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## Cellular mechanisms of $O_2$ sensing: the carotid body as combined $O_2$ and glucose sensor

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Oxygen sensing is part of the homeostatic biological processes necessary for adaptation of living organisms to variable habitats and physiologic situations. Cellular responses to hypoxia can be acute or chronic. Acute responses depend mainly on $O_2$-regulated ion channels which mediate adaptive changes in cell excitability, contractility and secretory activity. The main $O_2$ sensor mediating the acute responses to hypoxia is the carotid body, a minute bilateral organ located in the bifurcation of the carotid artery, which contains afferent nerve fibres that activate the brainstem respiratory centres to produce hyperventilation. Stimulation of the carotid body is also known to produce sympathetic activation and this organ has been postulated to be involved in glucose control (Alvarez-Buylla & de Alvarez-Buylla, 1988). The $O_2$-sensitive elements in the carotid body are the neuroectodermal-derived glomus cells. These are electrically excitable and have $O_2$-regulated $K^+$ channels in their plasma membrane. Hypoxia signalling in glomus cells involves inhibition of $K^+$ channels of the plasma membrane leading to cell depolarization, external $Ca^{2+}$ influx, and activation of neurotransmitter release, which, in turn, stimulates the afferent sensory fibers (for review, see López-Barneo *et al.* 2001). Despite the progress in the understanding of glomus cell electrophysiology and responsiveness to hypoxia, the molecular nature of the $O_2$ sensor remains unknown. We have developed a carotid body thin slice preparation in which the response of glomus cells to low $P_{O_2}$ can be studied in almost optimal physiological conditions (Pardal *et al.* 2000). Using this technique we have investigated whether sensitivity of intact glomus cells to hypoxia is altered by mitochondrial electron transport chain (ETC) inhibition. We have also studied whether glomus cells participate in blood glucose detection. The results indicate that, as hypoxia, mitochondrial ETC inhibitors evoke an extracellular $Ca^{2+}$-dependent secretory response from glomus cells. Sensitivity to lowering $P_{O_2}$ is not altered by blockade of mitochondrial electron flow in complexes I to IV, although responsiveness to hypoxia is selectively abolished by rotenone. Thus the data suggest that a rotenone binding protein is part of the $O_2$ sensor. In addition, we have observed that low glucose increases secretion from intact single glomus cells in a graded manner, and that this response depends on extracellular $Ca^{2+}$ influx and requires glucose metabolism but not changes in intracellular ATP (Pardal & López-Barneo, 2002). Inhibition of voltage-gated $K^+$ channels is the primary response to falling extracellular glucose, but sensitivity to low glucose is not altered by rotenone. Low glucose and hypoxia converge to raise cytosolic [$Ca^{2+}$] in glomus cells and to release transmitters, which stimulate afferent sensory fibres and evoke sympathoadrenal activation. The function of glomus cells as combined $O_2$ and glucose sensors, in which the two stimuli potentiate each other, is surely advantageous to facilitate activation of the counterregulatory measures in response to small reductions of any of the regulated variables.

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## Pro-amyloidogenic effects of chronic hypoxia on $Ca^{2+}$ homeostasis

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Adaptation to periods of prolonged hypoxia involves modulation of gene expression, which controls functional remodelling of a variety of different cell types. Classically, chronic hypoxia is known to cause increased production of erythropoeitin to stimulate red blood cell number and so the $O_2$ carrying capacity of blood. However, there are numerous detrimental effects of chronic hypoxia, amongst them an increased incidence of dementias, particularly Alzheimer's disease (e.g. Moroney *et al.* 1996).

At the cellular level, a major factor in Alzheimer's disease is the production of the plaque-forming amyloid $\beta$ peptides (A$\beta$Ps) which can cause cell death during the neurodegenerative progression of this dementia (e.g. Mattson, 1997). The mechanisms underlying cell death caused by A$\beta$Ps remain to be determined, but are believed to involve production of reactive oxygen species (ROS) and disturbances of $Ca^{2+}$ homeostasis (reviewed by LaFerla, 2002). We found that prolonged hypoxia (10 % $O_2$, 24 h) caused a marked potentiation of stimulus-evoked catecholamine release from model $O_2$-sensing PC12 cells, in part by inducing a $Cd^{2+}$ resistant $Ca^{2+}$ influx pathway. This effect was associated with (indeed, appeared to require) formation of A$\beta$Ps, and direct exposure to A$\beta$Ps exerted remarkably similar effects in these cells (Taylor *et al.* 1999). Subsequent electrophysiological studies revealed that hypoxia (via A$\beta$P formation) appeared to induce a small $Cd^{2+}$-resistant $Ca^{2+}$ influx pathway, and also selectively up-regulated L-type voltage-gated $Ca^{2+}$ channels in these cells (Green & Peers, 2001). These two separate effects were further distinguished, since inhibition of the transcription factor NFk-B prevented up-regulation of L-type channels, but not the hypoxic induction of the $Cd^{2+}$-resistant $Ca^{2+}$ influx pathway coupled to exocytosis (Green & Peers, 2002). However, both of these effects could be inhibited by a variety of antioxidants (Green & Peers, 2002; Green *et al.* 2002), indicating that formation of ROS, most likely from the A$\beta$Ps themselves, was an essential step in this pathophysiological response to hypoxia.

These observations in turn raise numerous further questions. For example, what switches on the production of A$\beta$Ps in hypoxia? A likely explanation is that there is a switch in the normal, non-amyloidogenic processing of amyloid precursor protein (APP) (which normally generates the neuroprotective, soluble fragment, sAPP$\alpha$) to the pro-amyloidogenic processing pathway. This may involve altered expression of secretases, the enzymes that cleave APP (Mattson, 1997). Another question of major importance is whether the above-described observations, all made using a continuous cell line, can be reproduced in cells of the central nervous system. We are currently addressing this latter question, and have recently shown that both hypoxia and A$\beta$Ps augment $Ca^{2+}$ currents in cerebellar granule neurones. Furthermore, we have reported that $Ca^{2+}$ signalling in primary cultures of astrocytes is disturbed following a period of chronic hypoxia, primarily because hypoxia appears to cause excessive $Ca^{2+}$ loading of mitochondria (Smith *et al.* 2002), and this effect is associated with pro-amyloidogenic APP processing. These observations are amongst the first to provide insights into the

cellular basis accounting for the increased incidence of Alzheimer's disease following hypoxic episodes. Such information is likely to be important in the future development of interventions designed to prevent the long-term, deleterious effects of hypoxia.

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## Oxygen sensing: control of HIF by enzymatic oxygen-dependent hydroxylation

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Hypoxia plays a central role in many common diseases including cardiovascular and cerebrovascular disease and cancer. The regulation of gene expression in response to variation in oxygen is essential in physiological control of vascular supply and metabolism and its pathological disturbance. This regulation of gene expression is mediated largely via the transcription factor HIF-1. It controls the expression of a wide variety of genes involved in erythropoiesis, angiogenesis, vascular tone and metabolism. The nature of the oxygen sensor which underlies regulation of HIF-1 has recently received further definition.

The regulation of HIF-1 by oxygen occurs principally through oxygen regulated degradation via the ubiquitin-proteasome system. In the presence of oxygen there is rapid degradation but in hypoxia HIF-1 is stabilised. This degradation requires recognition of HIF-1 by the von Hippel Lindau gene product VHL which then facilitates subsequent ubiquitylation and subsequent proteasomal degradation. The mechanism by which oxygen renders the HIF molecule available for VHL binding and subsequent destruction has recently been established. Two critical proline residues within the HIF-1 molecule are necessary for the binding of VHL. In the presence of oxygen these residues are hydroxylated to hydroxyproline. This process is enzymatic and requires the presence of ferrous ions and 2-oxoglutarate. The other well established role for prolyl hydroxylation is in the synthesis of collagen. The sequence of the collagen prolyl hydroxylase was used in database searches to identify homologous gene products which might function as HIF prolyl hydroxylases. Three highly homologous human proteins were identified which were able to convert HIF-1 to a VHL binding form by prolyl hydroxylation.

We undertook studies to examine whether these enzymes showed oxygen dependence of activity. The conversion of HIF-1 to a VHL binding form by these proteins (named PHD1, 2 and 3) was oxygen sensitive and also showed characteristic inhibition by desferrioxamine and cobalt. The ability of these enzymes to regulate HIF *in vivo h*as been confirmed by creating cells with inducible expression of each enzyme and utilising RNAi techniques. Recently a further oxygen-dependent modification of HIF has been defined – the hydroxylation of a specific asparaginyl residue which regulates the transcriptional activity of HIF and its interaction with p300. FIH has been identified as the 2-oxoglutarate-dependent oxygenase which mediates this hydroxylation and its structure has been determined.

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## Physiological hypoxia: concept, mechanisms of detection and responses

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Physiological hypoxia is a hypoxic hypoxia produced by a decrease in ambient $P_{O_2}$ as it happens naturally at high altitude due to a decrease in the barometric pressure. It is physiological because it proceeds without any pathology for the entire life span of individuals and from generation to generation. The upper limit to physiological hypoxia can be set at an altitude of ~4000 m above sea level, equivalent to a barometric pressure of ~465 mmHg, an inspired $P_{O_2}$ of ~88 mmHg and, before compensatory hyperventilation, an alveolar $P_{O_2}$ of ~40 mmHg. Nearly 15 million people live at an altitude close to 4000 m, and after corrections for racial and nutritional factors, no differences from sea level inhabitants in reproduction, growth or physical performance are found.

The normal functioning of the organisms in physiological hypoxia is possible because three cell systems, the chemoreceptor cells of the carotid body (CB), the erythropoietin-producing cells of the kidney and the pulmonary artery smooth muscle cells, are endowed with the ability to detect and to respond to physiological hypoxia. These three cell systems share several properties: they respond to hypoxia with a low threshold (i.e. mild hypoxia), their responses increase with the intensity of hypoxia, are sustained and reversible, and they are at the origin of regulation loops, aimed to restore $O_2$ availability to cells, i.e. the responses of these cells to hypoxia trigger local or reflex responses aimed to protect the entire organism from hypoxia. These cells are 'true' oxygen sensors. In adult mammals, the hypoxic threshold for the CB is an arterial $P_{O_2}$ of ~70–75 mmHg (arterial oxygen content > 94%), it responds with progressive higher intensity if the intensity of hypoxia increases, and the CB response to hypoxia does not adapt. The release of neurotransmitters in the CB chemoreceptor cells initiate a reflex hyperventilation aimed to restore $O_2$ availability for the entire organism. Contrary to this situation, most stirps of mammalian cells, including the neurons of the central nervous system, only respond to hypoxia if it is intense, and the responses generated are transient and reflect pathogenic mechanisms of the hypoxic damage or cellular protective mechanisms against hypoxia. Since the terms 'mild' and 'severe' might pose uncertainties due to the diversity of preparations, of methods of measurement or simply due to the lack of data on the thresholds of the hypoxic responses for most cell systems, it might suffice to state that cells endowed with sensitivity to physiological hypoxia respond to levels of hypoxia not threatening survival while the appearance of responses to hypoxia in other cell types, as for example in brain neurons, start only at much lower arterial $P_{O_2}$, which might represent a menace for the life of the animals.

These considerations, directed to distinguish physiological from pathological responses to hypoxia, are not intended to rest interest from the definition and characterization of the pathological responses to hypoxia and their mechanisms. On the contrary, their characterization would allow medical interventions to disrupt the pathogenic mechanism of the hypoxic damage or to potentiate the protective mechanisms of the cells. Even further, it is now clear that physiological and pathological responses share mechanisms. For example, the transcription factor HIF-1$\alpha$ controls the production of erythropoietin and via vascular endothelial growth factor directs normal vasculogonesis, yet, at the same time, it increases the

expression of vascular growth factor and glycolytic enzymes in tumoural cells securing tumour survival, growth and progression. Similarly, the inhibition of potassium channels is an early step in the activation of chemoreceptor cells and in the physiological pulmonary hypoxic vasoconstriction; the inhibition of the same channels in some brain areas might underlie their great susceptibility to hypoxic damage.

Aside from these general considerations on hypoxia, I shall present the most recent data of our laboratory dealing with the mechanisms of detection of hypoxia in the chemoreceptor cells of CB, including the significance of oxygen reactive species and of a putative plasma membrane-linked hemoprotein. I shall also present data supporting a role for regulatory $\beta$ subunits of the potassium channels as coupling elements between the oxygen sensing element and the conducting unit of the potassium channels. Finally, I shall discuss the molecular identity of oxygen sensitive potassium channel in the rabbit CB chemoreceptor cells.

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## Hypoxia inhibits the ligand-gated rat $P2X_2$ receptor cation channel

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In the nervous system purinergic excitatory synapses use ATP to mediate fast synaptic transmission via activation of P2X receptor cation channels. In the carotid body ATP is co-released with ACh during hypoxia, which activates $P2X_2$ and $P2X_3$ receptors on petrosal neurons to initiate corrective changes in ventilation (Prasad *et al.* 2001). However, the role of P2X receptors in this response is unclear. This study examined the response of cloned rat $P2X_2$ receptors to hypoxia.

Whole-cell currents were recorded from $P2X_2$ receptors stably expressed in HEK293 cells (Evans *et al.* 1996). Pipettes were filled with (mM) 10 NaCl, 117 KCl, 2 $MgSO_4$, 1 $CaCl_2$, 11 EGTA, 2 Na-ATP, 11 Hepes (pH 7.2), and cells were continuously perfused with 135 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 5 Hepes and 10 glucose (pH 7.4). Data are given as means ± S.E.M. and statistical analysis was peformed using Student's paired *t* test with $P < 0.05$ regarded as significant.

In the presence of external $Ca^{2+}$ and $Mg^{2+}$ perfusion of ATP elicited an inwardly rectifying current with an $EC_{50}$ of 26 ± 0.1 $\mu$M. At a potential of −70 mV, 5 $\mu$M ATP induced an inward current that showed little desensitization during repeated exposures under normoxic conditions (−50.5 ± 16.7 pA $pF^{-1}$ to −47.1 ± 18.4 pA $pF^{-1}$, mean of first three and last three exposures respectively, $n = 3$). Exposure to an hypoxic ATP (5 $\mu$M) solution ($P_{O_2}$ 25 mm Hg, EC solution bubbled with 100 % $N_2$) reduced the whole-cell current from −58.0 ± 11.2 pA $pF^{-1}$ to −31.1 ± 8.1 pA $pF^{-1}$ ($P < 0.01$, $n = 4$). Removal of external $Ca^{2+}$ and $Mg^{2+}$ plus buffering with 1 mM EGTA shifted the $EC_{50}$ to 0.2 ± 0.3 $\mu$M but did not prevent the reduction in current following a hypoxic challenge. Hypoxia reduced the inward current induced by ATP (0.1 $\mu$M) from −19.3 ± 4.4 pA $pF^{-1}$ to −13.7 ± 3.5 pA $pF^{-1}$ ($P < 0.01$, $n = 5$).

In summary, this is the first report indicating that hypoxia modulates the response of P2X receptors to ATP and suggests that P2X receptors are involved in mediating the response to hypoxia in the nervous system.

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