The possible role of GPI-ceruloplasmin in hypoxia de novo creation and maintenance

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Abstract: Ceruloplasmin is a member of the multicopper oxidases family (MCOs), multidomain proteins capable of oxidizing many structurally unrelated compounds reducing oxygen to water. While MCOs show great oxidative versatility, they can only transfer electrons to molecular oxygen, which is the obligate electron acceptor. Therefore, MCOs should also be considered as ''O_2 consuming enzymes''. The glycosylphosphatidylinositol anchored ceruloplasmin (GPI-Cp) isoform present on the surface of the plasma membrane, does not seem to be involved in copper and iron metabolism. Since hypoxia is also a common feature of many rapidly growing solid tumors, we postulate that the regulation of GPI-Cp could be the molecular event in the creation and the maintenance of hypoxia in tumor cells. By inhibiting the GPI-Cp expression, it would appear possible to attempt to overcome tumor hypoxia, thus improving the efficiency of radiotherapy.

Keywords: Hypoxia, Glycosylphosphatidylinositol-Ceruloplasmin, Multicopper Oxidases, Oxygen-Consuming Enzyme, Tumor

1. Introduction

Aerobic life survival depends on molecular oxygen, so when changes occur that result in an increase or a decrease of molecular oxygen, respectively hyperoxia and hypoxia, the body must cope with a crisis that leads to the depletion of energy reserves, changes in signal cascade, oxidative stress, cell death and tissue damage. Normoxia means a level of oxygen sufficient to take place within the normal physiological processes of cells, tissues and / or organs [1]. While have been established some guidelines, there are no values (pO_2) of partial pressure of oxygen (pO_2) to define the absolute '"hypoxia". Oxygen levels dictate a change in hypoxic direction depending on the system (cell / tissue / organ) [1]. In general, pO_2 less than or equal to 5% is indicative of hypoxia in the cellular systems. PO_2 levels of between 5 and 2% correspond to a condition of moderate hypoxia, while levels <2% represent a condition of marked hypoxia.

The O_2 supply is finely regulated in vivo, with respect to the cell and tissue type. The tissue O_2 concentrations are much lower than the atmospheric ones. One of the responses at the molecular level consists in the activation of specific transcription factors, or inducible by hypoxia, called HIF (Hypoxia Inducible Factors) [2]. HIF complexes are hetero-dimeric compounds from an α subunit, whose stability is oxygen-dependent and oxygen-independent β subunit. Have been identified three subunits α (1α, 2α and 3α) and a β subunit (also called ARNT, Aryl-hydrocarbon Receptor Nuclear Translocator), each with several splice variants [2- 3]. In normoxic conditions, in the cytoplasm, the α subunits undergo two hydroxylation events catalyzed by PHDs (prolyl hydroxylase domain proteins) and FIH (factor inhibiting HIF) enzymes, that causes the proteasome-mediated degradation [2]. Under hypoxic conditions, the PHDs and FIH enzymes are inactive due to the lack of oxygen necessary to the hydroxylation reactions. In this way, the α subunit is stabilized, and translocates to the nucleus to bind to the β subunit, forming a heterodimer (HIFα / HIFβ) which represents the active form of HIF, able to function as transcription factor.

Hypoxia is a critical component of the microenvironment of tumor cells: metabolic changes, drug resistance, protein overexpression, inhibition of apoptosis, induction of autophagy are all part of the cellular reprogramming triggered by HIFs [1, 4-5]. Interestingly, recent findings have also indicated that HIF-1α and HIF-2α play critical roles for the gain of more malignant phenotypes by highly tumourigenic

cancer stem/progenitor cells that are able to generate the bulk mass of heterogeneous and differentiated cancer cells within tumours and which are involved in primary cancer progression, metastases, resistance to current cancer therapies and disease relapse [6]. Hypoxia is a pivotal driving force of malignant progression [7]: it is well known that can induce tumor metastasis [1]. Most of human solid tumors contain hypoxic areas [8]; increased levels of tumor hypoxia have been associated with worse clinical outcomes in patients of Glioblastoma multiforme, the most malignant brain tumor [9], by compromising the effectiveness of chemotherapy [3].

The causes of tumor hypoxia are multifactorial, but at the present it is widely accepted that poorly oxygenated areas within solid tumors are due to aberrant blood vessel formation [10]. However, there is a growing body of experimental evidence that strongly suggests that tumor hypoxia is a more complex phenomenon and that in part it is programmed by the tumoral cell itself. Recently, it has been demonstrated that in human pancreatic cancer the severe hypoxia that characterizes this tumor is found not just in the center of the tumor mass, but also occurs at the invasion front [11]. Furthermore, it has been shown that microscopic tumors, less than 1 mm in diameter, are severely hypoxic [12].

The state of hypoxia is generally associated with pathological conditions, such as cerebral ischemia, disorders of the inflammatory response, diabetes and solid tumors, in which it is observed an increased cell proliferation associated with hypoxia [2, 4]. However hypoxic conditions are also observed in normal physiological processes, such as during fetal development, proliferation and cell differentiation, in the reparative processes of the tissues and in hematopoietic tissue [13]. In some tissues there are some real gradients of pO_2, such as in bone marrow, for which the cells closest to the vascular sinusoids are exposed to high pO_2 around 5-7%, while those present in the site furthest at the level of endosteum are exposed to much lower levels of pO_2, up to 0.1% [13]. Endosteal regions are rich in hematopoietic stem cells (HSCs) and the physiological condition of hypoxia is essential for the maintenance of stem cell of these cells. Fluctuations in the local concentration of oxygen induce the system - cell or body - to implement adaptation strategies that determine protection or limitation of damages. In the presence of low concentrations of oxygen, the cell reacts with a series of coordinated responses times to restore the ideal levels of O_2 [2, 3].

Beside pathophysiology, hypoxia also has a role in the function of normal tissues. In bone marrow, the hematopoietic stem cell (HSC) reside in well-defined microenvironmental domains called "niches" where they are maintained in a "quiescent state" by low oxygen concentration, ie, in the condition of hypoxia. The bone marrow appears to be the only one among the normal tissues to be physiologically in conditions of hypoxia [14]. Within the bone, anoxic regions exist (ie entirely without O_2) to protect HSCs from damage caused by oxygen free radicals . HSCs in the bone would also be organized in a manner dependent on the oxygen gradient: from a greater amount of HSCs within the bone, in areas with a lower oxygenation (hypoxia), it goes to drastically lower amount of CSE in most areas "superficial "as it increases oxygenation [14].

The adaptation induced by increased oxygenation of atmosphere conditioned the evolution of a complex circulatory and respiratory system to ensure an adequate O_2 supply for every cell type in organism. The complex mechanisms such as cellular respiration require O_2 as ultimate electron acceptor. But the need for O_2 as a metabolic substrate is opposed to the risk of cellular macromolecules oxidative damage (ROS generation). That is why its concentration within cells is maintained within a narrow range that optimally balances supply and demand. A cell could and has to fight against the ROS in excess. Therefore, it is really important for the cell, tissue or whole organism, to be able to finely adjust and calibrate the entry of oxygen, in order to maintain the optimal concentration.

2. Ceruloplasmin and GPI-Ceruloplasmin

Ceruloplasmin is a protein secreted in the serum; it is a copper-containing globulin that has a molecular weight of 151,000 Dalton. The protein contains 6 tightly bound copper ions, in 3 different types of binding site. The active site of each MCO contains four copper atoms per functional unit: one type 1 or ''blue'' copper site and three other atoms in a trinuclear cluster. Type 1 copper, with redox potentials ranging from 400 to 790 mV in the various MCOs, is the site where the oxidation of substrate molecules takes place [15]. The ''blue'' copper transfers electrons to the trinuclear cluster site which it then provides for the reduction of a molecule of oxygen into two molecules of water [16]. The redox potential for the type 3 is equal or to somewhat higher than the respective type 1 potential [15].

It is over 60 years since the discovery and isolation of the serum ceruloplasmin. In that time much basic information about the protein has been elucidated including its catalytic and kinetic properties as an enzyme, expression, sequence and structure. The importance of its biological role is indicated in genetic diseases such as aceruloplasminemia where its function is lost through mutation. Despite this wealth of data, fundamental questions about its action remain unanswered.

Despite the need for copper for the functions of ceruloplasmin, this protein plays no essential role in the transport or metabolism of the copper. The biological role of ceruloplasmin harboring a ferroxidase activity was demonstrated by Cartwright and colleagues in experiments using pigs with copper deficiency [17-19]. The copper depletion resulted in a marked decrease in the circulating serum ceruloplasmin in association with iron deficiency anemia that could only be corrected by

administration of copper and iron accumulation in parenchymal tissues, while the administration of exogenous ceruloplasmin resulted in the prompt release of tissue iron with subsequent incorporation into circulating transferrin. The essential function of ceruloplasmin is as a ferroxidase, utilizing the electron chemistry of bound copper ions to couple the oxidation of ferrous iron (Fe^{2+}) to the reduction of oxygen bound to the trinuclear cluster [20, 21].

In recent years, a ceruloplasmin isoform anchored by glycosylphosphatidylinositol (GPI-Cp) to the outer surface of the plasma membrane has been discovered in animal cells [22 - 23].

The GPI-Cp is generated by alternative RNA splicing of exons 19 and 20 [24]; originally found in astrocytes in the CNS, as well as in retina and hepatocytes. Recent studies revealed a much more ubiquitous expression of the GPI-Cp isoform, showing GPI-Cp detection in purified membranes of multiple organs of rats and mice [25]. Lately, it was demonstrated that both secreted Cp and membrane-bound Cp are coexpressed in several tissue, including immune cells as well as hepatocytes [26].

The expression of serum Cp and GPI-Cp in immune cells, namely lymphocytes and monocytes/macrophages, suggests a close functional relationship among immune system, oxidative stress and iron metabolism. In fact, Cp is an acute-phase protein with its plasma concentration increasing in many diseases including hepatitis, atherosclerosis, polyarthritis, cancer and many other inflammatory and infectious diseases [25].
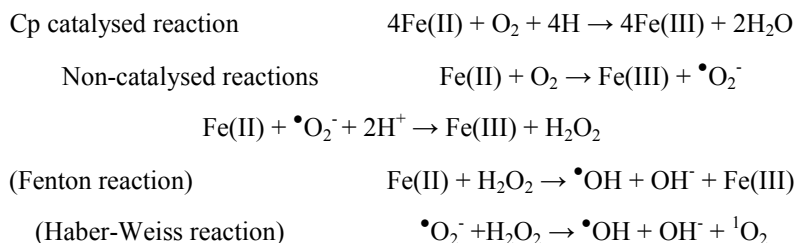
The proposed physiological functions of ceruloplasmin include copper transport, oxidation of organic amines, ferroxidase activity, regulation of cellular iron levels, glutathione peroxidase and ascorbate oxidase activities and an antioxidant activity. Furthermore, it has also been reported that ceruloplasmin may scavenge reactive oxygen species such as singlet ($1O2$), superoxide ($O2$) and hydroxyl radicals (OH). However, it has also been shown to have prooxidant activities, which involve a distinct active site from the antioxidant sites.

In the last few years, this protein appears to be involved in several disease: an insufficiency of ceruloplasmin synthesis or its decreased activity in the cells of the central nervous system is currently regarded as one of the mechanisms underlying the development of a number of neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [27, 28].

There is abundant evidence that ceruloplasmin plays a key role in iron metabolism. The importance of ferroxidase activity of Cp has been well demonstrated [29]. Humans with a mutation of the ceruloplasmin gene (aceruloplasminemia), which leads to inactivation of ferroxidase activity, are characterized by iron accumulation in the brain [30]. Several neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, have been associated with misregulation of iron metabolism in the central nervous system [31].

However, as stated above, it should be taken into consideration that Cp, while oxidizing Fe(II) to Fe(III), consumes molecular oxygen and that the subsequent drop in oxygen concentration (de novo created hypoxia) induces a decrease in the rate of non-enzymatic oxidation of Fe(II), because the affinity of the reaction for O2 is fairly low. Conversely, the drop in O2 concentration does not affect the ferroxidase activity of Cp because its affinity for O2 is very high. Decreasing the non-enzymatic oxidation of Fe(II) is a very useful event because this uncatalyzed oxidation is so potentially dangerous that it can trigger the formation of many ROS as described below:

Cp catalysed reaction $\qquad 4Fe(II) + O_2 + 4H \rightarrow 4Fe(III) + 2H_2O$

Non-catalysed reactions $\qquad Fe(II) + O_2 \rightarrow Fe(III) + {}^{\bullet}O_2^{-}$

$$Fe(II) + {}^{\bullet}O_2^{-} + 2H^{+} \rightarrow Fe(III) + H_2O_2$$

(Fenton reaction) $\qquad Fe(II) + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + Fe(III)$

(Haber-Weiss reaction) $\qquad {}^{\bullet}O_2^{-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + {}^{1}O_2$

3. Multicopper Oxidase Protein: An Oxygen-Consuming Enzyme

Ceruloplasmin is a member of the Multicopper oxidases family (MCOs). MCOs utilize the distinctive redox ability of copper ions, present in their active site, to drive electrons from the various electron donor substrates to molecular oxygen in a controlled way, i.e. forming two molecules of H_2O without release of reactive oxygen species (ROS) [32].

Being capable of oxidizing several structurally unrelated compounds, both inorganic [Fe(II), Cu(I), Mn(II)] and organic (ascorbic acid, organic amines) [33, 34], MCOs are involved in various metabolic pathways.

Furthermore, these enzymes can change their function in response to changes in substrate, composition of the reaction medium and differential cell localization or expression [17]. Since this multiplicity of actions does not conform to the ''one gene-one protein-one function'' paradigm, MCOs are called moonlighting proteins [34].

The multiplicity of actions of MCOs makes it difficult to decide which functions are important under what conditions. While MCOs show great oxidative versatility, they can only transfer electrons to molecular oxygen, which is the obligate electron acceptor [35]. Therefore, MCOs could also be considered as ''O2-consuming enzymes'' and this constitutes an important aspect of the role that these enzymes can play in the oxygen

management of aerobic organisms, in both physiological and pathological conditions.

Since molecular oxygen is not subject to active transport outside the vascular system, there is a general conviction that delivery of O_2 to the cells is driven by the concentration gradient set up by O_2 consumption occurring in the cells and their compartments [36]. Oxygen traffic and distribution outside the vascular system is a more complex process that could be regulated, in part, by the activity of multicopper oxidases.

Until now nearly all research on MCOs has been focused on the electron donor(s) side of the reaction. Ceruloplasmin is involved in many biological processes (copper transport, amino acid neurotransmitter metabolism, nitric oxide synthase modulation, antioxidant activity) [37]. However, after 70 years of research, the main known function of Cp remains its ability to oxidize the more toxic ferrous ions (Fe^{2+}) to the less toxic ferric form to (Fe^{3+}) [38]. Remarkably, it has been almost completely disregarded that Cp, while catalyzing iron oxidation, also act simultaneously as oxygen-consuming enzyme.

4. Ceruloplasmin and Oxygen Relationship

There are several reports in literature that show a direct link between oxygen concentration and ceruloplasmin. The ceruloplasmin amount increase in hypoxic condition; HIF is known to promote the ceruloplasmin expression in HepG2 cells. The ceruloplasmin promoter indeed contain an hypoxia-responsive element (HRE) recognized by HIFα and HIFβ, respectively, and is indeed activated by Hypoxia-inducible factor-1 [39, 40].

Prolonged hypoxia can induce the mRNA ceruloplasmin transcription in mouse, and ceruloplasmin mRNA expression is also induced in the liver of hypoxic mice in vivo [41].; furthermore, in macrophages, the ceruloplasmin activity (iron release) was absolutely dependent on the presence of hypoxic condition [42].

Patients with chronic obstructive pulmonary diseases that induce hypoxia have a 35–100% increase in serum Cp [43]. In addition, exposure of healthy adults to high altitude hypoxia significantly increases serum Cp [44].

Furthermore, copper concentration can enhance ceruloplasmin expression, and is also able to activate HIF 1: thus, there are several different experimental data that support a functional relationship between oxygen levels and ceruloplasmin expression [41].

Experimentally, the ceruloplasmin expression is induced by raising the concentration of O2 in vivo, while dietary iron status has little effect on the expression of the enzyme [38, 45]. Recently it has been demonstrated that hypoxic treatment in syncytiotrophoblasts culture enhance ceruloplasmin expression by approximately 25-fold [46]. It has been shown that lower GPI-Cp did not correlate with tissue iron retention; furthermore, this Cp isoform does not seems to have any role in the iron metabolism [25].

On the basis of these data, indicating that in vivo activity of ceruloplasmin is regulated in response to oxygen availability, GPI-Cp could be part of a dynamic mechanism operating whenever cells have to control oxygen availability.

5. A New Target to Improve Tumor Radiotherapy

Cancer is presently a major cause of mortality in developed country: worldwide, it accounts for 7.6 million deaths (around 13% of all deaths) in 2008, according to the World Health Organization report. Radiotherapy is almost always used to treat various forms of cancer. Ionizing radiation works by damaging the DNA of exposed tissue leading to cellular death: shaped radiation beams are aimed from several angles of exposure to intersect at the tumor, providing a much larger absorbed dose there than in the surrounding, healthy tissue. The radiation acts only on the part of the body that is exposed to the radiation. Radiation used for cancer treatment is called ionizing radiation because it forms ions (electrically charged particles) in the cells of the tissues it passes through. It creates ions by removing electrons from atoms and molecules. Unfortunately, solid tumors with hypoxic areas are the most aggressive and resistant to radiotherapy and this limits the radio curability of many tumors [47]; although several oxygenation approaches have been utilized, none of them is capable of restoring normoxia in the hypoxic areas present in tumors [48].

New therapeutic strategies such as guanine-rich oligodeoxynucleotides (G-rich ODNs), chemical compounds and chemotherapeutic drugs that target HIF-1α and/or HIF-2α proteins and repress the expression of their target genes in various cancer cells have been shown to counteract primary cancer progression and metastases at distant sites, and reverse treatment resistance under normoxic and hypoxic conditions [49, 50].

The understanding of how hypoxia drives tumour progression is attracting substantial investigation; however, a lot remains to be done to clarify the molecular mechanism involved.

It is conceivable to hypothesize that tumor cells maintain a low oxygen concentration through the expression of GPI-anchored ceruloplasmin on their plasma membrane. It is possible that the major role of this GPI isoform of Cp is to regulate the entrance of oxygen into the cell.

A new line of research to improve the efficiency of tumor radiotherapy could target the GPI-ceruloplasmin. By regulating the expression/activity of this Cp isoform, the level of O2 in cells could be modulated; using small interfering RNA to inhibit GPI-Ceruloplasmin expression, it looks possible to overcome the state of hypoxia in

tumor areas, increase partial O2 pressure, thus improving the efficiency of radiotherapy.

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