

OXYGEN, HYPOXIA AND STRESS

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ABSTRACT: Since cyanobacteria began to photosynthesise and introduced the colourless and odorless gas oxygen into the earth's atmosphere some 2.5 billion years ago, human evolution has been intrinsically linked to this critical molecule. Initially, the electrophilic chemical properties of oxygen rendered it a formidable toxic challenge to organisms however, eukaryotic cells, following the incorporation of bacterial-derived mitochondria, evolved to make beneficial use of the chemical properties of molecular oxygen as the final electron acceptor in the highly efficient production of cellular energy supplies in the form of adenosine tri-phosphate (ATP). Because of both its necessity for eukaryotic life and its reactive chemical nature, however, a delicate balance exists between the supply of oxygen to a cell/tissue/organism and the beneficial or harmful outcome. In this minireview, we shall discuss the role of oxygen in metabolism with a particular emphasis on outcomes when oxygen supply is significantly altered. Furthermore, we will describe endogenous mechanisms which have evolved to protect cells and tissues during such adverse conditions and may prove useful as novel therapeutic targets in a range of disease states where oxygen-related stress occurs.

KEYWORDS: OXIDATIVE STRESS, HYPOXIA, ADAPTATION.

INTRODUCTION

Oxygen in metabolism. Eukaryotic cells generate ATP primarily through the oxidative metabolism of glucose obtained from carbohydrates in food. Glucose is converted to pyruvate by the glycolytic pathway in the cellular cytoplasm. These pyruvate molecules are converted into CO₂ and a 2-carbon acetyl group which combines with coenzymeA (CoA) to form acetyl CoA which feeds into the mitochondrial Krebs cycle in the generation of the

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electron carrier NADH which donates its electrons to the process of oxidative phosphorylation. Oxidative phosphorylation occurs in the mitochondrial membrane and involves the activity of four protein complexes (Complex I-IV). Electrons are passed down through a series of cytochromes resulting in the generation of a proton gradient across the inner mitochondrial membrane which serves as the driving force for the synthesis of ATP. This process involves the consumption of oxygen by cytochrome c oxidase (complex IV) and the generation of ATP and H₂O₂. Collectively these three processes (Glycolysis, the Krebs cycle and oxidative phosphorylation) are extremely efficient and yield a total of 38 molecules of ATP for each molecule of glucose consumed. Notably, this process is not 100% efficient and involves the generation of reactive oxygen species which if excessive, may act as physiologic signals or mediators of cell damage and death.

In the steady state, approximately 90% of available oxygen is consumed by mitochondria during the production of ATP through oxidative phosphorylation (1). The ATP produced in this process goes on to fuel the vast majority of active cellular processes. Thus, because the chemical reduction of molecular oxygen is the primary source of metabolic energy for virtually all eukaryotic cells, a constant oxygen supply is critical for continued cell function and survival (2). Thus hypoxic stress, which occurs when oxygen demand exceeds supply, represents a severe threat to continued cell, tissue and organism survival. Conversely, as outlined above, a significant consequence of oxidative phosphorylation is the generation of reactive oxygen species. For this reason, excessive or discontinuous oxygen consumption can also have significant pathologic implications through the generation of such reactive oxygen species. Thus, fluctuating oxygen levels in tissues can represent a significant threat to continued cellular function. Below we will outline the various conditions where adverse reactions to oxygen-related stress may occur.

OXYGEN AND DISEASE

Hypoxia: In the steady state (normoxia), the vast majority of oxygen consumed by a cell is utilized by the mitochondria in the generation of ATP by oxidative phosphorylation. This leads to the generation of sufficient cellular ATP levels to facilitate normal physiologic function. The remainder “spare” oxygen is used for non-mitochondrial processes and is consumed by non-mitochondrial dioxygenases. When the oxygen supply to a tissue is compromised (or demand is sufficiently increased), a situation occurs where insufficient ATP levels prevent a cell from carrying out its normal function leading to a state of metabolic crisis (2).

Hypoxic stress occurs in a wide range of conditions and may be acute or chronic in nature. Perhaps the purist form of whole animal hypoxia occurs in altitude sickness where rapid exposure to decreased atmospheric oxygen leads to a whole body hypoxia. A similar condition occurs in carbon

monoxide poisoning where the oxygen carrying capacity of hemoglobin is significantly altered. Surgical ischemia due to the temporary ligation of major blood vessels can also result in tissue hypoxia. Tissue hypoxia is also present in a diverse range of more clinically relevant disorders. For example vascular diseases including stroke and atherosclerosis cause decreased blood supply to tissues resulting in hypoxia. Chronic hypoxia can also occur chronic inflammatory disease where extensive and prolonged inflammation and fibrosis can lead to a breakdown in the microvascular architecture with subsequent decrease in perfusion efficiency and subsequent hypoxia. The relationship between hypoxia and inflammation will be further discussed at this symposium by Cormac Taylor. A number of encephalopathies are a result of prolonged ischemia leading to hypoxic stress in brain tissue. Finally, as a solid tumor develops, it outgrows the local blood supply resulting in significant tumor hypoxia, an event which may be critical in the development of the tumor (13). This topic will be further discussed during the symposium by Jacques Pouyssegur. Thus hypoxic stress is a pathologically relevant stimulus which occurs in a diverse range of disease states.

Hyperoxia: Hyperoxia arises when excessive levels of oxygen are delivered to a cell or tissue and is primarily associated with the administration of exogenous oxygen in a clinical setting. This is primarily the case when suprathysiologic oxygen supplementation is used in the prevention or treatment of hypoxemia and acute respiratory failure. It is clear however, that prolonged exposure to hyperoxia can cause significant pathology particularly in the pulmonary epithelium where cells are exposed to the highest PO₂ levels (3). Hyperoxic stress results in the induction of inflammatory pathways in the lung characterized by elevated pro-inflammatory cytokine levels, increased leukocyte infiltration, impaired gas exchange and pulmonary oedema resulting in injury and death in pulmonary epithelial cells. Critically, it is thought that in spite of the superior pulmonary anti-oxidant capacity, the generation of reactive oxygen species including superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (HO) as a result of increased mitochondrial oxygen consumption and/ or NADPH oxidase activity is an initiating signal in this pathophysiologic response. ROS produced during hyperoxia may directly chemically damage cellular macromolecules leading to cellular damage and death. Alternatively, ROS-dependent transcriptional responses can lead to the induction of inflammatory pathways which exacerbate this toxic response. For example, the activation of inflammatory pathways by hyperoxia-induced ROS can lead to the signals necessary to increase leukocyte infiltration which may serve as a further source of ROS generation. The net effect of these events is the induction of multiple cell death pathways including necrosis and apoptosis in pulmonary epithelial cells leading to the pathophysiologic outcome of pulmonary disease. The intracellular signaling pathways involved in the

hyperoxia-dependent induction of cell death have been expertly reviewed elsewhere.

Hypoxia/Reoxygenation: Reoxygenation occurs when the delivery of oxygen to a tissue which has been interrupted is rapidly re-introduced (4-5). Reoxygenation-dependent injury is typically associated with conditions where blood flow is diminished or removed and then rapidly re-introduced including thrombolytic therapy in myocardial infarction, acute renal failure and post-transplantation injury. Collectively, this response is referred to as reperfusion injury and has been associated with the reoxygenation-dependent induction of Fas-ligand-dependent cell death pathways, increased production of reactive oxygen species and irreversible mitochondrial dysfunction all leading to enhanced cell death through apoptosis and necrosis. The source of ROS production in reoxygenation injury may be from either resident cells or inflammatory cells which have infiltrated a tissue during the ischemic period. Interestingly, non-lethal reoxygenation/reperfusion can lead to a state of protection for a tissue against subsequent bouts of reperfusion injury termed ischemic preconditioning.

Intermittent hypoxia: Intermittent hypoxia occurs when a cell or tissue is exposed to repetitive cycles of hypoxia and reoxygenation and is primarily associated with the repetitive nocturnal oxygen desaturations experienced by patients suffering from obstructive sleep Apnea Syndrome (OSAS;6). It has recently become appreciated that OSAS is a major risk factor for cardiovascular disease through increased expression of systemic inflammatory mediators such as TNF α and as such, the molecular mechanisms underlying this effect has become an area of intense investigation. Recent studies have demonstrated the the intermittent hypoxia associated with OSAS is associated with the production of reactive oxygen species during repetitive bouts of reoxygenation.

Thus, while the primary pathological insult associated with hypoxia is associated with the decreased availability of oxygen (and subsequently ATP) to fuel physiologic processes leading to metabolic crisis and loss of function, hyperoxia, reoxygenation and intermittent hypoxia are more likely associated with the generation of chemically potent reactive oxygen species (ROS). Because both oxygen deprivation and ROS production are encountered often in both physiologic and pathophysiologic states, it is not surprising that over the course of evolution, we have developed the ability to respond to such environmental stresses with the induction of specific protective pathways. Some of these pathways will be outlined below.

ENDOGENOUS PROTECTIVE MECHANISMS

Adaptation to hypoxia

Hydroxylases and the HIF-1 pathway: Over the course of evolution, we have developed the ability to adapt to hypoxia through the induction of a specific transcriptional pathway governed by the hypoxia inducible factor-1 (HIF-1), a transcription factor which regulates the expression of genes promoting angiogenesis, vasodilatation, oxidative phosphorylation, glycolysis and erythropoiesis (7). The induction of such genes leads to increased tissue perfusion and anaerobic metabolism thus maintaining ATP levels and forming a critical adaptive pathway in dealing with a hypoxic threat.

The mechanism by which HIF-1 is activated in hypoxia is relatively well understood (2). HIF-1 α is constitutively synthesised at a high level in normoxia, but its level is repressed by members of the 2-oxoglutarate-dependent dioxygenase superfamily, namely the prolyl hydroxylases (PHDs). Three PHD isoforms which regulate HIF-dependent transcriptional activity have been described to date (PHD1, PHD2 and PHD3). Oxygen-dependent modification of specific proline residues within consensus LxxLAP motifs (Pro402 and Pro564) in HIF-1 α by these enzymes, primarily the PHD2 isoform, results in targeting of HIF-1 α for ubiquitination via an E3 ligase complex initiated by the binding of the Von Hippel Lindau protein (pVHL) and subsequent proteasomal degradation. A further hydroxylation of Asn803 in the transactivation domain of HIF-1 α by Factor Inhibiting HIF (FIH), an asparagine hydroxylase, represents a second mechanism of oxygen-dependent repression through inhibition of transactivation uncovered by Murray Whitelaw who will present at this symposium(8). Similar mechanisms exist for HIF-2 α . The hypoxic sensitivity of the HIF pathway is achieved by the absolute requirement of hydroxylases for molecular oxygen as a co-substrate (with iron and the Krebs cycle intermediate 2-oxoglutarate). Therefore, these enzymes act as true oxygen sensors. Inhibition of this pathway in hypoxia with the resultant stabilisation and transactivation of HIF- α subunits represents a paradigm for oxygen sensing, and hypoxia responsive alterations in gene expression. Thus, the rapid hypoxia-dependent activation of the HIF-1 pathway leads to adaptive changes which promote tissue survival during hypoxia.

ATP-dependent signalling: A second protective mechanism which has evolved to enhance survival during hypoxia involves the initiation of signaling pathways which sense an alteration in cellular energy status as reflected by ATP levels. Hypoxia decreases mitochondrial ATP generation through decreased respiratory activity. ATP depletion is accompanied by a rise in its precursor AMP thus increasing the AMP:ATP ratio leading to activation of AMP activated kinase (AMPK) through phosphorylation at Thr172 (9). The kinase upstream of AMPK primarily responsible for its

phosphorylation / activation is the tumor suppressor LKB1. AMPK is a critical regulator of cellular energy homeostasis, the activation of which promotes catabolic pathways including glucose transport, gluconeogenesis, respiration, the use of alternative energy sources to oxygen as well as downregulating anabolic pathways. These events are critical in maintaining cellular ATP levels. It has been hypothesised that the AMPK pathway may be involved in the beneficial effects of exercise and may represent a new therapeutic target in metabolic diseases such as diabetes.

Adaptation to oxidative stress

Anti-oxidant systems: An important mechanism by which cells adapt to oxidant stress is to transcriptionally upregulate a distinct array of cytoprotective genes responsible for buffering the cells antioxidant capacity. These genes act to maintain glutathione content and conjugational activity and are also responsible for the detoxification of damaging electrophilic by-products of oxidant stress and include glutathione S-transferases (GSTs), aldehyde dehydrogenases (ALDH) and NAD(P)H:quinone oxidoreductase 1 (NQO1). A master regulator of this specific anti-oxidant phenotype is the transcription factor Nrf2 (10). This transcription factor is held in the cytoplasm by a cytoskeletal associated specific inhibitory protein KEAP1 under conditions of normal cellular redox state, where Nrf2 is continuously targeted to proteasomal degradation. Under conditions of oxidative stress cysteine residues within the hinge region of KEAP1 become modified through mechanisms which involve thiol oxidation, resulting in a conformational change in KEAP1 with the loss of Nrf2 binding and proteasomal targeting. Nrf2 then accumulates and localizes to the nucleus where it heterodimerizes with specific co-factors including members of the maf protein family, and co-ordinates the upregulation of cytoprotective genes through the initiation of transactivation at Antioxidant Response Elements (AREs) within the regulatory regions of these genes.

A second important regulator of the cells anti-oxidant armoury is the AP-1 transcription factor family member junD which regulates the basal expression of a number of antioxidant genes and reduces angiogenesis. Indeed junD^{-/-} cells accumulate H₂O₂ reducing the availability of FeII of the HIF- prolylhydroxylases (PHDs) that target HIF-1 α for degradation. This mechanism accounts for the enhanced VEGF-A expression and increased angiogenesis in junD deficient cells (14).

OXYGEN AND REDOX SENSING

A key question which remains incompletely answered is how cells sense a change in oxygen levels or oxidative stress and respond by initiating the adaptive / protective responses outlined above. While this remains an area of

intensive investigation, some recent studies have shed light on cellular oxygen and redox sensing mechanisms. While the direct dioxygen-dependence of the hydroxylase enzymes represents a clear and direct link between the hypoxic environment and the activation of the HIF system, there are complicating factors which likely modify this sensing mechanism including the co-dependence of the hydroxylases on Krebs cycle intermediates, Fe²⁺ and potentially reactive oxygen species. Furthermore, intracellular oxygen gradients are determined by the rate of mitochondrial oxygen consumption by cells, a factor which can be manipulated by inhibitors of respiration such as nitric oxide (11). Thus, while this oxygen sensing system represents a direct link between the local oxygen levels and activation of the HIF pathway, these co-regulating factors likely allow the level of oxygen at which cells respond to hypoxia differ between tissues.

The oxygen sensing pathways outlined above relate primarily to conditions where there exists a sustained period of hypoxia allowing transcriptionally-mediated adaptive and protective responses to occur. A separate critical response to arterial hypoxaemia involves the induction of an acute physiological response resulting in increased rate and depth of pulmonary ventilation. A central event in this process involves excitation of the carotid body which signals to the respiratory centres located in the brain stem. While it is clear that a hypoxia-dependent derepression of potassium channel activity in cells of the carotid body is central to this response, the nature of the oxygen sensor in this system remains an area of hot debate (12). In fact, it is unlikely in this system that a single oxygen sensing pathway exists. Candidate sensors which will be discussed during this symposium (by Paul Kemp) include mitochondria, AMPK and haemoxygenase-2.

THERAPEUTIC POTENTIAL OF OXYGEN SENSING PATHWAYS

As outlined above, recent advances have shed significant light on our understanding of how cells respond to stress associated with insufficient oxygen supply or oxidative stress. This information is allowing the development of novel pharmacological intervention strategies in a range of disease states. For example, the HIF-1 pathway is an attractive therapeutic target in ischemic disease where the promotion of HIF-1-dependent adaptation with pharmacologic hydroxylase inhibitors would be predicted to enhance tissue survival. Conversely, a developing tumor which becomes hypoxic as it outgrows the local blood supply utilizes the HIF-pathway to facilitate tumor development. Clearly, inhibiting the HIF pathway in cancer is of therapeutic potential. Finally, the Nrf-2 pathway can be activated by organic compounds such as sulforaphane which may be used to enhance the

expression of endogenous anti-oxidant mechanisms which may be protective in disease states associated with extensive oxidative stress.

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