition to methods evaluating oxygen transport,[53] more and more studies are devoted to defining a good hypoxia marker usable in immunomicroscopic studies. The use of 2-nitromidazole drugs that specifically bind to hypoxic cells has been largely advocated, pimonidazole and EF5 being the most well known. Reductive enzymes metabolize these drugs in the presence of oxygen, while when oxygen is absent, the extra electrons are not removed, and the drugs are converted to highly reactive free radical molecules that

covalently bind to proteins and DNA. The drug-protein adduct can then be detected by specific antibodies. Studies like the one from Evans et al[54] nicely demonstrated the suitability of this method. However, these drugs present the disadvantage that they need to be administered before sampling the tissue. Therefore, the search for possible surrogate markers for hypoxia allowing retrospective studies is still growing.

The discovery that HIF-1α is specifically up-regulated under hypoxia and rapidly degraded in the presence of oxygen raised the hope that this protein was potentially such an endogenous marker. Several works evaluating HIF-1α as an endogenous marker for hypoxia have confirmed spatial colocalization of HIF-1α and EF5 or pimonidazole,[55] but also evidenced differences in pO_2 value threshold for EF5 binding and HIF-1α expression, the former occurring at lower oxygen concentration but also in temporal localization: EF5 or pimonidazole binding representing a summation of the "history of hypoxia" over the incubation period while HIF-1α, since rapidly degraded on reoxygenation, providing a "snapshot" of hypoxia at the time of surgery.[56] It has to be noted that it might not be straightforward to employ HIF-1α as a reliable hypoxia marker, since HIF-1α levels are also regulated by factors other than hypoxia for example resulting from oncogenic mutations.[57] This issue thus deserves further investigation.

Since HIF-1 transcriptional activity may be more tightly regulated than HIF-1α level, the expression of HIF target genes would be a convenient alternative to assess tumor hypoxia. Studies demonstrating colocalization of carbonic anhydrase 9 and GLUT-1 expression with pimonidazole staining support this hypothesis.[58,59]

Not only are these approaches using pimonidazole staining, HIF-1α protein level, or HIF-1 target gene expression useful to define hypoxic areas in tumors, but they have also been successful in other pathological ischemic situations and have yielded interesting results.[60,61]

Conclusion

Better delineation of hypoxic areas in ischemic tissues or in growing tumors as well as expanding knowledge of the cellular and molecular responses to hypoxia leads to possible applications in the treatment of major diseases associated with tissue hypoxia. Targeting HIF for cancer therapy[62] or injecting VEGF or VEGF-expressing vectors to restore vascularization in ischemic organs are such possibilities, among others yet to be developed.

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