

**Title:**

**Oxygen sensors of the peripheral and central nervous system.**

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**Abstract:**

Neural systems exposed to diminished oxygen availability have a compromised metabolism that leads to pathophysiological changes or neuronal death, depending on the severity and duration of oxygen deprivation. A distributed network of oxygen sensors responds to protect cells by slowing or ameliorating pathophysiological changes and forestalling neuronal death via short-term and/or long-term changes involving gene expression and the modification of sensors and effectors. In mammalian systems such protective changes are not sufficient to prevent damage under extreme conditions, unlike some hypoxia- and anoxia-tolerant vertebrates which demonstrate an oxygen dependent and reversible reprogramming to protect vital organs such as the brain and heart.

This review examines (1) the nature of the signal for oxygen sensors; (2) the molecules used to sense oxygen; (3) how the primary signal is generated, converted and used in an oxygen-dependent manner; (4) how effector systems function in different cell types; and (5) how oxygen sensing pathways are interconnected to more general protective stress responses which confer cross-protection for a number of physiological stressors.

While future therapies may focus on the activation of HIF and its downstream gene products, selected gene products could be administered to reduce neuronal loss after acute insults due to ischemic events and degenerative diseases of the brain and retina and improve recovery. Activation of neuroprotective pathways by oxygen sensors and other physiological stressors could be used as pre-treatment to minimise neurotrauma associated with neurosurgical procedures and as an ancillary treatment during early stages of rehabilitation.

**Key words:** Oxygen sensors, oxygen-dependent, haem molecules, myoglobin, cytoglobin, neuroglobin, *cytochromes*, NADPH oxidase, prolyl and asparagine hydroxylases, AMP kinase, exchangers and co-transporters, voltage sensitive ion channels, HIF, HRE, redox-responsive transcription factors, mitochondria, ROS, redox-sensitive, stress pathways, oxygen-sensitive effector systems, oxygen dependent gene expression, xenobiotically induced gene expression pathways, oxygen- and temperature-dependent responses, erythrocyte, carotid body, neuroepithelial bodies, gill neuroepithelial cells, neuroprotection, preconditioning, exercise.

**1.1 Introduction.**

Diminished oxygen availability is a physiological stressor that compromises metabolism by slowing or halting aerobic ATP production and oxygen-dependent enzymatic reactions. In any neural system this can result in pathophysiological changes or neuronal death, depending on the severity and duration of oxygen deprivation. Oxygen-dependent responses mediated by oxygen sensors via their effectors can elicit neuroprotection in the face of significantly decreased oxygen levels, such that pathophysiological changes are ameliorated and neuronal death forestalled. Disruptions to oxygen availability in humans can range from a severe acute insult occurring as a result of heart attack, stroke and traumatic brain injury to a chronic mild insult occurring as a result of sleep apnoea and early stage cardiovascular disease. The prognosis for short-term survival of these events depends upon how well and how quickly the neural systems can be protected or how fast they can recover. There are clearly several functionally related questions that pertain to oxygen-dependent phenomena. (1) What is the nature of the signal sensed by oxygen sensors? (2) Which molecules and molecular pathways are used to sense oxygen? (3) How is the primary signal generated, converted and used in an oxygen-dependent manner? (4) How do effector systems function in different cell types, particularly in neural systems? (5) How are oxygen sensing pathways connected to more general protective stress responses which confer cross-protection for a number of physiological stressors?

For the purpose of this chapter, we define oxygen sensors as molecules and proteins that respond to changes in tissue oxygen levels with a direct change in their structure that results in changes in the tertiary structure and function of existing proteins and/or the up-regulation oxygen dependent genes (ODG) often as a result of transcription factor function. Thus, we consider the whole sequence of events from primary oxygen sensing to oxygen-dependent effects and their significance to equate to oxygen sensors. Consequently, we cover, e.g., haem-containing proteins (globins and cytochromes), membrane bound ion channels, mitochondria and their subtypes which appear to be multifunctional (sensing oxygen dependent signals, generating signals to be sensed by other oxygen sensors and as an oxygen-dependent effector site), cellular systems, and specialized organs such as the carotid body. It is also apparent that oxygen sensors are located in a number of cellular compartments ranging from extracellular sensors (including erythrocytes as sensing cells) to cytoplasmic compartments (neuroglobin) and even intranuclear domains as exemplified by cytoglobin

distribution in the mammalian brain. Also, evidence for interactions between oxygen-dependent and other stress pathways are discussed.

Since molecular oxygen is rarely sensed as a pure signal this review will discuss what additional signals can be sensed when oxygen level approaches a critical threshold, and how changes in the level of some molecules (metabolites) such as reactive oxygen species (ROS including hydroxyl radicals), and nitric oxide (NO) as well as changes in NADPH, ATP, pH and general redox status can modulate the activity of the oxygen sensors and their downstream effectors. We will also examine the extent of cross-talk between oxygen-dependent pathways and pathways originating from other sensors detecting physiological status because it is difficult to separate them entirely, for example energy-dependent pathways are profoundly altered when oxygen levels fall below a critical threshold.

There are two important points to note throughout. First, while single cell organisms such as bacteria and yeast contain a single oxygen-sensitive molecular pair to regulate the expression of oxygen sensitive genes (reviewed by Bunn and Poyton, 1996) no universal oxygen sensor has been identified in vertebrates (reviewed by Cummins and Taylor, 2005; Lahiri *et al.*, 2006; Lopez-Barneo *et al.*, 2001; Wenger 2000, Lutz and Prentice, 2002). Instead, there appear to be several different primary oxygen sensors which are linked to a number of different effector systems that synergistically mediate oxygen-dependent responses in which neurotransmitters and modulators may or may not be involved.

Second, most studies on oxygen sensors have used hypoxia as a stressor to elicit oxygen-dependent changes. Usually the physiologically effective degree of hypoxia has not been characterized in detail because the actual oxygen level experienced by the studied cells has not been measured. The oxygen tension experienced by any cell in an organism depends on its location, especially its distance from an arterial blood vessel, and on its oxygen consumption. Consequently, there are marked differences of cellular oxygen tensions between cell types in normoxic body. For example, at normal ambient oxygen levels, arterial PO<sub>2</sub> is approximately 100mmHg while the PO<sub>2</sub> in muscle interstitium *in vivo* has been measured at 3.3-24.2mmHg and the PO<sub>2</sub> at the muscle mitochondria was 4-20 mmHg (Richmond *et al.*, 1997). The mean cerebral PO<sub>2</sub> is higher than that of muscle, at around 20 mmHg and the PO<sub>2</sub> at the renal medulla is close to 50 mmHg (Johannes *et al.*, 2006). These PO<sub>2</sub> values can be considered to reflect normoxia for the respective sites because this is the [O<sub>2</sub>] at which the animal displays its routine metabolic rate. Thus, it is clear that normoxia is very different depending on whether the frame of reference is a particular tissue or the mitochondria within that tissue (see also review by Lutz and Prentice, 2002). Furthermore, the oxygen tensions

experienced by, e.g., lung epithelial cells and hepatocytes are markedly different. Since vertebrates characteristically display a heterogeneous oxygen map, perhaps one should define hypoxia as an oxygen tension that is below the tension at which aerobic metabolism becomes limited, i.e. the critical  $PO_2$ . In this paradigm, muscle hypoxia would be below 2.4-2.9 mmHg, its critical  $PO_2$  (Richmond *et al.*, 1997) and hypoxia for the mitochondria would be below its critical  $PO_2$  of 1 mmHg (Rosenthal *et al.*, 1976). Similarly, *in vitro* work on established cell lines should take into account the origin of the cell type used in the definition of hypoxia. In terms of oxygen-dependent responses, it is also important to differentiate between decreased oxygen level (hypoxia) and anoxia, i.e., total lack of oxygen (Wenger and Gassmann, 1996).

In defining hypoxia as the  $[O_2]$  at which aerobic metabolism becomes limited, one also needs to consider how limited the aerobic metabolism needs to become, before oxygen sensors initiate signal cascades which result in the up-regulation of oxygen dependent genes and whether general physiological stress *per se* can turn on what are currently thought of as oxygen dependent genes. We discuss the possibility that whenever anoxic conditions prevail, a response attributed to the involvement of oxygen sensors could be a more general stress response, which has little to do with changes in oxygen tension.

## **2.0 The nature of the signal sensed by oxygen sensors.**

Among the systems that are usually classified as oxygen-sensitive, only ones that include haem-containing molecule (Lopez-Barneo *et al.*, 2001) or prolyl/asparagine hydroxylase use molecular oxygen as a substrate (Berra *et al.*, 2006). In addition, the level of reactive oxygen species may be the primary signal that is affecting the activity of oxygen-dependent systems. While earlier on it was thought that reactive oxygen species are mainly conferring oxygen toxicity, recent findings indicate that at low levels they are important signalling molecules (Finkel, 1998; Weir *et al.*, 2002; Werner, 2004; Wolin *et al.*, 2005). The effects of ROS are often considered to be mediated via effects on conserved cysteine residues (Michiels *et al.*, 2002). Especially hydrogen peroxide and hydroxyl radicals appear to be important in signalling (Gloire *et al.*, 2006). Hydrogen peroxide may be important, since it is quite stable, membrane permeant (Lesser, 2006) and can affect the activity of tyrosine phosphatases by oxidizing cysteines in the catalytic centre (Gloire *et al.*, 2006). Hydroxyl radicals can also carry out the oxidation, and their use in cellular signalling would introduce spatial resolution in the system, because the short life time of the molecule ( $10^{-7}$  s) restricts the diffusion distance to 4-5 nm (Lesser, 2006). Notably, it is very difficult to separate hydrogen peroxide and hydroxyl radicals, since hydrogen peroxide is converted to hydroxyl

radicals in the Fenton reaction, if adequate iron (or copper) ion stores are available (Lesser, 2006; Bogdanova and Nikinmaa, 2001). While NO has been recognized as an important signalling molecule, less emphasis has been paid to the fact that NO can affect the oxygen affinity of, e.g., mitochondrial function (Koivisto *et al.*, 1997). Also, NO can react with superoxide anion, and the formed peroxynitrite anion is a powerful membrane-permeant oxidant (Fridovich, 1986; Marla *et al.*, 1997) with a lifetime near 0.1 s. Consequently, NO-dependent pathways may play a role in any effect that is considered oxygen-sensitive. Two other gaseous molecules may play a role in oxygen-dependent signalling, namely CO (carbon monoxide) and H<sub>2</sub>S (hydrogen sulphide). Heme oxidase enzyme, which has CO as one end product, is regulated by hypoxia (Lee *et al.*, 1997). CO may control the neural discharge from rat carotid body (Lahiri and Acker, 1999). Hydrogen sulphide is involved in the oxygen-dependent regulation in vascular tone (e.g., Olson, 2005). Any marked decrease in oxygen availability leads to a decrease in cellular ATP concentration (Lutz and Nilsson, 2004) which appears to be the first step in induction of neural death (e.g., Lipton, 1999) and does not occur in the very anoxia-tolerant crucian carp (Lutz *et al.*, 2003) or the tropical epaulette shark (Renshaw and Dyson, 1999). Thus, any mechanism detecting disturbances in the energy balance with oxygen depletion would be highly useful for maintaining cellular function in general and neural function in particular. Although from energetic point of view the ADP/ATP ratio is the primary regulated function, the AMP/ATP ratio varies as a square of ADP/ATP ratio thus enabling a more sensitive regulation of the energy balance, if it is sensed and energy producing/consuming systems consequently adjusted (Hardie, 2003). The AMP kinase senses changes in AMP and, consequently, AMP/ATP ratio, (Hardie, 2003; Hardie *et al.*, 2006), which can thus function both in oxygen- and energy-dependent signalling.

### **3.0 Primary oxygen sensors.**

#### **3.1 Haem based molecules.**

Proteins containing a haem moiety that are capable of binding molecular oxygen have been identified in diverse taxa from bacteria to vertebrates. Haem based proteins sense oxygen by binding it reversibly and thereafter initiating a number of signalling cascades with several second messenger molecules sometimes leading to altered gene expression where specific transcription factors are utilized (Wenger 2000). The blockade of the oxygen-dependent responses by carbon monoxide, which binds with very high affinity to the oxygen-binding site of many haem proteins, is taken as confirmation that haem proteins act as oxygen

sensors (Zhu and Bunn 1999). Similarly, if the ferrous iron in the haem group is replaced by cations which do not bind oxygen, such as cobalt, the effect of such replacement on second messenger systems and transcription mimics that of exposure to hypoxia (reviewed by Wenger, 2000). Also, the suggestion that oxygen sensing is haem based is supported by the observation that treatments of cells with iron chelators such as desferrioxamine, result in a hypoxic response (Ho and Bunn, 1996; Wang and Semenza, 1993).

Simple haem-containing oxygen sensing molecules that respond to environmental changes are present in microbial symbionts (of plants), regulating genes associated with nitrogen fixation. The rhizobial FixL/FixJ system consists of a protein histidine kinase and its response regulator FixJ, which act as an oxygen-sensitive switch. Oxygen binding to the haem moiety of FixL inactivates its kinase activity. This prevents the phosphorylation of FixJ and inhibits its downstream signal transduction pathway (Nakamura *et al.*, 2004).

Thermodynamic studies have shown that both ferrous and ferric forms of the oxygen sensor FixL have a significantly lower oxygen affinity than myoglobin (Rodgers and Lukat-Rodgers, 2005). The evolution of high affinity extra-cellular and intra-cellular oxygen sensors in higher organisms may have facilitated formation of more complex body plans which include a nervous system and the occupation of a wider variety of niches.

### ***3.2 The globin family of haem proteins.***

Circulating oxygen carriers are used by many invertebrates (hemerythrin, haemocyanin and haemoglobin) and by vertebrates (members of the globin family) to facilitate oxygen transport from environment to tissues. Oxygen causes a direct change in the structure of these proteins and, thus, they can be considered as oxygen sensors. The globins bind oxygen to a Fe-containing porphyrin ring. The binding is often cooperative, if the molecule containing globin(s) is composed of subunits. Of the different globin molecules, myoglobin, cytoglobin and neuroglobin have been detected in the brain and other neural systems and thus may have different neuroprotective functions.

#### ***3.2.1 Myoglobin.***

Myoglobin is not only found in muscle, but its isoforms are also expressed in other tissues which have a high metabolic rate and correspondingly high oxygen demand such as liver, gill, and brain (Fraser *et al* 2006). Interestingly, expression of the unique myoglobin isoform in neural tissue did not change in response to environmental hypoxia (Fraser *et al* 2006) unlike the hypoxia sensitive up-regulation of myoglobin in the heart (Roesner *et al*

2006). It has been suggested that constitutive levels of myoglobin isoforms may have other functions in non-muscle tissues. For example, myoglobin may act as a cytoprotective agent to forestall injury during hypoxia/ischemia and reoxygenation/reperfusion by scavenging free radicals (Mammen *et al.*, 2006). Importantly, myoglobin also plays a role in intracellular oxygen diffusion from cell surface to mitochondria (Roesner *et al.*, 2006; Wittenberg and Wittenberg, 2003)

### 3.2.2 Cytoglobin.

Cytoglobin has been localised to the nucleus in the cells of several tissues (Geuens *et al.*, 2003). The cytoglobin gene contains both Hypoxia Response Elements (HREs) and mRNA stabilisation sites characteristic of an oxygen-regulated gene. Furthermore, real-time quantitative PCR has confirmed that cytoglobin is regulated by HIF-1 $\alpha$  (Fordel *et al.*, 2004).

While cytoglobin is up-regulated in response to hypoxia in hippocampal cells *in vitro* (Fordel *et al.*, 2004), it is not uniformly upregulated throughout the brain *in vivo* and it is expressed in different brain regions from those in which neuroglobin is found (Mammen *et al.*, 2006). There is no increased expression of cytoglobin mRNA or protein in the neocortex after either chronic or intermittent hypoxia (Li *et al.*, 2006). The brain regions expressing significantly elevated levels of cytoglobin in response to hypoxia, are the areas of the archicortex that are sensitive to hypoxia and oxidative stress, namely the hippocampus, thalamus, and hypothalamus. This provides evidence that cytoglobin is an oxygen responsive globin *in vivo* (Mammen *et al.* 2006). Cytoglobin is strongly expressed in the developing mammalian central nervous system and it has been suggested that its localisation in brain areas sensitive to oxidative stress could be related to a myoglobin-like role in scavenging free radicals associated with the metabolism of oxygen and nitrogen (Mammen *et al.*, 2006).

### 3.2.3 Neuroglobin.

Another recently discovered and characterised intracellular globin, neuroglobin, is expressed in the mammalian central and peripheral nervous system and has recently been detected in cultured astrocytes from newborn mouse brain (Chen *et al.*, 2005). In ischemic astrocytes apoptosis was increased when cultured cortical astrocytes were treated with neuroglobin antisense (Chen *et al.*, 2005). It has been suggested that neuroglobin is not only associated with areas of high metabolic rate (as indicated by their elevated mitochondrial density) in the brain but also in other neuronal compartments (Hankeln *et al.*, 2005). Neuroglobin mRNA and protein can be detected in neuronal perikarya, axons and synapses

(Hankeln *et al.*, 2005). Neuroglobin makes up less than 0.01% of the protein in the brain (Mammen *et al.*, 2002).

Mammen *et al.*, (2002) suggested that neuroglobin expression is correlated with regions of the CNS involved in adaptive stress response pathways. Neuroglobin is found in brain areas that have high levels of nitric oxide (Mammen *et al.*, 2002). Since one of the cytoprotective actions of myoglobin is to detoxify nitric oxide in the heart, it has been suggested that neuroglobin may act similarly in the brain (Burmester and Hankeln, 2004; Mammen *et al.*, 2002). While neuroglobin is strongly expressed in the subthalamic nucleus, only low levels are found in the hypoxia-sensitive cerebellum and hippocampus (Pesce *et al.*, 2004). Data with radiolabelled RNA probes and *in situ* hybridization suggest that neuroglobin expression is related to oxygen-dependent functions. There is a constitutive level of neuroglobin present in brain areas that respond to changes in oxygen levels, for example: the locus coeruleus, the parabrachial complex and the periaqueductal grey (reviewed by Mammen *et al.*, 2002). The highest levels of neuroglobin have been reported in the mitochondria-rich photoreceptors of the retina (Hankeln *et al.*, 2005).

Neuroglobin has an oxygen affinity characterized by a  $P_{50}$  value of 2 torr, which is twofold higher than that of myoglobin but much lower than that of most haemoglobins. Neuroglobin has been implicated in the storage and/or intracellular transport of oxygen in highly metabolically active tissues (Burmester *et al.* 2000; Couture *et al.* 2001; Trent *et al.* 2001) and in facilitating  $O_2$  diffusion into mitochondria (Burmester *et al.*, 2000). It seems likely that it could function as an intracellular oxygen sensor as well as participate in the metabolism of reactive species such as NO and ROS. Burmester and Hankeln (2004) suggested that neuroglobin could be involved in the destruction of ROS especially in hypoxic conditions or resulting from oxidative stress that follows hypoxia and reperfusion. However, this suggestion appears not to hold for retina, since there was not a significant increase in neuroglobin mRNA and protein in zebrafish (*Danio rerio*) retina following hypoxia even though brain neuroglobin level increased 5-fold (Roesner *et al.*, 2006). The lack of neuroglobin upregulation in the retina following hypoxia may be species-specific since some hypoxia-tolerant animals such as crucian carp can turn off visual processing at low oxygen levels (Johansson *et al.*, 1997). It has also been suggested that neuroglobin could function as a terminal oxidase to regenerate  $NAD^+$  (Milton *et al.* 2006) and thereby maintain ATP production when oxygen levels are diminished. Neuroglobin has been shown to have neuroprotective properties demonstrated by reduced neuronal damage following stroke (Sun *et al.*, 2003). Furthermore it is up-regulated in the brain of the anoxia and hypoxia tolerant

turtle and it has been suggested that it mediates neuronal survival under anoxia (Milton *et al.*, 2006). This protective effect was diminished by the inhibition of neuroglobin expression with an antisense oligodeoxynucleotide, and enhanced by neuroglobin over-expression (Sun *et al.*, 2003). Fago *et al.*, (2006) suggest that since neuroglobin reacts with ferric cytochrome c with rapid kinetics it may well have a role in preventing apoptosis following periods of neuronal stress that cause a surge in the release of cytochrome c.

Neuroglobin induction may also be a part of the stress response initiated by haem based transcription factors and/or their second messengers, because a transient increase in neuroglobin level was observed *in vitro* cultures of cortical neurons after they were exposed to priming conditions that usually result in HIF-1  $\alpha$  upregulation such as severely diminished oxygen levels or the addition of cobalt chloride or the iron chelator, desferroxamine (Sun *et al.* 2003). However, neuroglobin may be regulated by response elements other than HREs (see Hankeln *et al.*, 2005 for a review). Neuroglobin mRNA can be induced directly by hemin in a time- and concentration-dependent manner via a non-hypoxia dependent second signal transduction pathway which can be blocked by the protein kinase G inhibitor KT5823 (Zhu *et al.*, 2002) suggesting that neuroglobin may be a multifunctional protein that is upregulated by hypoxia and downregulated by protein kinase G. The upregulation of neuroglobin by hemin may represent a HIF-1 $\alpha$  independent pathway and further research is needed to clarify the interaction of HIF with hemin-induced neuroglobin upregulation. Furthermore, Zhu *et al.*, (2002) demonstrated that the hypoxic induction of neuroglobin could be prevented by the mitogen-activated protein kinase inhibitor, PD98059, revealing that neuroglobin expression is regulated by more than one signal transduction pathway. Notably, mitogen-activated protein kinases are involved in redox-dependent signalling.

### 3.3 Cytochromes.

The involvement of cytochromes in oxygen sensing has been indicated in many studies (Ehleben *et al.*, 1998; Duranteau *et al.*, 1998; Guzy *et al.*, 2005; Guzy and Schumacker, 2006; Porwol *et al.*, 2001). The cytochromes involved have been suggested to be mitochondrial (Guzy *et al.*, 2005; Guzy *et al.*, 2006) and non-mitochondrial, e.g., cytochrome aa3 (Porwol *et al.*, 2001) in origin. In both cases, it appears that ROS are the actual transducing molecule for the cytochrome signal. It also appears that CO may significantly regulate the oxygen-dependent cytochrome function (Porwol *et al.*, 2001).

### 3.4 NADPH Oxidase.

Some of the cytochromes suggested to take part in oxygen sensing are parts of the NADPH oxidase enzyme. NADPH oxidase is a heterodimeric flavocytochrome of gp91-phox and p47-phox, which is capable of recruiting the polypeptides p67-phox, p47-phox and p40-phox to form a membrane bound, multi-subunit structure (Dahan *et al.*, 2002). Recent findings indicate that there are several isoforms of NADPH oxidase in many cell types and that components of some isoforms can act as a putative oxygen sensor (reviewed by Acker, 2005). The signalling is mediated via the oxygen regulated ROS formation. NADPH oxidase inhibitors blocked the response of carotid chemoreceptor discharge to hypoxia (Cross *et al.*, 1990) and blocked  $K^+$  and  $Ca^{2+}$  currents in type 1 cells of the carotid body (Wyatt *et al.*, 1995). Similarly, mRNA for NADPH oxidase and voltage gated  $K^+$  channels have been co-localised to hypoxia responsive pulmonary neuroepithelial bodies and it has been suggested that this tight association represents an oxidase linked  $K^+$  channel, that acts as an oxygen sensor (Wang *et al.*, 1996). Furthermore, gene knock out studies have shown that gp91-phox null mice had a significantly impaired hypoxic ventilatory response due to the decreased sensitivity of pulmonary neuroepithelial bodies (Kazemian *et al.*, 2001). However, the gp91-phox knock outs show no impairment of carotid body function (Roy *et al.*, 2000) or pulmonary vasoconstriction (Archer *et al.*, 2000) indicating that this single polypeptide is not pivotal in all effector tissues and it is likely that effectors may contain multiple oxygen sensors so if one malfunctions then compensatory changes can be made by other oxygen responsive sensors. Since NADPH oxidase appears to be involved in oxygen sensitive responses both in plants and in animals, it may be a widespread evolutionarily conserved oxygen sensor.

### 3.5 Prolyl and asparagine hydroxylases.

Transcriptional regulation by oxygen is mainly achieved via the function of hypoxia-inducible factor. Its function is, to a large extent, regulated either by affecting the stability of the protein by hydroxylation of conserved prolines (proline 402 and 564 in the human protein) and consecutive degradation of the molecule, or by affecting the interaction of the molecule with p300 and consecutive DNA binding as a result of hydroxylation of a conserved asparagine residue (Asp803), although it now appears that in hypoxia-tolerant animals also the transcription of HIF may play a role in the regulation of the HIF pathway (see, e.g., Shams *et al.* 2004, Rissanen *et al.* 2006b, Law *et al.* 2006). In the case of both prolyl and asparagyl hydroxylation, the hydroxylases catalysing the respective reactions, i.e., prolyl and asparagyl

hydroxylases use molecular oxygen as a substrate. Their function is oxygen-dependent and thus they function as oxygen sensors affecting hypoxia-inducible gene expression as first demonstrated by Ivan *et al.* (2001) and Jaakkola *et al.* (2001). The function of prolyl hydroxylases has recently been reviewed by Fandrey *et al.* (2006). Three types of oxygen-dependent proline hydroxylases (PHD1-3) has been described, although the presence of a fourth (PHD4) has been deduced on the basis of genomic information (Oehme *et al.*, 2002). While hydroxylation of conserved prolines (which have the LXXLAP sequence) is achieved by the proline hydroxylases, it appears that several other residues are important for the proper functioning of the PHDs (Fandrey *et al.*, 2006). This indicates that in addition to the properties of enzymes themselves, e.g., the three-dimensional structure of HIF-1 $\alpha$  affects the hydroxylation. Notably, hydrophobicity plots of various vertebrate HIF-1 $\alpha$ s show that the conserved proline residues are in a highly hydrophobic environment, suggesting that the residues are folded within the protein (Unpublished results, Rytönen K, Vuori KAM, Primmer CR and Nikinmaa M., 2007). Because of this, one possibility for the effect of ROS and calcium on the HIF-1 $\alpha$  function is that they, by affecting some of the residues important for HIF-1 $\alpha$  protein folding, affect the three dimensional structure of the protein, thereby affecting the accessibility of the conserved prolines towards proline hydroxylases. This mechanism would add another way for regulating HIF-1 $\alpha$  function by oxygen, since ROS levels can be oxygen-dependent. It would also explain, why HIF-1 function has been shown to be ROS-dependent in several studies (reviewed by Haddad, 2002; Acker *et al.*, 2006; Kietzmann and Gorch, 2005), although the enzymatic hydroxylation and consecutive proteasomal breakdown of the protein do not require ROS (Fandrey *et al.*, 2006).

Oxygen-dependent regulation of the DNA-binding of HIF is achieved via the function of asparagine hydroxylase (FIH= factor inhibiting hypoxia-inducible factor; Kaelin, 2005). Because the prolyl and asparagyl hydroxylases have different oxygen affinities, it is possible that different genes are regulated by the two enzymes (Dayan *et al.*, 2006). Also, it is possible that the two enzymes regulate HIF function at different oxygen levels.

### **3.6 AMP Kinase.**

The function of AMP-activated protein kinase has been reviewed recently, e.g., by Hardie (2003). The enzyme is composed of three subunits, the catalytic  $\alpha$  subunit, and the regulatory  $\beta$  and  $\gamma$  subunits. While the system is activated by AMP, it remains inactive even in the presence of this allosteric effector, if not phosphorylated to a critical threonine residue

(e.g. Hardie 2003). Because the enzyme is activated by decreasing energy charge, it is activated, in addition to hypoxia and anoxia, by any form of stress that affects energy production/consumption. Thus, much research on AMPK has been directed towards glucose/glycogen/diabetes and fat metabolism studies (e.g., Kim *et al.*, 2005; Yun *et al.*, 2005). AMPK function is also affected by ROS (e.g, Choi *et al.*, 2001), and NO (e.g., Lei *et al.*, 2005), adding to the possibilities of interaction between different regulatory pathways. Since AMPK is involved in regulating cellular energy balance, its activation switches off energy consuming and switches on energy producing pathways. One of the major oxygen consuming processes in the cells involves mRNA translation to proteins. Notably, it is inhibited by AMPK in hypoxia also independently from HIF regulation (Liu *et al.*, 2006), showing the importance of energy sensing in hypoxia regulation.

#### **4.0 Effector systems.**

There are basically two types of effector systems involved in oxygen-dependent phenomena, those that exert their oxygen-dependent effect immediately, and those that mediate slower oxygen-dependent changes in, e.g., transcription. The former, often coupled to ion channels and consecutive immediate neural functions exert acute effects on, e.g., the function of respiratory neurons and may be involved in peripheral oxygen sensing associated with ventilatory regulation. The slower, chronic effects can, on the other hand, regulate neural function in such a way that either anaerobic energy production is facilitated or energy is spared usually via oxygen-dependent gene regulation. Often such gene regulation involves hypoxia-inducible factor. Notably, oxygen-dependent effects may in addition to molecular oxygen also rely on signals in the form of ROS and energy metabolites, so that interactions between oxygen, oxidative stresses and energy metabolism may occur both in acute and chronic oxygen responses.

#### **4.1 Oxygen-dependent ion transport systems.**

##### **4.1.1 Exchangers and co-transporters.**

Oxygen-dependent co-transporters and exchangers have been studied in most detail in erythrocytes (Gibson *et al.*, 2000) with only sporadic information on other cell types (Tuominen *et al.*, 2003). In erythrocytes, it appears that a major factor regulating oxygen-dependent ion transport is the hydroxyl radical, which has been suggested to increase the activity of KCl cotransport (Bogdanova *et al.*, 2001) and decrease the activity of  $\text{Na}^+/\text{H}^+$

exchange (Nikinmaa *et al.*, 2003). While data in mammalian and bird erythrocytes have generally been compatible with haemoglobin being the proximal oxygen sensor (Drew *et al.*, 2004; Flatman, 2005; Honess *et al.*, 1996; Muzyamba *et al.*, 1999; Muzyamba *et al.*, 2000), work on hypoxia-intolerant teleost fish species, rainbow trout, indicated that in the species bulk haemoglobin could not be the oxygen sensor (Berenbrink *et al.*, 2000). Interestingly, studies on the erythrocytes of the hypoxia-tolerant crucian carp indicate the presence of two different oxygen sensors, one of which has oxygen affinity similar to (bulk) haemoglobin, and the other much lower (Berenbrink *et al.*, 2006). Thus, the data are compatible with minimally two different oxygen sensors of which one may use molecular oxygen as the sensed molecule and the other may use oxygen radicals (Berenbrink *et al.*, 2006; Bogdanova *et al.*, 2001).

#### 4.1.2 Ion channels.

Most work on the oxygen-dependent ion channels has been done on excitable cells. Some voltage-gated ion channels, such as  $K^+$ ,  $Ca^{2+}$  and  $Na^+$  channels, have their conductance regulated by oxygen-dependent factors with the result that the excitability of a cell is oxygen-regulated. Fast adaptive changes that compensate for diminished  $O_2$  levels are mediated by oxygen responsive ion channels and are manifested as changes in excitability, secretion and/or contractility depending on the cell type (Lopez-Barneo *et al.*, 2001). Cysteine-rich voltage gated ion channels respond to changes in reduced/oxidised redox pairs such as  $NADH/NAD^+$  so that they close when oxygen levels drop favouring the formation of more  $NAD^+$  (Archer, 2000).

A decrease below the threshold  $PO_2$ , normally close to 50 Torr, in glomus cells of the carotid body usually experiencing arterial  $PO_2$ , or the neonatal ductus arteriosus results in the inhibition in the level of the tonic  $K^+$  current. Oxygen regulated inhibition of  $K^+$  channels, which may be mediated by mitochondria-derived hydrogen peroxide (Archer *et al.*, 2004) results in an increase in cellular excitability, increased  $Ca^{2+}$  influx and a resultant increase in the level of  $Ca^{2+}$  in the cytosol (reviewed by Lopez-Barneo *et al.*, 1999).

Neuroepithelial bodies found in mammalian airways have oxygen-sensitive  $K^+$  currents and are putative airway chemoreceptors which detect changes in the level of  $O_2$  in the airway lumen (Wang *et al.*, 1996). Exposure to acute hypoxia *in vivo* (Lauweryns *et al.*, 1987) or *in vitro* (Fu *et al.*, 2002) results in serotonin release which may have a role in controlling pulmonary vascular tone. The oxygen sensitivity of these channels may result from their close localisation to membrane-bound cytochromes (Youngson *et al.*, 1997). It is clear that the

closure of voltage gated  $K^+$  channels by hypoxic exposure has implications for neuroprotection as demonstrated by  $K^+$  channel arrest in hypoxia tolerant turtles (Pek and Lutz, 1997; Bickler and Buck, 1998; Bickler and Donohoe, 2002; Hochachka and Lutz 2001) which reduces  $Ca^{2+}$  influx (Bickler and Buck, 1998). In some anoxia tolerant species, neuronal energy is not only conserved by ion channel arrest but also by ATP-sensitive mitochondrial  $K^+$  channel arrest (reviewed by Buck and Pamerter, 2006). This may have implications for clinical interventions.

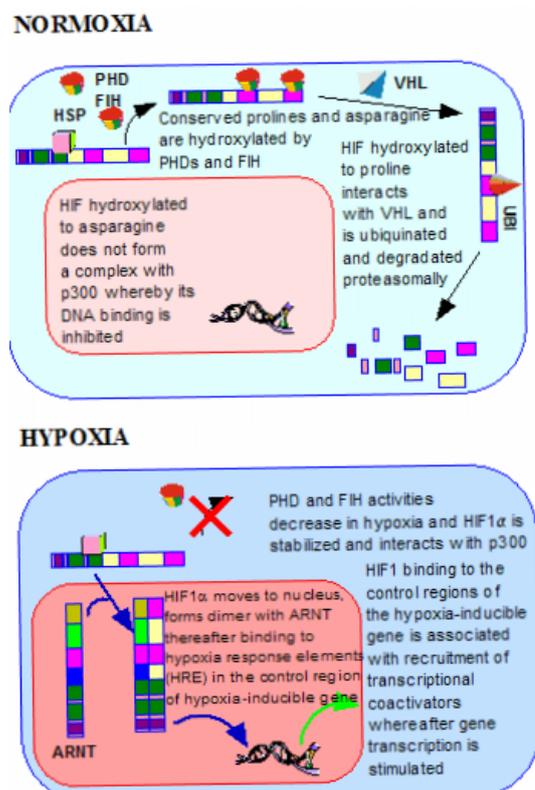
In the carotid body, hypoxia causes increased  $Ca^{2+}$  influx through voltage gated  $K^+$  channels. This results in an increased afferent input to the brain stem and consequent neurosecretion of catecholamines. Conversely, in the ductus arteriosus a rise in  $PO_2$  triggers increased  $H_2O_2$  production. This inhibits voltage gated  $K^+$  channels and leads, in turn, to vasoconstriction and the closure of the ductus arteriosus (Archer *et al.*, 2004). Calcium entry also occurs through L-type  $Ca^{2+}$  channels involving PKC and/or phosphatase-sensitive pathways (Summers *et al.*, 2000). It is this  $Ca^{2+}$  entry through voltage-gated  $Ca^{2+}$  channels that mediates hypoxia-responsive neurosecretion by the carotid body and the hypoxic response in the carotid body can be blocked by  $Ca^{2+}$  channel blockers (reviewed by Lopez-Barneo *et al.*, 2001). Chronic exposure to hypoxia upregulates T-type voltage gated  $Ca^{2+}$  channels as one of the actions of hypoxia inducible transcription factors (Del Toro *et al.*, 2003).

Neurons in the caudal hypothalamus appear to be oxygen responsive. They alter their firing rates in hypoxia to contribute to the control of cardiorespiratory responses to hypoxia (Horn and Waldrop 1997). Oxygen sensitive  $Na^+$  channels appear to regulate neuronal excitability in the caudal hypothalamus, since, as oxygen levels drop below threshold, the  $Na^+$  current is inhibited by a PKC-dependent mechanism (O'Reilly *et al.*, 1997). Prolonged hypoxia can result in  $Na^+$  channel excitability (Xia *et al.*, 2000), depending on the frequency, intensity and duration of the hypoxic episodes (Zhao *et al.*, 2005). Furthermore, a subset of voltage-gated  $Na^+$  channels, which are tetrodotoxin-sensitive, are not inhibited by hypoxia and generate “persistent” sodium currents which could cause irreversible neuronal damage as  $Na^+$  homeostasis, is lost (Hammarstrom and Gage, 2002). Under experimental conditions the “persistent” sodium current can be inhibited by reducing agents such as dithiothreitol and reduced glutathione (Hammarstrom and Gage, 2002) suggesting that the current is, in fact, redox sensitive.

#### 4.2 Hypoxia-Inducible Factor Dependent Pathway.

In 1990s oxygen-dependent gene expression was conclusively shown to take place. The early studies concentrated on erythropoietin pathway (Semenza and Wang, 1992), but later studies have indicated that oxygen directly affects the expression of many, maybe a hundred, genes (Lahiri *et al.*, 2006). The master regulator of oxygen-dependent gene expression is the hypoxia-inducible transcription factor (HIF), which regulates transcription in the following fashion (Bracken *et al.*, 2003; Wenger, 2000; see Figure 1.) The active factor is a nuclear dimer of HIF $\alpha$  and ARNT (HIF $\beta$ ). In the dimer, HIF $\alpha$  is the component conferring hypoxia sensitivity and ARNT is constitutively expressed. HIF $\alpha$  confers hypoxia sensitivity to the function of the protein dimer, since, although it is continually produced at all oxygen tensions, it is rapidly broken down in normoxia. HIF $\alpha$  degradation involves prolyl hydroxylases (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), which bind at conserved proline residues in the oxygen-dependent degradation domain. It appears that the actual core of the oxygen-dependent degradation domain [including proline564 and proline402 (human nomenclature) which undergo oxygen-dependent hydroxylation by prolyl hydroxylases (Semenza, 2001) is invariable in HIF-1 $\alpha$ s across vertebrates (Rytkönen K, Vuori K.A.M., Primmer C.R. and Nikinmaa M., unpublished observations).

**Figure 1**



**Figure 1.** A representation of HIF (hypoxia-inducible factor) function. In normoxia, HIF-1 $\alpha$  is broken down after prolyl hydroxylation and its interaction with p300 is diminished after asparagyl hydroxylation. In hypoxia, hydroxylase enzymes are inhibited, whereby the DNA binding of HIF may occur and oxygen-sensitive genes can be induced.

The regulation of oxygen-dependent degradation may, however, also require residues in the vicinity of ODD, which affect the tertiary structure of ODD core. Prolyl hydroxylation enables the interaction of HIF $\alpha$  and the von-Hippel-Lindau protein, subsequent ubiquitylation and proteasomal degradation. Recent results suggest that although prolyl hydroxylation does not require ROS, it can be under redox control by hydroxyl radicals (Liu *et al.*, 2004). In hypoxia, prolyl hydroxylation does not occur, whereby HIF $\alpha$  protein is stable, is transported from cytoplasm to nucleus, where it forms a dimer with ARNT and recruits the general transcriptional activator CBP/p300. Recruitment of transcriptional activators depends on hydroxylation reactions of conserved asparagine (Mahon *et al.*, 2001). Thereafter HIF (HIF $\alpha$  + ARNT) binds to hypoxia-response elements present in the promoter/enhancer region of the hypoxia-inducible genes, and gene transcription is stimulated. Since both the stability of HIF and its transcriptional activity are affected by oxygen-dependent enzymes, the oxygen affinity of the gene expression depends on the oxygen affinities of both enzymes. Oxygen availability may further affect the three dimensional structure of HIF protein, since both the DNA binding and the transcriptional activation by HIF appear to be under redox control (Bracken *et al.*, 2003; Lando *et al.*, 2000): serine-to-cysteine mutation at a specific residue in the DNA binding domain confers redox sensitivity of DNA binding, and the nuclear redox regulation Ref1 potentiates the hypoxic induction of a reporter gene (Lando *et al.*, 2000). At present, while the principles of how HIF regulates gene expression are clear, the fact that oxygen can affect HIF function at several different places and the lack of measurements means that there is currently no decisive information on the possible differences of oxygen affinities for oxygen-dependent gene expression between tissues and species. Also, the HIF pathway can be stimulated in normoxic conditions by various growth factors, hormones, and cytokines (Kodama *et al.*, 2003; Ma *et al.*, 2004; Page *et al.*, 2002; Richard *et al.*, 2000). In most cases the basis of the stimulation is not known.

#### ***4.3 Hypoxia response element.***

In addition to the properties of HIF itself, the hypoxia response elements (HREs) especially in the promoter/enhancer regions of the transcribed gene affect gene expression (HREs may also be present in the introns of oxygen-dependent genes; Rees *et al.*, 2001). The minimal consensus HRE is A/GCGTG (Camenisch *et al.*, 2002). In some cases the presence of HREs alone is not sufficient for hypoxic induction of the genes (Firth *et al.*, 1995), but additional

elements such as binding sites for AP1, ATF1/CREB1, HNF4 or Smad3 may be required (Bracken *et al.*, 2003).

#### ***4.4 Redox-responsive transcription factors.***

While hypoxia-inducible factor has been studied most with regard to hypoxia-inducible gene expression, several other transcription factors also appear to be hypoxia-sensitive (Cummins and Taylor, 2005). These include, e.g., NF $\kappa$ B, which is a redox-sensitive transcription factor (Fan *et al.*, 2003; Fratelli *et al.*, 2005), and consequently responds to variations in ROS, the level of which is dependent on oxygen level (Bogdanova *et al.*, 2003). Observations suggest that while HIF induces gene expression especially in hypoxia, NF $\kappa$ B exerts its major influence in hyperoxia (Michiels *et al.*, 2002). Other redox-sensitive transcription factors include PPAR $\gamma$ , Nrf2, AP-1, STAT and p53 (Kim and Surh 2006). Many of these show interaction with hypoxia-inducible factor (Pan *et al.*, 2004) and can be affected by prostaglandins (Kim and Surh, 2006).

#### ***4.5 Mitochondrial function as an oxygen-sensitive effector system.***

Mitochondria provide the fuel for life processes by generating ATP but can also provide the signals for death via apoptosis if their membrane potential is compromised. Also, as indicated above, mitochondrial cytochromes are suggested to be involved in primary oxygen sensing (Chandel and Schumacker, 2000; Guzy *et al.*, 2005; Guzy *et al.*, 2006; Wilson *et al.*, 1994; Zhu and Bunn 1999). The oxygen sensor function of mitochondrial cytochromes requires that their ROS production is oxygen sensitive. Both an increase and a decrease in ROS production with decreasing oxygen tension are feasible. When the mitochondrial electron transport chain is slowed down or arrested there is a decreased generation of ROS coupled with an increase in reducing equivalents so either of both of these could act as signals generated by mitochondria in response to low oxygen. An increase in ROS production in hypoxia can occur if oxygen affects the lifetime of the ubisemiquinone radical in complex III in the mitochondrial inner membrane, its ability to access the ubisemiquinone radical or the relative release of mitochondrial ROS to the matrix vs. intermembrane space (Guzy *et al.*, 2006). Hydrogen peroxide can both mimic hypoxia (Canbolat *et al.*, 1998) and counteract the hypoxia responses as shown by the facts that it inhibits the induction of erythropoietin in response to hypoxia (Fandrey *et al.*, 1994) and that the blockade of Epo expression could be reversed by the addition of cobalt chloride and iron chelation (Fandrey *et al.*, 1997). It is

possible that mitochondria from different cells (and conditions) differ from each other (Michelakis *et al.*, 2002).

With regard to the oxygen dependence of mitochondrial energy production, isolated mitochondria are capable of producing energy aerobically at much lower oxygen concentrations *in vitro* than are lethal *in vivo* (Gnaiger *et al.*, 1998; Krumschnabel *et al.*, 2000). Notably, the energy production is decreased already at relatively high oxygen tensions (more than 30 mmHg) (Rissanen *et al.*, 2006a). Notably, the oxygen affinity of mitochondrial function is affected both by NO (Koivisto *et al.*, 1997) and by CO (D'Amico *et al.*, 2006), both of which are molecules associated with oxygen sensing. In addition to the mitochondrial energy production, also apoptosis (programmed cell death) is oxygen-sensitive, and affected by mitochondrial function (Araya *et al.*, 1998; Banasiak *et al.*, 2000). The hypoxic induction of apoptosis often involves release of cytochrome C from mitochondria, which initiates consequent caspase activation (Chae *et al.*, 2001). It appears that ROS are involved in the hypoxic apoptosis signalling (Kim and Park, 2003). Also the dependency of apoptosis on oxygen level shows interaction with NO, CO and glucose indicating interdependence of different cellular effector pathways (Madesh *et al.*, 1999; Malhotra and Brosius, 1999; Tofighi *et al.*, 2006).

#### ***4.6 Interactions between oxygen-dependent and other effector pathways.***

Considering the interconnected nature of biochemical pathways, it is not surprising that antagonistic and synergistic effects of other effector pathways on oxygen sensors have been reported. In many cases a number of stressors are acting simultaneously and the integrated response may differ in direction or intensity from that caused by a single stressor either diminishing the oxygen sensitive response or enhancing it. Furthermore the effect of cross-protection by stressors needs to be taken into account when considering physiological responses to a specified stressor because oxygen sensors may be affected by more than one type of stress. For example, physiological stress caused by exercise can increase the generation of both ROS and NO which are key signalling molecules in the oxygen-dependent pathways as discussed above. It has recently been demonstrated that exercise at physiologically relevant levels could result in significant elevations in HIF-1 $\alpha$  protein levels coupled with decreased von Hippel-Lindau tumour suppressor protein levels, responsible for its turnover (Ameln *et al.*, 2005). So it is conceivable that the neurogenerative and neuroprotective effects of exercise may be mediated by activation the oxygen sensing pathway.

Both NO and CO may affect (and be involved in) oxygen-dependent phenomena. Accordingly, *in vitro* experiments have demonstrated that elevated levels of NO or CO can diminish effects of hypoxia on gene transcription. These molecules both activated the internal oxygen dependent degradation domain and repressed the C-terminal transactivation domain of HIF resulting in less HIF-1 $\alpha$  that can bind to DNA (Huang *et al.*, 1999). On the other hand, *in vivo* experiments have demonstrated that NO is one of the modulators of the hypoxic ventilatory response mediated by the rostral mediolateral medulla (de Paula and Brancho, 2003). It is not clear at present, whether different cell types respond in a different manner.

Activated microglia in the CNS express high levels of inducible nitric oxide synthase (iNOS) and occur in high numbers in brain ageing and inflammatory pathologies such as stroke and neurodegenerative diseases (Mander *et al.*, 2005). In addition, exposure to hypoxia and oxidative stress can further increase the number of activated microglia *in vivo*. Cerebellar granule cells in culture were more susceptible to hypoxia-induced neuronal death when there were increased levels of NO present (Mander *et al.*, 2005). Thus NO may potentiate the deleterious effects of hypoxia by blocking the action of HIF-1 $\alpha$  on the induction of neuroprotective genes via the diminished levels available to bind to DNA, as discussed above (Huang *et al.*, 1999).

The brain, approximately 2% of the body mass, has a high metabolic demand which accounts for approximately 50% of total body glucose utilization. Cross-talk between diminished oxygen and glucose levels occurs for at least two reasons: first, glucose sensing is activated by a signalling pathway that is common with oxygen sensors – the ROS system. The neurons of the arcuate nucleus which modulate insulin release show increased activity in response to glucose or in response to increased ROS as a result of treatment with mitochondrial complex blockers such as rotenone and antimycin, which can be reversed by treatment with antioxidants (Leloup *et al.*, 2006). Second, glucose sensing occurs in some cells which also sense oxygen levels, for example, in the carotid body. While peripheral glucose levels are monitored and responded to by the liver, low glucose levels also inhibit voltage-gated K<sup>+</sup> channels in the glomus cells, as occurs when oxygen levels fall below a threshold (Pardal and Lopez-Barneo, 2002). Since glomus cells respond to both low glucose and low oxygen, it has been suggested that this strategically placed multifunctional sensor regulates oxygen and glucose homeostasis to protect the brain (Pardal and Lopez-Barneo, 2002). Also, interaction between two transcription factors, HIF and glucose-dependent transcription factor has been described, in which the respective response elements in DNA

can function both as glucose response elements and hypoxia response elements (Kietzmann *et al.*, 2002).

The interaction between oxygen-dependent pathways and more general stress sensing pathways can be seen at an intracellular level, for example in the upregulation of molecular chaperones such as heat shock proteins (Hsp) which assist in refolding damaged proteins (Burston and Clarke 1995; Lund 1995) and maintaining the tertiary structure of proteins during metabolic stress (Gonzales *et al.*, 1991). Hsps are upregulated from their constitutive level in response to a diverse array of physiological stressors (Basu *et al.*, 2002). These stressors include exposure to psychoactive drugs (Miller *et al.*, 1991); neurodegenerative disease (Harrison *et al.*, 1993), cellular injury (Liang and MacRae, 1997), hypoxia, ischemia (Welch, 1992; Locke and Noble, 1995) and acute temperature change (Airaksinen *et al.*, 1998). In fact, many studies have shown the accumulation of heat shock proteins in hypoxia/anoxia (Hammerer-Lercher *et al.*, 2001; Patel *et al.*, 1995). In many of the studies which show hypoxic/anoxic accumulation of heat shock protein, it is difficult to say, if the response is a general response to stressful conditions or specific to oxygen limitation. The theory of parsimony predicts that a broad range of triggers probably converge on a single molecular target, which once activated serves to up-regulate Hsp production and while this remains to be fully tested, there is evidence that induction of Hsp70 is linked to both an oxygen sensor and/or an energy sensor since Hsp70 promoter activation responds to low oxygen (Madamanchi *et al.*, 2001), as well as to decreased cellular energy charge (Kiang and Tsokos, 1998). Furthermore, energy sensors and oxygen sensors appear to act synergistically to regulate Hsp70 levels and it was suggested that a metabolic sensor may be involved in further upregulating the level of Hsp70 above the level that could be induced by anoxia alone (Renshaw *et al.*, 2004). Similarly, cross-protection occurs: when animals are exposed to a sublethal physiological stressor, they are protected from a subsequent stressor of the same or a different modality. In the mammalian heart, heat treatment activates HIF-1 $\alpha$  and its target genes including EPO which may then be responsible for the observed cross-protection to infarction after ischemia reperfusion (Maloyan *et al.*, 2005). In the brain, exposure to brief ischemia made the hypoxia sensitive CA1 neurons of the hippocampus tolerant to levels of ischemia that were normally lethal (Kitagawa *et al.*, 1990; Kirino, 2002). In addition, non ischemic insults which resulted in the induction of Hsps in the brain and retina have been associated with resistance to a variety of insults including ischemia (Franklin *et al.*, 2005). Taken together these data illustrate the multifunctional nature of oxygen sensitive molecules

and suggests that many may be part of the repertoire of responses to stress in general rather than to a specific stressor.

Interaction between oxygen-dependent and other responses occur both in rapid and slower responses. For example, the rapid oxygen-dependent regulation of membrane transport appears quite often to be regulated by reactive oxygen species, and thereby redox-state. Thus, any redox disturbance will affect oxygen-dependent ion transport. As indicated above, gene expression – a slower oxygen-dependent system – is also affected by redox-state dependent transcriptional regulation.

#### ***4.7 Oxygen-dependent and xenobiotically induced gene expression pathways.***

With regard to the function of hypoxia-inducible factor, its dimerization partner ARNT is also a dimerization partner for many other transcription factors, among them the aryl hydrocarbon receptor (AhR; dioxin receptor). When it was observed that both the xenobiotically induced and hypoxic gene expression used the same dimerization partner, several studies investigated the possibility that one affected the other. While some studies have not been able to show interaction, several others have indicated interaction which may be cell type specific (Chan *et al.*, 1999; Gassmann *et al.*, 1997; Gradin *et al.*, 1996; Nie *et al.*, 2001). There may also be differences in which pathway is preferred (Hofer *et al.*, 2004). In addition to the interaction between aryl hydrocarbon receptor and HIF pathways, studies have indicated an interaction with HIF-pathway and the pathway involved in the generation of diel rhythmicity (Chilov *et al.*, 2001).

#### ***4.8 Oxygen- and temperature-dependent responses.***

Interactions between temperature responses and hypoxia-induced responses may also occur. The possibility of this interaction is of minor importance for the central nervous system (CNS) of homeothermic animals, since CNS temperature is usually tightly regulated. If effects in homeotherms are observed, they will be apparent in the peripheral nerves of animals living in cold climates, since there the temperature may be decreased. In contrast, the temperature of all the tissues of poikilothermic animals may show large fluctuations. However, even in mammals temperature affects HIF-1 $\alpha$  expression. Increased amounts of HIF-1 $\alpha$  protein with concomitant induction of Hsp90 and Hsp70 have been observed in mice exposed to heat (Katschinski *et al.*, 2002), upon heat-acclimation in rat (Maloyan *et al.*, 2001) and in human hepatoma cell lines exposed to heat (Katschinski *et al.*, 2002). In poikilotherms, increased expression of HIF occurs during the heat acclimation of

*Caenorhabditis elegans* (Treinin *et al.*, 2003). Association of HIF- $\alpha$  with the heat shock protein hetero-complex Hsp90-Hsp70, stabilizes HIF-1 $\alpha$  by protecting it from degradation in both normoxia and hypoxia (Minet *et al.*, 1999; Katschinski *et al.*, 2002; Katschinski *et al.*, 2004). It appears that Hsp90 binds to PAS-B domain of HIF- $\alpha$  and exerts a stabilizing influence on the protein (Katschinski *et al.*, 2004). Whereas the above studies have indicated interaction of the heat shock response and the HIF response, recent studies (Rissanen *et al.*, 2006b) have indicated that acclimation to a reduced temperature is associated with an increased level of Hsps of the 70 and 90 classes, and increased HIF function, as shown by increased DNA binding of the transcription factor, in a poikilothermic vertebrate, crucian carp. The close association between increased Hsp expression and increased HIF reveal the interaction of temperature and/or stress sensitive pathways with oxygen sensitive pathways.

## **5. Cellular systems described for oxygen-dependent phenomena.**

### ***5.1 Non-excitabile cells: erythrocyte as an example.***

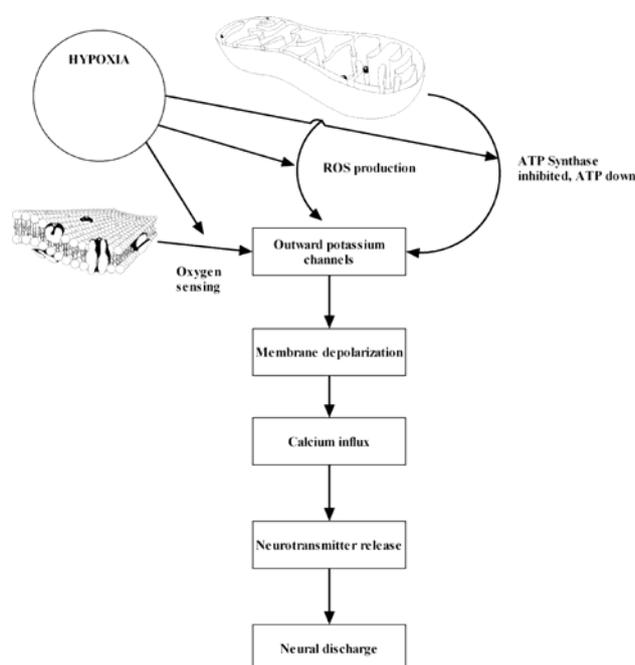
Among non-excitabile cells, erythrocytes have been studied in most detail with regard to oxygen-dependent ion transport, and the function of the ion transport pathways has been reviewed by Gibson *et al.*, (2000; see also section 4.1 above). As to the physiological role played by the oxygen-dependent pathways, they regulate oxygen transport in hypoxia, and possibly in hyperoxia (Nikinmaa, 2003). While data do not allow firm conclusions to be made about the role that erythrocytes may play in oxygen sensing, the interaction between NO and haemoglobin, and the possibility of consequent blood flow regulation (Stamler *et al.*, 1997), open up the possibility that regulation of erythrocytic oxygen transport is utilized in the regulation of oxygen-dependent responses.

### ***5.2 Excitable cells: carotid body glomus cell as a primary example.***

Carotid body glomus cells are cells that sense oxygen in the arterial chemosensory organ. Because of their behaviour as oxygen sensors of the peripheral nervous system, they are involved in the control of breathing, and have become probably the most important single excitable cell type in which oxygen sensing has been studied. Oxygen sensing by carotid body glomus cells and consecutive mechanisms behind oxygen-dependent nervous signalling to CNS have been the subject of several reviews (e.g., Acker and Xue, 1995; Bisgard, 2000;

Gonzalez *et al.*, 1995a; Gonzalez *et al.*, 1995b; Lahiri *et al.*, 2001; Lahiri *et al.*, 2006; Lopez-Barneo, 2003; Prabhakar and Overholt, 2000). Basically, in acute hypoxia, changes in oxygen level are sensed, the activity of potassium channels depends on the oxygen level sensed, leading to an increased efflux of potassium and membrane depolarization in hypoxia. Consequently, calcium influx occurs via calcium channels, neurotransmitters are released and increased neural discharge occurs in hypoxic conditions (Lahiri *et al.*, 2006). The sequence of oxygen-dependent responses of carotid glomus cells is schematically shown in Figure 2.

Figure 2



**Figure 2.** A schematic representation of how a decrease in oxygen tension (hypoxia) may affect carotid body glomus cell function. In the mitochondrial model hypoxia affects either reactive oxygen species (ROS) production or ATP production of mitochondria. Both of these may affect the outward flux of potassium via the potassium channel with the downstream effects shown in the diagram. In the membrane model, the ROS production by membrane-bound molecules (cytochromes) is oxygen-sensitive and thereby affected by hypoxia. Thus, these membrane-bound molecules function as proximal oxygen sensors, and cause effects on potassium channels with the downstream effects described in the figure and in the text.

However, although the sequence of events is well characterized and although a Nobel prize was awarded in 1938 to Heymans, a Belgian physiologist, for showing that the carotid body function is responsible for hypoxic hyperventilation, the actual mechanism of oxygen sensing is not clear. Both mitochondrial and membrane models for oxygen sensing have been presented, and several intracellular messenger systems of carotid body glomus cells respond to hypoxia. In the mitochondrial model, the flux of electrons to the final electron acceptor, oxygen, is reduced, whereby the mitochondrial membrane is depolarized leading to calcium efflux which stimulates the secretion of excitatory neurotransmitters, and leads to increased activity of afferent nerve fibres (Lahiri *et al.*, 2006). In the membrane model, hypoxia decreases the flux of ions via plasma membrane potassium channels thereby depolarizing the cells, and leading to the influx of calcium from extracellular space (Lahiri *et al.*, 2006). Experimental evidence supporting both models is available. Several inhibitors of

mitochondrial function also inhibit the hypoxia response of glomus cells (Lahiri *et al.*, 2001; Lahiri *et al.*, 2006). The mitochondrial cytochrome a3 has been suggested as a primary oxygen sensor in the glomus cell (Wilson *et al.*, 1994). On the other hand extramitochondrial or cell membrane-associated cytochromes have also been implied as primary oxygen sensors (Acker *et al.*, 1996; Porwol *et al.*, 2001). In both mitochondrial model and membrane model redox changes and reactive oxygen species are involved (Lahiri *et al.*, 2001; Lopez-Barneo, 2003; Porwol *et al.*, 2001), but whereas hypoxia is associated with a decrease in ROS in the case of extramitochondrial control (Porwol *et al.*, 2001), an increase occurs at low oxygen levels in mitochondria (Guzy and Schumaker, 2006). Also, based on the present data it appears that both NO and CO can influence the effect of hypoxia on neural discharge from the carotid body glomus cells (Lahiri *et al.*, 2006). Notably, NO influences the oxygen dependency of mitochondrial respiration (Koivisto *et al.*, 1997). In addition, interaction between oxygen-dependent and pH-dependent phenomena occur in glomus and other excitable cells (Miller *et al.*, 2004; Peers, 2004). It is, furthermore, possible that a globin, neuroglobin, is involved in oxygen sensing, since it has been characterized also in the carotid body (Di Giulio *et al.*, 2006). In sustained hypoxia, it appears that gene expression in the carotid body cells is modified via the hypoxia-inducible factor dependent pathway as in other cell types (Fung and Tipoe, 2003; Lopez-Barneo, 2003).

### ***5.3 An example of other excitable cellular systems: Gill neuroepithelial cells.***

Cells that were classified as neuroepithelial cells were microscopically observed in fish gills in early 1980s (Dunel-Erb *et al.*, 1982). Since breathing rate in fishes is mainly regulated by oxygen, several studies have investigated the possible effects of oxygen on these cells. The neuroepithelial cells share characteristics with both the carotid body glomus cells and lung neuroepithelial cells (Burlison *et al.*, 2006), and have high levels of biogenic amines (Burlison *et al.*, 2006; Dunel-Erb *et al.*, 1982). The branchial neuroepithelial cells are situated in the primary lamellae between water and blood, but appear to have no direct contact with environmental water (Dunel-Erb *et al.*, 1982). Burlison *et al.*, (2006) cultured the putative neuroepithelial cells to evaluate whether they had oxygen-sensitive potassium channels, which are a characteristic of oxygen-sensing cells of the carotid body (Donnelly, 1997; Donnelly, 1999; Lopez-Barneo *et al.*, 1999). The results indicated this to be the case. Notably, the function of oxygen sensing cells in the gills of fishes have so far been studied only in a couple of species – notably the zebrafish (Jonz *et al.*, 2004) and the channel catfish (Burlison *et al.*, 2006).

## 6.0 The importance of oxygen sensing in neural function.

When the PO<sub>2</sub> in the medulla falls below threshold, putative oxygen sensing neurons increase sympathetic activity (Solomon, 2000) triggered by the release of ATP to stimulate an “adaptive increase” in breathing (reviewed by Gourine 2005). The oxygen-chemosensitive cell of the central nervous system is encompassed in a distributed network of neurons in the brainstem (reviewed by Neubauer and Sunderram, 2004). Central oxygen responsive neurons mediate short- or long-term adaptations to hypoxia which provides both retaliatory and pre-emptive neuroprotective responses. The increased ventilatory response provides a neuroprotective function by pre-empting energy failure due to a mismatch of oxidative metabolism with energy expenditure. While many of the short-term rapid responses to PO<sub>2</sub> levels below threshold can be mediated by local peripheral oxygen-sensitive sensors including the carotid body and effectors, long term acclimatisation to hypoxia requires alterations in sympathetic outflow in the associated brainstem nuclei (reviewed by Guyenet, 2000). Using a chemodenervated rat model to remove afferent input from the carotid body Roux *et al.*, (2000) investigated the direct effects of hypoxia on neurons of the tractus solitarius and ventrolateral medulla and provided evidence that tyrosine hydroxylase (TH) mRNA levels increased in a similar manner to the sham operated group and some degree of ventilatory acclimatization still occurred, revealing that neurons in these two nuclei acted as oxygen sensors in their own right. Furthermore, neurons in the rostral ventrolateral medulla also demonstrated intrinsic oxygen sensitivity: since cultured neurons demonstrated hypoxia mediated excitation they have been proposed as putative oxygen sensors (Mazza *et al.*, 2000).

It has been known for some time that TH protein expression is oxygen-sensitive and shows a graded response to the duration of hypoxic exposure in the rat medulla. A short 3-day exposure resulted in a 26 - 50% increase in TH depending on the subpopulation of medullary nuclei examined. In contrast, 14 days of hypoxic exposure resulted in 31-41% increases in TH, after which the level of TH returned to baseline (Schmitt *et al.*, 1993). While oxygen sensors in the medulla appear to regulate the level of TH without input from the carotid body, the hypoxia induced increase in turnover rate of noradrenaline (NE) requires input from the carotid body (Soulie *et al.*, 1992).

Microdialysate collected from the phrenic nerve to measure oxygen-sensitive neurotransmitter release from the network of brainstem nuclei affecting phrenic nerve output demonstrated that there was a time-dependent change in neurotransmitter release in response to hypoxia, which was biphasic with an initial decrease in taurine followed by a sustained increase and the initial increase in phrenic nerve activity was consistent with a rapid elevation

in the excitatory neurotransmitter glutamate accompanied by a more gradual increase in the inhibitory neurotransmitter GABA (Hoop *et al.*, 1999). Such hypoxia-induced respiratory depression parallels neuronal hypometabolism in brain stem regions involved in respiratory and cardiovascular control in hypoxia intolerant mammals (LaManna *et al.*, 1996) and in the hypoxia and anoxia-tolerant tropical reef shark, *Hemiscyllium ocellatum* (Mulvey and Renshaw, 2000). However, neuronal hypometabolism in the anoxia-tolerant reef shark is not related to brain failure since the brain energy charge was maintained above critical levels even after a 50 min exposure to anoxia (Renshaw *et al.*, 2002).

Hypoxia induced increases in cerebral blood flow provides a second neuroprotective pre-emptive defence strategy. Putative oxygen sensors which regulate cerebral blood flow were investigated in rats with bilateral lesions of the rostral ventrolateral medulla. Exposure to a PaO<sub>2</sub> of 36 ± 1mmHg significantly increased cerebral blood flow in control animals up to 204%, but not in animals with bilateral lesions (Underwood *et al.*, 1994). However, in animals with bilateral lesions there was a significantly lower cerebral blood flow response to hypoxia due to a 50-69% decrease in cerebral vasodilation, demonstrating that neurons of the rostral ventrolateral medulla have a specific oxygen sensitive role in controlling vascular tone in response to hypoxia, these changes were not detected in response to hypercapnia (Underwood *et al.*, 2003).

## 7.0 Clinical implications.

Asphyxia, sleep apnoea, vascular cognitive impairment, stroke, some neurodegenerative diseases and cardiovascular disease are just a few of the conditions that can result in brain damage as a consequence of reduced oxygen levels. A few minutes of oxygen deprivation is enough to cause neuronal death in the mammalian brain (reviewed by Lutz *et al.*, 2003; Acker and Acker, 2004). This is not so for all vertebrates. The challenge of how to protect vulnerable organs, such as the energetically expensive heart and brain, from hypoxia-induced damage depends on a number of adaptations, including those of oxygen sensing, and has been solved several times during vertebrate evolution. Hypoxia and anoxia tolerance has been retained by some fish and turtles. Examination of hypoxia tolerant species has revealed that they can reversibly reprogram gene expression to achieve a “protected phenotype” displaying a suite of retaliatory and pre-emptive mechanisms to forestall cell death (reviewed by Lutz *et al.*, 2003). An understanding of the mechanisms involved in the reversible switch to a protected phenotype may provide an insight into potential intervention strategies that can be used in clinical settings to minimise ischemia-reperfusion injury following stroke and heart

attack. Comparative physiology is the nursery ground of several potential treatment strategies in clinical settings.

Oxygen levels regulate the pattern of gene expression in health and disease via a master switch, HIF-1 $\alpha$  (reviewed by Nikinmaa and Rees, 2005). While HIF-1 $\alpha$  acts as a ubiquitous transcription factor to increase cell survival during hypoxia and all organisms studied so far express HIF-1 $\alpha$ , there are only a few vertebrates that can survive prolonged periods of hypoxia or anoxia, so the ability to change the level of HIF-1 $\alpha$  expression *per se* does not automatically trigger an expression of a hypoxia or anoxia tolerant phenotype. An understanding of why HIF-1 $\alpha$  prolongs survival in some vertebrates and not in others is likely to lead to new treatments which may involve manipulating HIF-1 $\alpha$  levels and/or its lifespan.

Oxygen regulated gene expression via HIF-1 $\alpha$ , as a master regulator, results in the up-regulation of a number of neuroprotective proteins such as EPO which can protect neurons from apoptotic cell death via its action on the PI-3-k/AKT pathway (Weishaupt *et al.*, 2003). It has been shown that administration of EPO or its induction by hypoxia significantly reduces infarct size in the mammalian brain (Gassmann *et al.*, 2003) and EPO has recently been used successfully in a number of clinical trials (see reviews by Ehrreich *et al.* 2004; Grasso *et al.* 2004; Ren and Finklestein, 2005). As discussed above, the oxygen sensor pathway is closely interlinked with several other stress pathways so preconditioning with a stimulus which causes mild cell injury including exposure to low oxygen levels or exercise (Ameln *et al.*, 2005) activates a suite of protective physiological mechanisms (reviewed by Dirnagl *et al.*, 2003) which confer a naturally protected phenotype. For example stressors that activate the Hsp family of molecular chaperones not only protect cells from subsequent ischemia and block cell death by apoptosis but may also affect a number of neurodegenerative diseases such as Alzheimer's (reviewed by Franklin *et al.*, 2005). The interaction of the oxygen sensing pathway with other stress pathways opens up the possibility of cross-protection by targeting key neuroprotective chaperones and transcription factors. This is illustrated by the effect of physiological stressors such as heat (Christians *et al.*, 2002) or exercise (Ameln *et al.*, 2005) on elevating the level of Hsps and/or HIF-1 $\alpha$  respectively. Understanding how to manipulate these neuroprotective pathways is expected to lead to strategies designed to preempt injury and facilitate functional recovery.

Future therapies may focus on the activation of HIF-1 $\alpha$  and its downstream gene products, for long enough to gain the neuroprotective benefits but not long enough HIF to ultimately initiate neuronal death via apoptosis (reviewed by Acker and Acker, 2004). It is

also likely that selected gene products, such as neuroprotective globins, that are turned on by intermittent hypoxia (Di Giulio *et al.*, 2006) could be administered to reduce neuronal loss and improve recovery after acute insults due to head trauma, ischemic events and during chronic diseases such as neurodegenerative diseases of the brain, including the retina.

## 8.0 References.

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