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OXYGEN SIGNALLING IN METABOLIC REGULATION : SHORT, INTERMEDIATE, AND LONG TERM ROLES

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ABSTRACT

We are led at least tentatively to conclude from this analysis that O2 sensing and signal transduction pathways are critically involved.

(i)in the regulation of acute changes in ATP turnover rates in response to increasing or decreasing oxygen availability (or to change in work and perfusion rates),

(ii)in the regulation of intermediate term responses to hypoxia (such as EPO biosynthesis, *cfos* and *cjun* activation, or suppression of gluconeogenic pathway enzymes).

(iii)in the regulation of longer term responses to moderate hypoxia (for example, up regulation of tissue mitochondrial volume densities, of oxygen carrying capacitites, or of oxygen efficient metabolic pathways), and

(iv)in the regulation of long term responses to extreme hypoxia(for example, down regulation of tissue mitochondrial volume densities, adjustments in anaerobic/aerobic metabolic capacities, or adjustments in oxygen efficiency of metabolic pathways).

Perhaps even more evident than the above is the conclusion that oxygen sensing, signal transduction, and expression pathways in metabolic adjustments to hypoxia are still largely unexplored, especially in the comparative field. Although current models for coupling O2 sensing to regulation of ATP turnover are still a long way from being complete or confirmed, they are, in principle, useful and attractive for several reasons: (i)they can account for the often observed relationship between VO2 and perfusion, (ii)they develop a mechanistic basis for O2 conformity (VO2 varying with [O2] or with O2 delivery) over broad concentrations ranges usually very much higher than the Km for O2 for mitochondrial metabolism, (iii)they supply a mechanism for coordinate and near-instantaneous regulation, upwards, of all components in ATPase and ATP synthesis pathways with change in O2 availability, (iv)they supply a mechanistic explanation for up or down regulation of ATP turnover rate with minimal change in concentrations of intermediates in pathways of ATP utilization or of ATP production, and (v)they display interesting potential for strategic transfer to clinical situations, because they seem to be generally applicable to most tissues. Whereas for such obvious reasons these models may be useful, it is not yet clear whether or not they will turn out to be correct. For this further research is clearly required.**(Acta Andina 1996, 5:41-56)**

RESUMEN

Podemos tentativamente concluir a partir de este análisis que las vías sensoras y las de transducción de las señales de oxígeno están críticamente involucradas en las siguientes funciones:

(i) en la regulación de los cambios agudos en las tasas de recambio de ATP en respuesta al aumento o disminución de la disponibilidad de oxígeno (o cambio en las tasas de trabajo y perfusión).

(ii) en la regulación de las respuestas intermediarias a la hipoxia (como la biosíntesis de EPO, la activación de *cfos* y *cjun*, o la supresión de las enzimas de la vía gluconeogénica)

(iii) en la regulación de las respuestas prolongadas a la hipoxia moderada (por ejemplo, el "up regulation" de las densidades volumétricas mitocondriales, ajustes en las capacidades metabólicas anaeróbico/aeróbicas, o ajustes en la eficiencia de las vías metabólicas).

Quizás, aún algo más evidente es la conclusión que el sensor de oxígeno, la transducción de la señal, y las vías de expresión en los ajustes metabólicos a la hipoxia todavía no han sido explorados a profundidad, especialmente en el campo comparativo. Aunque los modelos corrientes para el acoplamiento del sensor de oxígeno a la regulación del recambio de ATP aún no han sido confirmados, ellos son, en principio, útiles y atractivos por diversas razones: (i) pueden dar explicaciones a la relación frecuentemente observada entre VO2 y perfusión, (ii) pueden desarrollar las bases mecánicas para la conformación de 02 (variación de VO2 con la "O2" o con la entrega del 02) sobre rangos amplios de concentraciones usualmente mucho más altos que el Km para O2 para el metabolismo mitocondrial, (iii) proporcionan un mecanismo para la regulación coordinada y estrecha de todos los componentes en las vías sintéticas de la ATPasa y ATP con los cambios en la disponibilidad de O2, (iv) ellos proporcionan una explicación para el "down" y "up regulation" de la tasa de recambio de ATP con cambios mínimos en las concentraciones de los intermediarios en las vías de utilización de ATP o de la producción de ATP, y (v) muestran interesante potencial para la transferencia estratégica a situaciones clínicas, porque parecen ser generalmente aplicables a muchos tejidos. Si bien para tales razones obvias.estos modelos pueden ser útiles, aún no está claro si ellos serán correctos. Para ello se requiere de ulteriores investigaciones..**(Acta Andina 1996, 5:41-56)**

Introduction

A short time ago, two Canadian adventurers in Nepal were asked to assist in a rescue operation; a climbing team caught in a storm and on its way down from the peak of Mt. Everest was in dire straits and its location was uncertain. Along with three Sherpas, the Canadians were assigned the task of trying to locate the climbing team's position from a small plane. When the plane reached about 3000 m, one of the Canadians (unacclimated to altitude) put on his mask and began breathing supplementary oxygen. At about 4000 m, the second Canadian (who had been at altitude in Nepal for some weeks and was thus acclimated) did the same. The plane continued to climb, then finally began circling the peak of Mt. Everest at altitudes over 9000 m; they circled at these altitudes for approximately an hour, but at no point in the reconnaissance did the Sherpas bother to put on their masks and breath supplementary oxygen, so well tuned were they to life under chronic hypobaric hypoxia.

This anecdote, in a nut shell, describes all three aspects of human hypobaric hypoxia problem which form the basis of this paper. In biology, it is almost axiomatic that the response of organisms to varying [O_2] depends upon the time available for the adaptation. Most studies in this area concentrate on the way bioenergetic processes are organized in response to O_2 availability and, traditionally, the literature recognizes three categories (Hochachka and Somero, 1984): (i) short or near-instantaneous response, (ii) intermediate term or acclimation response, and (iii) long term or adaptational response. The boundaries between these three time courses are not rigidly defined and may vary between cells, tissues, and organisms. For example, very soon after an acute response to an hypoxic stress is stabilized (with appropriate adjustments in O_2 fluxes to aerobic pathways of metabolism), the cell may activate or silence batteries of genes in order to begin orchestrating longer term backup defenses against extended O_2 limitation. Although *in vivo* it may take days to weeks to reach a new steady state, the entire process is included in the term acclimation the important point to bear in mind is that in response to intermediate time courses of exposure (acclimation times of hours to days to weeks), cells and tissues are able not only to adjust overall ATP turnover rates, but they can also reorganize pathways of ATP demand and supply (by selective activation or repression of specific genes) so as to improve function (or improve chances of organismal survival) under hypoxic conditions. Populations exposed to hypoxia over phylogenetic time (generations) have even greater cell-level adaptation options in terms of maximizing survival chances under chronic hypoxia.

Although the empirical effects of short, intermediate, and long term exposure to hypoxia are extensively described, in none of this literature are the roles of O_2 sensing and O_2 signal transduction in metabolic adaptation and regulation emphasized; indeed, they usually are overlooked. In this paper, we shall mainly focus on recent developments on the roles of these two processes in acute and acclimatory responses to hypoxia; however, we will briefly consider their roles in long term metabolic adaptation as well.

Our recent analyses of short term (essentially instantaneous) responses to varying availability of O_2 arose almost by chance in the context of evaluating metabolic regulation concepts (Hochachka et al, 1991; Hochachka and Matheson, 1992; Hochachka , 1994). Our evaluation led to several conclusions:

(i)For over 30 years metabolic biochemists have been searching, without success, for metabolite signals which might account for large scale changes in ATP turnover rates in tissues. Most current metabolic regulation theories are based on feedback and feedforward control loops where substrates and products serve to activate or inhibit key enzymes in pathways of ATP u tilization or ATP production; ADP and Pi are often central to these models (Balaban, 1990; Connett, 1988; Connett and Honig, 1989; From et al, 1990; Funk et al, 1990; Kushmerick et al, 1992; Nioka et al, 1991; Rumsey et al, 1990).

(ii)A major problem, not adequately addressed by the above regulation models, is that for many enzymes in pathways of ATP utilization and of ATP synthesis, there is a notable absence of large changes in substrate or product concentrations during up or down regulation of ATP turnover rate. We argue that this leaves effective enzyme concentration as the regulatable parameter. In this view, the traditional roles of metabolite concentrations changes are modified and are assumed to play fine tuning regulatory functions ather than major on-off switching functions (Hochachka and Matheson, 1992).

Hypometabolism means masking of catalytic potentials.

(iii)At this time, only one metabolite 'signal' -oxygen delivery - correlates directly with (upwards or downwards) change in ATP turnover rate and since its regulatory effects clearly occur at concentrations well above the apparent Km for mitochondrial metabolism, it is postulated (Hochachka, 1994; Thurman et al, 1993) that an O_2 sensing system must be involved in transduction of the O_2 signal and in regulation of ATP turnover

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(iv)O_2 sensing and transduction mechanistic details are not known; however, two key conditions (near instantaneous transmission to all parts of the cell and near simultaneous activation of multipe enzymes in pathways of ATP turnover) must be satisfied for this kind of control to be workable. Finally,

(v)A parsimonious model satisfying these two critical constraints assumes that O_2 signal transduction is coupled (presumably through a secondary signal or signals such as Ca++ ions) to changes in the physical state of the milieu which changes accessibility of enzymes to their substrates, in this way unmasking otherwise 'latent' catalytic power (Hochachka, 1994). Our appreciation of these special functions for O_2 arose from recent studies in two different lines of research - both pointing to the possibility of such roles for O_2 and O_2 sensing systems in regulating aerobic metabolism.

Metabolic Regulation Requires [Oxygen] Sensing

The first of these two study lines initially was concerned mainly with hypoxia acclimation (not acute responses), specifically with hypoxic stimulation of erythropoietin (EPO) synthesis, and this work simultaneously may have exposed a universal O_2 sensing system. Earlier studies had already confirmed the ocurrence of an O_2 sensor located primarily in special kidney cells, but also to some extent in liver cells; the experimental evidence was consistent with a heme protein as the O_2 sensor, which in its deoxy form triggers an activationpathway culminating in increased transcription of the EPO gene. Later studies suggested that (i) an O_2 sensing system and (ii) an enhancer sequence regulating gene transcription are much more widespread than had been previously believed (Tsuchiya et al, 1993; Eckardt et al, 1993; Maxwell et al, 1993; Wang and Semenza, 1993). These studies, based on the development of a molecular reporter system allowing rapid scanning of many cell types *for enhancer-specific hypoxia sensitivity, showed that O_2 sensitive gene expression is nearly universal* (Maxwell et al, 1993). When O_2 is limiting (Figure 1), the signal (reduced O_2) mediates the production of an hypoxia-inducible factor (HIF-1) which binds to a specific 'hypoxia induction site' on DNA and accelerates transcription of hypoxia regulated genes (including the EPO gene and possibly genes for at least some glycolytic enzymes and the GLUT1 isoform of the glucose transporter (Bashan et al, 1992)). Even if most studies of this complex O_2 sensing system are directed mainly towards hypoxic regulation of EPO production (an intermediate time-course response), by serendipidy they may have revealed a universal molecular pathway for sensing declining O_2 availability at the cell level.

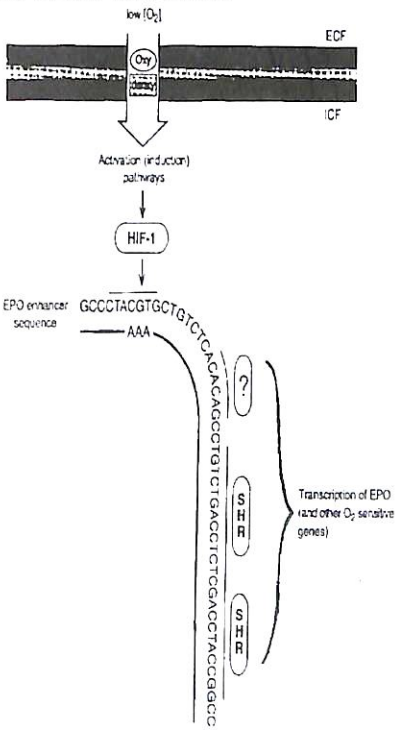


Figure 1. Diagrammatic structure of the hypoxia-inducible enhancer of the EPO gene. Under hypoxic conditions, the percent deoxygenation of the putative O2 sensor increasesand leads to the induction of HIF-1 (hypoxia inducible factor-1). Only a part of the 50 nucleotide sequence of the EPO enhancer froms the HIF-1 responsive (binding) element. Transcription of reporter genes (e.g. the gene for chloramphenicol acetyltransferase) containing the EPO gene enhancer sequence is induced by hypoxia in many (and possibly all) cell types. Since mutations which eliminate HIF-1 binding eliminate hypoxia induction, this factor is considered pivotal in this pathway of O2 sensitivity. Modified from Wang and Semenza (1993).

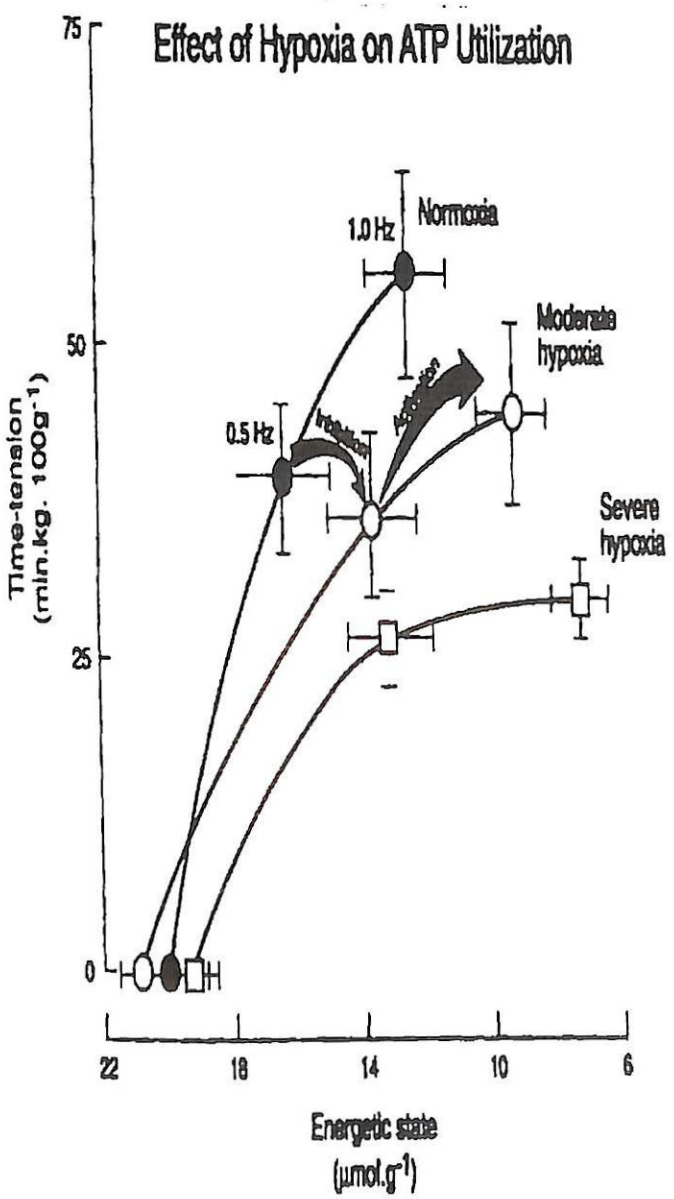


Figure 2. Effect of hypoxia on muscle ATP utilization (proportional to time-tension integral) as a function of the utilizable (high energy) phosphate (defined as the energetic state). Data in each case shown for resting conditions (high energetic state), for 0.5 Hz stimulation (intermediate energetic state) and for 1 Hz stimulation (lowest energetic state). Note that under normoxia, increasing stimulation frequency (decreasing energetic state) increases the work output. At any given conditions, hypoxia decreases contraction-linked ATPase activity (shown by arrow moving from normoxia to moderate hypoxia). This is a regulated response because increasing stimulation from 0.5 to 1.0 Hz increases ATPase to near normoxic levels (shown for moderate hypoxic conditions by arrow moving from intermediate to low energetic state). These kinds of data are consistent with ATP demand being set by stimulation frequency but being modulated by O2 availability. Data from Arthur et al (1992) and Hogan et al (1992).

sensing system powerfully influenced subsequent interpretations in the second of the above two lines of research, which was designed to tease out the roles played by O_2 availability in control of ATP turnover during muscle work under normoxia, moderate hypoxia, and extreme hypoxia. A dog muscle preparation was used in order to bring the system as much under the experimenters' control as possible (Arthur et al, 1992; Hogan et al, 1992). These studies confirmed that none of the usual putative regulatory metabolites (such as ADP or Pi) can account for the changes in ATP turnover rates observed-*except possibly for oxygen itself.* The roles of O_2 are complex and are best explained by looking at ATP demand (proportional to ATPase) first, then at the metabolic response.

Under normoxia (Figure 2), increasing stimulation frequency from 0.5 to 1 Hz leads to increasing ATPase activity (increasing work), which, of course, is expected. What is somewhat surprising is that moderate hypoxia at 0.5 Hz brings about a *regulated* decline in ATPase activity. We Know this is an hypoxia-regulated rate because if the muscle is stimulated harder (at 1 Hz), its ATPase activity reaches that characteristic of normoxic conditions at 0.5 Hz. A similar if more extreme situation occurs at extreme hypoxia. In strictly empirical terms, these data show that the ATP demand during mechanical work ***is set by the ATPases but regulated at least in part by oxygen availability***, and the question arises of how the ATP supply side of the system responds.

ATP supply mechanisms (Figure 3) respond to increasing stimulation wtih an 18 fold increase in O_2 consumption at 1 Hz under normoxic conditions. Again, at 0.5 Hz, moderate hypoxia brings about a *regulated* decline in ATP synthesis rate. As before, we know this is a regulated rate because at double the stimulation frequency the metabolic rate in hypoxia increases to normoxic 0.5 Hz values. In empirical terms, these results show that the ATP supply side of the system is also regulated by O_2 availability. What is more, the regulation is coordinated, for the % of change in ATP demand is almost perfectly balanced by % change in ATP supply; that is why a nearly 20-fold change in O_2 consumption rate occurs with but a 2-3 fold change in free ADP concentrations; although ADP is often considered as a major regulatory metabolite, the above discordance is widespread (Hochachka and Matheson, 1992). In contrast, and because of this coordination, ATP turnover rate in dog muscle is a linear function of O_2 delivery In this preparation, as in many other systems studied

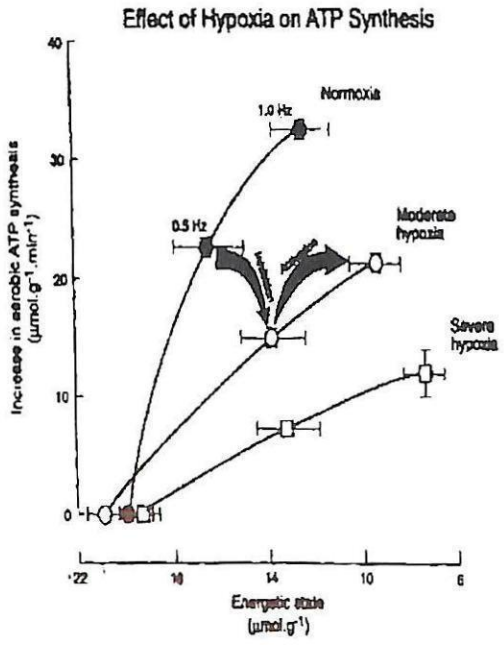


Figure 3. Effect of hypoxia on ATP synthesis in dog muscle gastrocnemius at various work levels. Data are shown for resting conditions (highest energetic state), for 0.5 Hz stimulation (intermediate energetic state, and for 1 Hz stimulation (lowest energetic state). Under normoxia, increasing stimulation (decreasing energetic state) correlates with increasing ATP synthesis rates. On imposition of hypoxia at 0.5 Hz, ATP synthesis rates decline (shown by arrow going from normoxia to moderate hypoxia). That this too is a regulated response is indicated by the fact that at higher stimulation frequency (1Hz), ATP synthesis rates increase to normoxic range , despite the sustained hypoxia. These data are consistent with ATP synthesis rates being set by the ATP demand but being modulated by O2 availability. Data replotted from Arthur et al (1992) and Hogan et al (1992).

by other workers, ***the only currently known 'signal' which is large enough and which is directly proportional to the observed change in ATP turnover is in fact O_2 availability.***

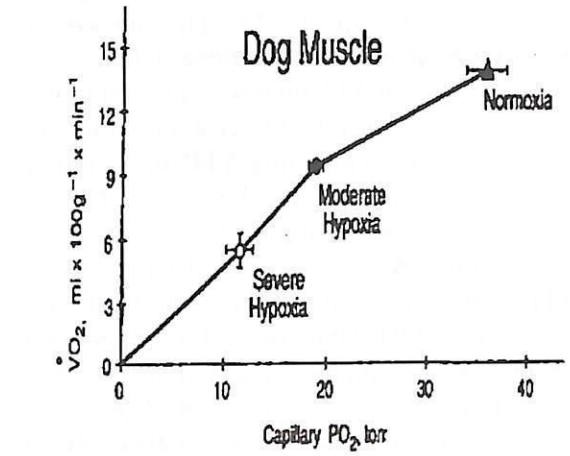
An important point to emphasize is that in all these Kinds of systems, O_2 regulation is not mediated simply by O_2 as a substrate for mitochondrial metabolism. Because of (i) a very high O_2 affinity of mitochondrial cytchrome oxidase and (ii) very shallow gradients between cytosol and mitochondria (Gayeski and Honig, 1986; Connett and Honig, 1989), cytochrome oxidase could not possibly act as an O_2 sensor over physiological O_2 tensions. In dog muscle (Figure 4), as in other tissues where similar effects have been observed (Hochachka and Guppy , 1987; Thurman et al, 1993), O_2 regulation cuts in at extracellular concentrations that are in the range of 20-30 torr; under these conditions, intracellular O_2 is estimated to be about 5 torr which would be fully saturating for mitochondria (Gayeski and Honig, 1986). This means that mitochondrial metabolic pathways (as well as other enzymes in ATP turnover) are responding to O_2, but not only to O_2 as a substrate. Put another way, O_2 is affecting ATP turnover rates at concentrations well above the Km for mitochondrial metabolism per se. To accomodate such striking and often-observed dependence of ATP turnover on [O_2], we and others (see Hochachka, 1994; Thurman et al, 1993) postulate ***an O_2 sensor which displays a much lower apparent O_2 affinity than typical of mitochondria and which (directly or indirectly)modulares both ATP demand and ATP supply pathways in a simultaneous coordinated way*** (Figure 4).

Rather than linked to HIF-1 induction, the O_2 sensor in this case would have to be linked to an intracellular, long-range, multitarget activation pathway. In outline, such a model can be summarized as follows: ischemia or hypoxia -> declining O_2delivery to cells --> increasing [deoxy form]/[oxy form] of the sensor --> --> masking of many catalytic potentials --> down regulation of ATPases and of ATP producing pathways. The reverse is postulated for improved O_2delivery, with increasing oxygenation state of the putative O_2 sensor and with enzyme unmasking causing ATP turnover rates to accelerate.

From our present understanding, there are two minimal requirements which this kind of model must satisfy: (i) it needs to account for the simultaneous activation of both ATP demand and ATP supply pathways during activation of cell work and O_2 delivery, and (ii) it needs to account for intracellular long range activation; i,e., for a mechanism to 'spread' the signal to

Current Concepts in O2 Sensing and Signalling Pathways

The process of O_2 sensing of course has already been examined by physiologists and biochemists, mainly in the context of carotid body chemoreception. At the 1993 International Union of Physiological Sciences conference in Glasgow, for example, three O_2 sensing models were reviewed in detail: a high Km mitochondrial model, a nitric oxide (NO) based model, and an O_2-derived free radical model. Other evidence has pointed in the direction of an unique outwardly



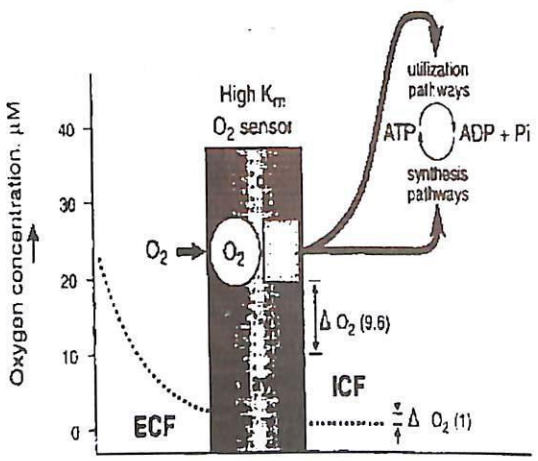


Figure 4. Potential roles for an O2 signalling mechanism in the regulation of metabolism at varying O2 availability. A. **Upper panel.** Changing O2 consumption rates (VO2 in ml O2 100g-1 min-1) as a function of capillary O2 concentrations in working dog gastrocnemius (Hogan et al, 1992). It is important to emphasize that the relationship is between VO2 and extracellular PO2; under these conditions, intracellular PO2 is thought to be fully saturating to mitochondrial metabolism (Gayeski and Honig, 1986). A diagrammatic interpretation of how an O2 sensor might be involved in regulating ATP turnover rates is shown in B, **Lower Panel.** To relate this model to experimental data such as those in the upper panel, the oxygen profile from the ECP to the ICF refers to oxygen concentration (uM units), not to oxygen partial pressure (recall that PO2 = [O2]/solubility coefficients). The problem of sensing O2 for a working cell is complex since the higher solubility of O2 in the lipid bilayer of membranes creates higher O2 concentrations and gradients. Under the conditions described, the difference in concentration across the lipid bilayer is about 9.6 times the difference between the two aqueous compartments; [O2] in aqueous solution at 1.23 x 10-6 M torr-^{1} corresponds to [O2] in the lipid bilayer at about 1.18 x 10-5 M torr-^{1}. What part of this complex situation is sensed by cells is unclear at this time; in this diagram the sensor is positioned in the membrane (where the external to internal gradient is maximized) to emphasize the correlation between extracellular O2 and VO2 (shown in upper panel). [O2] values given in lower panel are arbitrary, chosen only to quanlitatively illustrate the situation O2 signal transduction is linked to regulation of both ATP demand and ATP supply pathways to account for the oft-observed relationship between O2 and ATP turnover rates.

the scope of our analysis here. In the present context, it is sufficient to point out that to this point research into the possible role of O_2 sensing in regulation of ATP turnover can be categorized into direct or indirect models. The first assumes an O_2 sensing system analogous or homologous to that involved in EPO regulation. An alternate framework for O_2 sensing in regulation of ATP turnover considers an indirect pathway which is based on studies showing that nitric oxide inhibits both ATP production, indirectly measured as O_2 uptake rates, and ATP utilization, measured as mechanical work (King et al, 1993). Based on these kinds of observations, an indirect O_2 'sensing' pathway might be summarized as follows: ischemia and /or hypoxia--> reduced O_2 delivery--> increasing intracellular Ca++/calmodulin concentrations --> nitric oxide synthase (NOS) activation--> increasing NO flux --> several effects including simultaneous inhibition of ATPases and ATP synthesis pathways. Increased NO availability also could act via guanylate cyclase activation and cGMP to down regulate energy demand and energy supply pathways, as postulated for vascular smooth muscle (Knowles and Moncada, 1993; Toda and Okamura, 1992).

The flipside of this model postulates a reverse set of processes to regulate rate transitions during improved perfusion and oxygenation characterizing muscle activation. Preliminary evidence favoring such an indirect O_2 response pathway comes from administration of agonists or antagonists designed to specifically block or enhance NO production; perfusion, O_2 consumption and work output are proportionately up or down regulated (King et al, 1993; Sun and Reis, 1992). There are serious difficulties with this indirect model of the role of O_2 in metabolic regulation (NO has a notably finite life time and is destroyed on encounter with hemoglobin); however, it has the distinctly favorable features of being predictive and testable Unfortunately, neither of the above two approaches confronts the issue of a long range intracellular 'signalling' system to instantaneously 'spread' the activation signal to all parts of the cell. A quick search of popular texts and reviews will indicate that approaches to intracellular signalling are currently dominated by target-specific second messenger concepts. Indeed, we have to keep open the possibility that some potent second messenger systems are in fact pivotal in turning on cell ATP .turnover processes; Ca++ has clearly been identifed as playing such a pivotal role in regulation

of actomyosin function. A fundamental limitation of all such second messenger frameworks is the requirement for numerous highly specific intracellular targets. The complex biological reality in most (and probably all) tissues is that every protein in every step in ATP demand and ATP supply pathways must be almost simultaneously activated, and it is hard to visualize a second messenger hitting so many targets so rapidly. In some cases, the time course for metabolic change is awesome. For example, there is more than a 2000 fold activation of Na+ K+ ATPase within less than 300 ms during electric organ discharge (Blum et al, 1990; 1991); there is a nearly 1000 fold, essentially square wave activation of mitochondrial metabolism in insect flight muscle (Sacktor, 1976; Wegener et al, 1991), and a similar (Dobson and Hochachka, 1987; Dobson et al , 1988; Hochachka et al, 1991; Moyes et al, 1992; Suarez et al, 1990)) more than 100 fold activation of ATP turnover during maximum work of fast twitch muscles in vertebrates! The number of targets, their speed of activation, and their degree of activation would seem to stretch second messenger frameworks to the breaking point. Thus it appears that something is missing in target specific second messenger views of cell activation, especially in tissues like muscle where response time in evolutionary terms may influence survival of the organism. We are considering the hypothesis that the missing element in current models of control of ATP turnover may be the intracellular milieu itself.

Intracellular Fluids Are Not Simple Aqueous Solutions

The insight that the physical state of the cytosol may play a role in regulating metabolism arose when we turned to barnacle muscle cells to try get estimates of true resting concentrations of metabolites such as ADP (Hochachka, P.W., unpubl. observations). The giant barnacle is well known to have giant muscle cells (1-3 mm in diameter and 10-30 mm in length!) and our initial goal was to obtain small (picoliter or nanoliter quantities) of cytosol from the resting cell. To our surprise, the cytosol could not be micropipetted; nor, when the muscle fibers were cut, did it leak out; instead, the cytosol behaved more like a gel than a sol. (This has been well known for over 30 years by neurophysiologists studying the giant squid axon; they routinely roll out axoplasm gel toothpaste out of a tube, but the metabolic and regulatory significance of this unexpected physical state of the ground substance in these cells has been pretty well ignored.) Many years ago, researchers such as Heilbrunn (1956) knew of this behaviour, but methods for studying the properties of the cytoplasm were not advanced enough to evaluate its physical state in vivo. Today the situation is different. Using a variety of new and penetrating approaches, we now know that cytoplasm is remarkably viscous, in the range of 10-15 cP, not the 1 cP we normally assume which is typical of water (Dix and Verkman, 1990).

Under in vivo conditions, diffusion rates may be very different from those expected in simple solutions. Using a reference phase technique, workers in this area find (i) that many proteins are diffusive, many are not, and (ii) that even small solutes (disaccharides are often used as metabolic models) may show varying diffusion rates (Mastro et al, 1984). Concentrations of small molecules and metabolites may be similar in different regions of the cell or may vary (Horowitz and Miller, 1984). Even more relevant to our analysis is the observation that all of the above, especially microviscosities in different regions of the cell, are not static physical states; instead, they change with the activity or metabolic status of the cell (Paine, 1984), and the same situation is even more exaggerated in the mitochondrial matrix. The viscosity of the inner compartment of mitochondria (i) varies with the metabolic state of the mitochondria (*more* viscous in state 3 than in state 4!) and (ii) can be up to *40 times* higher than the viscosity of dilute buffers (Scalettar, et al, 1991). These observations raise the possibility that transitions in the physical state of intracellular fluids could alter substrate accessibility to enzymes (the catalytic power of enzymes with no access to substrate is 'latent'; providing substrate accessibility is potentially a simple but effective way of 'unmasking' enzyme catalysis) and this information could be instantaneously transmitted throughout the cytosol to all reaches of the cell during large scale upwards or downwards shifts in ATP turnover rates in tissues like muscles. If this were linked to O_2 supply (for example, through Ca+ +) it would account for two of the chief experimental observations that frequently arise in *this field: the direct relationship between O_2 availability and the ATP turnover rate and the near-instantaneous spread of the activation or deactivation 'signal' to essentially all protein*

components involved in integrated cell and tissue function at once.

In that the above model makes clear cut for further research. However, from this discussion it will be evident that the roles of acute O_2 sensing and transduction mechanisms in mediating the metabolic regulatory effects of this metabolite, although recognized, are poorly understood and are barely described. In contrast, oxygen signalling pathways over intermediate time courses are better worked out.

Intermediate Term (Acclimatory) Responses to Hypoxia

Based on early studies on hypoxia sensitive systems, it was at first believed that, when under O_2 limiting conditions, cells and tissues would make up energy deficits by activating anaerobic pathways. However, in its simplest form, this strategy is to down regulate ATP turnover rates. In early acclimation stages, anaerobic metabolic pathway fluxes may remain unchanged (which by virtue of the inefficiency of anaerobic glycolysis leads to a decline in ATP turnover rates) or may gradually decline, especially after acclimation time is extended. Because of sustained O_2 limitation, however, a large fraction of overall ATP turnover necessarily depends upon anaerobic glycolysis, which is why in such cells and tissues, the ratio of anaerobic/aerobic metabolic pathway capacities may well be regulated upwards (further discussion of strategies of hypoxia acclimation is available in Hochachka and Randall, 1978; Hochachka and Somero, 1984; Hochachka and Guppy, 1987).

Similar but less exaggerated processes appear to be found during hypoxia acclimation in mammals. In rats, acclimation to hypobaric hypoxia increases capillarity and the glucose transporter capacity of the brain (Harick et al, 1991); presumably these adjustments are coordinated with simultaneous up regulation of overall tissue glycolytic capacities to allow for sustained ATP synthesis rates despite chronic O_2 limitation (LaManna et al, 1992; Harick et al, 1991; 1994). Earlier studies of other tissues in animals undergoing high altitude acclimation suggested increases in capillarity and in oxidative capacities; these results are consistent with between-species studies of high vs low altitude animals. However, later studies found decreases in mitochondrial [enzymes] with little or no change in capillarity (see Hoppeler and Desplanches, 1992). Part of the explanation of the confusion in this research may be methodological. For example, exposure to hypoxia leads to reduced exercise capacity and often to caloric imbalance and gradual loss of body (especially muscle) mass. Hence, changes observed in muscle aerobic metabolic capacities might be due to changes in activity (or training) state, to caloric imbalance, or to hypoxia *per se* - or all of the above, since all three factors in theory could influence cell metabolic organization. Takahashi et al (1993) recently reexamined the issue trying to correct for activity; they found that hypoxia acclimation in rats led to a measurable decrease in total malate dehydrogenase (MDH) activity per g of muscle, but to no change in hydroxyacyl CoA dehydrogenase (HOAD). MDH is a poor marker of mitochondrial metabolism since most of total MDH reflects cytosolic activity; thus the lack of effect on HOAD implies little or no effect on muscle mitochondrial oxidative capacity. Even more convincing is a recent study of three months of 3800 m acclimation on heart enzymes in non-pregnant sheep, in near-term mothers, and in the sheep fetus. Citrate synthase, as a marker of mitochondrial oxidative capacity, increased about 50% in the adults and nearlycoupled with training programs (Terrados et al, 1990; Terrados, 1990). However, this effect is not seen when elite endurance -trained athletes are acclimated for 2 weeks at modest altitude (Swedish cross country skiers were used, known to have one of the highest maximum aerobic metabolic rates thus far measured in humans! (Mizuno et al, 1990)). In contrast, similar exposure of humans to extreme altitudes (over 5000 m and up to 8800 m) consistently leads to a down regulation of oxidative capacities (and to an increase in the ratio of anaerobic/aerobic metabolic capacities) at least of skeletal muscles (Cerretelli et al, 1990; Hoppeler and Desplanches, 1992). Unfortunately, the basis for the striking difference between extreme and moderate hypoxic challenge in humans is not yet understood.

However, even if the phenomenological description of acclimatory responses of human muscles to hypoxia are incomplete, it seems fair to conclude that at least during acclimation to relatively extreme hypoxia, one notable effect involves an up regulation of the ratio of anaerobic/aerobic pathway capacities. Similar adjustments of anaerobic/aerobic pathways are to be expected for other tissues, such as kidney (Halasz et al, 1974) and liver (Longmuir, 1992; Tran-Thi et

al, 1994; Yoshino et al (1991), although it is beyond our mandate to review these effects in detail here. Suffice to emphasize that, at the cellular level, O_2 sensing and transduction processes probably form an integral part of the regulation of these complex acclimaticion processes to extreme hypoxia. For illustration, we will discuss their nature and roles (i) in anoxia acclimatory responses in turtle hepatocytes, (ii) in the hypoxia acclimatory response of gluconeogenesis and glycolysis to hypoxia in rat hepatocytes and (iii) in the *cfos* and *cjun* hypoxia acclimation responses in rat cardiac myocytes. All of these *in vitro* studies used extreme O_2 limitation and thus would be most comparable to *in vivo studies* of humans acclimating to extreme (not moderate) hypoxia.

O2 Sensing, Signal Transduction, and Hypoxia Acclimation

One of the experimentally more useful outcomes of the EPO studies to the hypoxia field in general is the development of protocols useful in identifying the occurrence of O_2 sensing systems. Three such protocols are particular widely used (for literature in this area, see Land and Hochachka, 1995): (i) the O_2 sensing response is independent of the ocurrence of oxidative metabolism (for example, a cyanide block is insufficient signal for the induction of hypoxia-associated genes), (ii) nickel and cobalt, by locking heme proteins in deoxy conformation, should mimic the effects of anoxia on heme protein based O_2 sensing pathways, (iii) carbon monoxide, by locking heme proteins in the oxy conformation, should reverse anoxia effects, and (iv) inhibitors of heme protein synthesis pathways should inhibit or abolish heme protein based O_2 sensing. We used these probes to identify the ocurrence of a heme protein based O_2 sensing and signal transduction system in turtle hepatocytes (Land and Hochachka, 1995). In these cells, the short term (acclimatory) response to anoxia involves sensing of O_2 lack by a heme protein based pathway and the pathway and the subsequent example, a cyanide block is insufficient signal for the induction of hypoxia-associated genes), (ii) nickel and cobalt, by locking heme proteins in deoxy conformation, should mimic the effects of anoxia on heme protein based O_2 sensing pathways, (iii) carbon monoxide, by locking heme proteins in the oxy conformation, should reverse anoxia effects, and (iv) inhibitors of heme protein synthesis pathways should inhibit or abolish heme protein based O_2 sensing. We used these probes to identifi the ocurrence of heme protein based O_2 sensing. We used these probes to identify the ocurrence of a heme protein based O_2 sensing and signal transduction system in turtle hepatocytes (Land and Hochachka, 1995). In these cells, the short term (acclimatory) response to anoxia involves sensing of O_2 lack by a heme protein based pathway and the pathway an the subsequent preferential pression of genes for glycolytic enzymes while the expression of genes for gluconeogenic enzymes is decreased (Figure 6) In turtle hepatocytes (Buck et al, 1993; Land and Hochachka, 1995), these gene-expression responses to hypoxia are thought to defense adaptations that are well known charateristic of this: (i) down regulation of ATP utilization pathways (e.g. Na+ K+ATPase, protein turnover, gluconeogenesis, ureagenesis, etc.) coordinately with the down regulation of O_2 dependent ATP synthesis pathway, (ii) the decreased conductivity of cell membranes (referred to as 'channel arrest' in the literature), and (iii) the maintenance of conditions which protect the cells against recovery and reperfusion damage (this latter hypoxia defense strategy is largely assumed to be self-evident since these species clearly survive any ppossible 'reperfusion' damage after extreme hypoxia and ischemia; it is, however, a poorly understood and largely unexplored area in research on hypoxia tolerant species (for metabolic aspects of this problem, see Schulte et al, 1992). In other cells, hypoxia-mediated induction of detoxification pathways such as catalyzed by heme oxigenases (Murphy et al, 1991) are thought to protect cells and tissues from free radical damage during recovery. However, it is not known if similar intracellular messenger system whose primary defense strategies are utilized in hypoxia tolerant species.

Similar, acclimatory (or intermediate time course) responses to low O_2 tensions in rat cardiac myocytes have been explored by Webster and his colleagues (1994). Their observations also indicate the ocurrence of an O_2 sensing and signal transduction system that is involved in regulating the expression of two oncogenes, *cfoc* and *cjun*. In this case, the signal transduction pathway seems to involve protein kinase C and the phosphorylation of target proteins which regulate the expression of *cfos* and *cjun*; the products of the two latter genes are separately

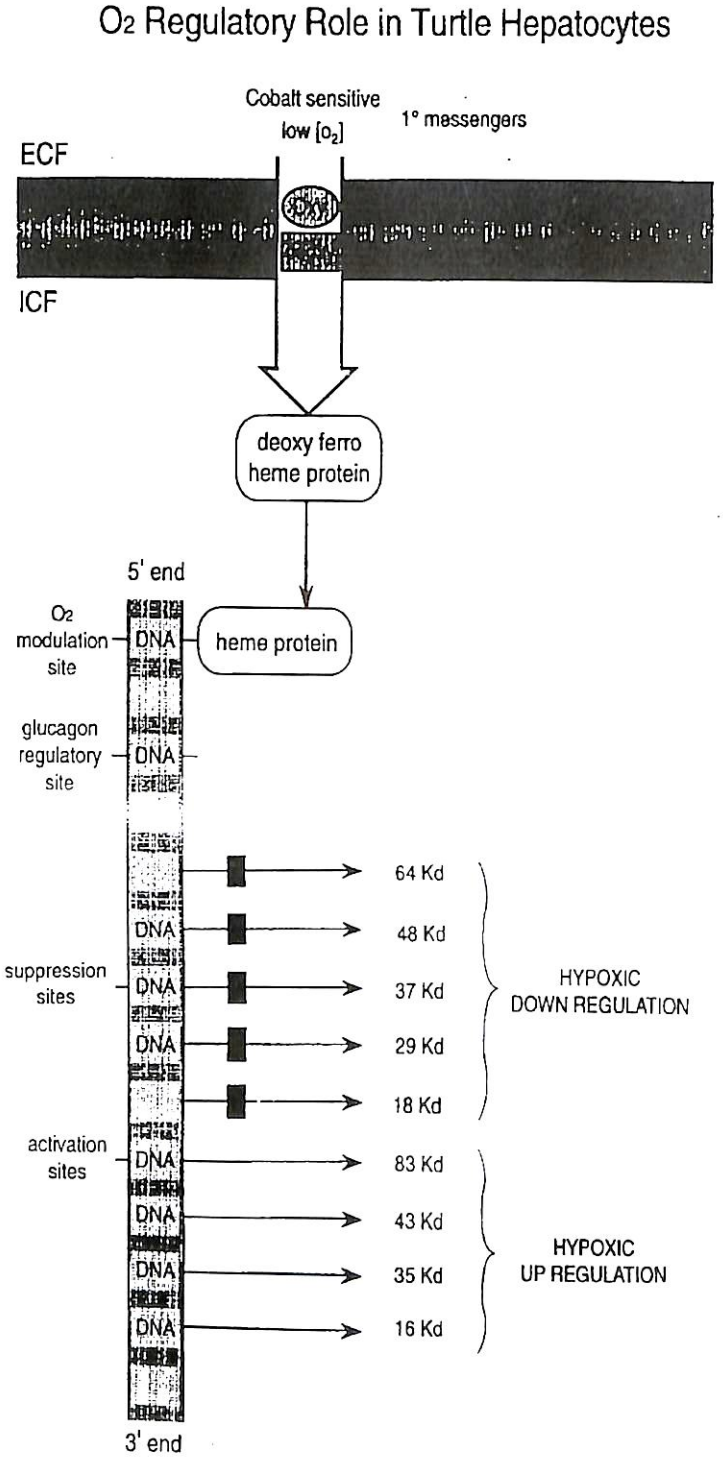


Figure 5. A diagrammatic summary of the postulated role in an hipoxia tolerant cell (turtle hepatocyte) of a heme protein based 02 sensing system that through a currently unknown signal transduction pathway leads to the accelerated expression of several genes (some of which, on the basis of comparable studies with other cells, may be glycocytic enzymes) with the simultaneous suppression of several other genes. At this stage, the regulated proteins are only identified according to size. Summary is based upon Land and Hochachka (1995).

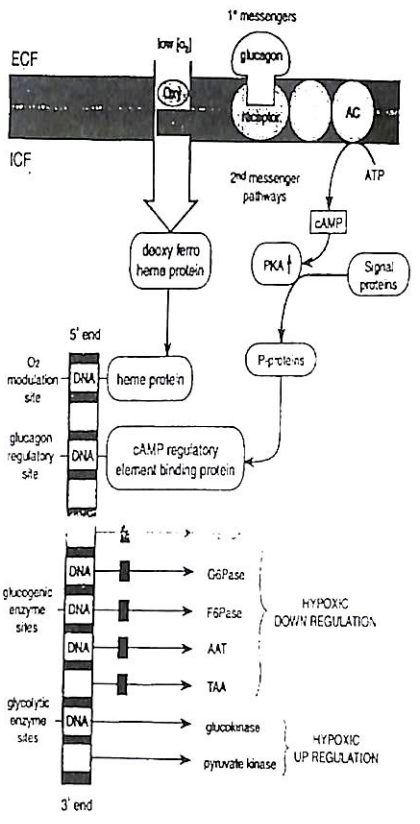


Figure 6. A diagrammatic summary of a heme protein based O2 sensing system whose primary role appears to be the modulation of glucagon (and insulin?) regulation of genes specifying enzymes in glucose homeostasis. Summary is based on studies of rat liver hepatocyte cultures by Keitzmann et al (1992; 1993).

inactive, but self-assemble into heterodimers that serve as a Kind of 'tertiary' function is to regulate the expression (through the AP1 site) of several additional genes (Figure 7). Although the physiological functions of the latter gene products are not fully worked out, it is probable that they too are involved in orchestrating the hypoxia defense adaptations that these cells undergo during hours-todays exposure to reduced atmospheric oxygen: overall reduction in ATP turnover (suppressed ATP utilization pathways with simultaneous adjustments in the membrane structure-function adjustments mentioned above, although not directly demonstrated in hypoxia-acclimated rat heart myocytes, may be orchestrated by *cfos* and *cjun* regulated proteins.

Metabolic Adaptations to Long Term Exposure to Hypoxia

Just as phenomenological short term responses to O_2 availability are well described, so too are long term adaptational responses. Thus, if one

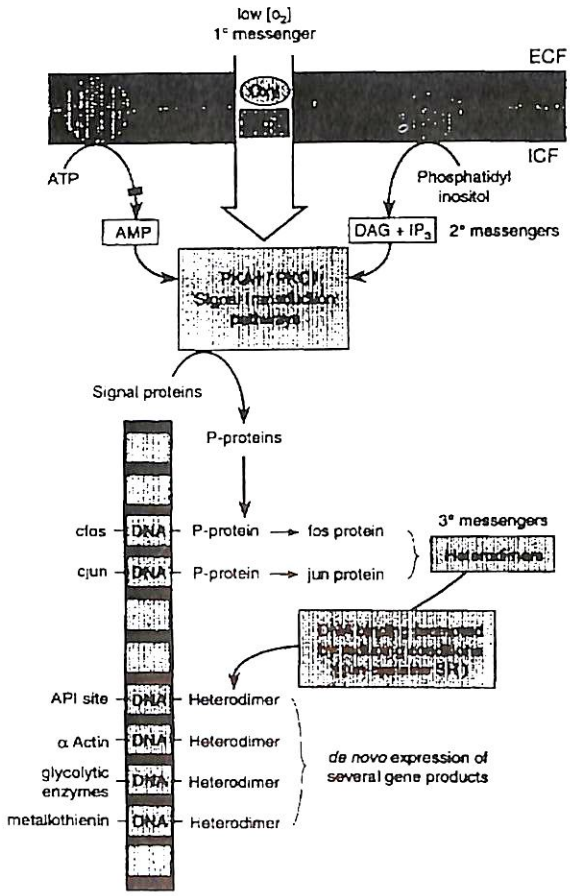


Figure 7. A diagrammatic summary of an O2 sensing and signal transduction system in rat cardiac myocytes. The transduction pathway in these cells appears to depend largely upon protein kinase C phosphorylation of currently unidentified 'signal' proteins involved in regulation of two oncogenes, *cfos* and *cjun*. Alone, the products of these two genes are inactive; however, they self assemble into heterodimers which serve as tertiary messengers: by binding to the AP1 site they are crucially involved in regulating the expression of several additionalgenes. The protein products of these latter genes in turn appear to be involved in orchestrating hypoxia defense mechanisms of cardiac myocytes exposedc for hours to days to extreme hypoxia. Summary based on Webster et al (1994).

compares a given homologous tissue among the vertebrates, say skeletal muscle, one observes about a 500 fold range over which the oxidative capacities vary among species. With citrate synthase (CS) as a mitochondrial marker enzyme, muscle catalytic capacities range from less than 1 in white muscles of some Amazon fishes (see Hochachka and Randall, 1978) to nearly 500 umol per g per min in hummingbird flight muscles (Suarez et al, 1990)- a capacity range presumably arising at least in part as a function of the chronic availability of molecular O_2 at the cellular level. In mammals, the effect of hypoxia on cell oxidative capacities seems to vary with the intensity of the stress. In moderate hypoxia, tissues seem to adapt by increasing capillarity, O_2 carrying capacity in myoglobin containing tissues, and concentrations of enzymes in aerobic pathways of

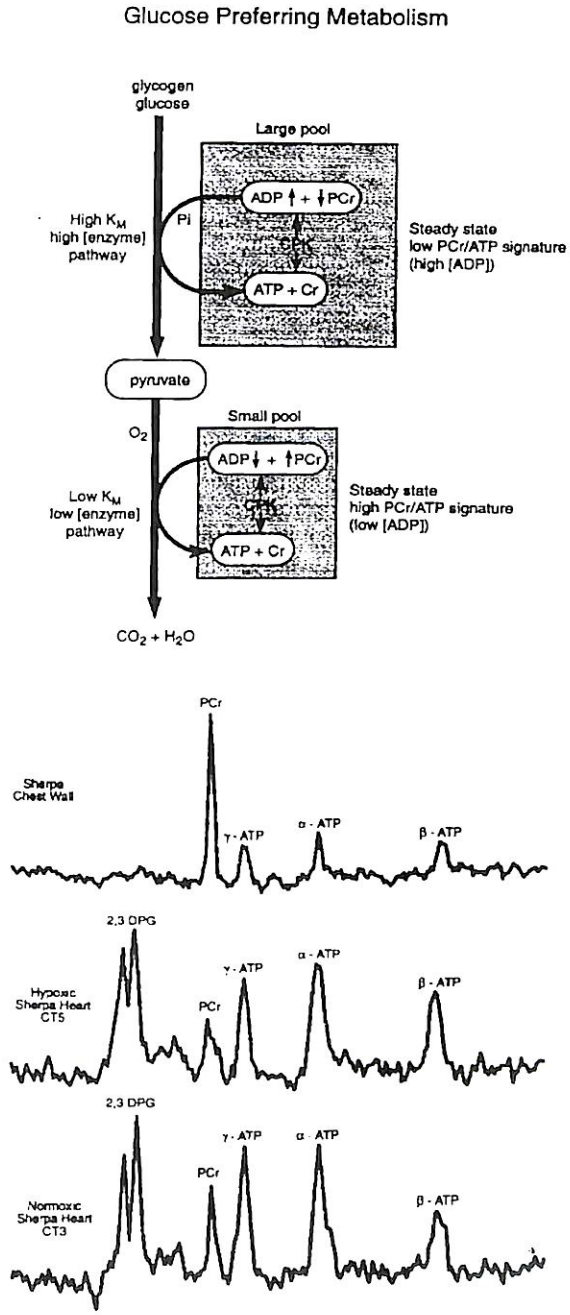


Figure 8.**Upper Panel**: The participation of an active aerobic glycolysis forces heart creatine phosphokinase to equilibrium at reduced [PCr], stable [ATP], elevated [Cr] and elevated [ADP]. This situation is the reason why a low [PCr]/[ATP] is taken as a signature of an O2 efficient, carbihydrate based fuel preference in heart metabolism. **Lower Panel.** ^{31}P M agnetic Resonance Spectra from the heartof an individual Sherpa at 4 tesa during normoxia (CT3) and hypoxia (CT5), compared to the chest wall (phosphate peaks mainly from chest skeletal muscle). The 2,3 diphosphoglycerate (2,3 DPG) peaks arise from red blood cells. The phosphocreatine (PCr) and ATP peaks arise from the left ventricle and the septal wall. Using the gamma phosphate of ATP for reference, the saturation correct heart PCr/ATP ratio in this subject was 0.8 under normoxia and 1.0 under hypoxia, substantially less than the 1.8 average found in hearts of lowlanders and from the value of 6.0 found for the chest wall muscles. The chest wall PCr/ATP ratios in Sherpas are similar to those found in lowlanders. Modified from Hochachka et al (1995).

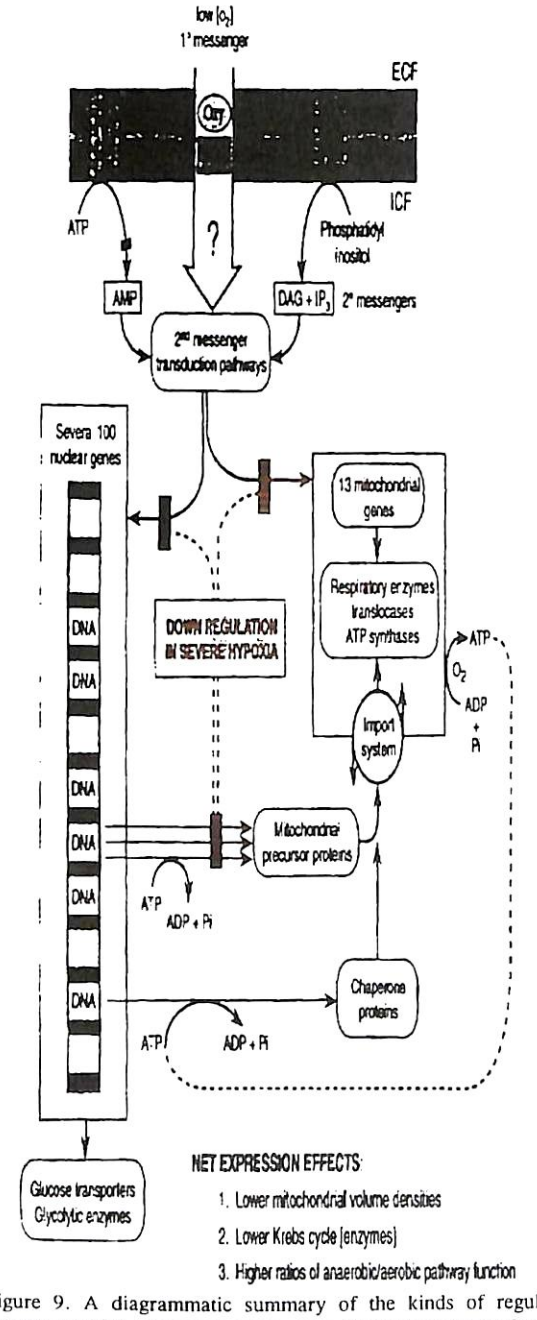


Figure 9. A diagrammatic summary of the kinds of regulatory processes which are presumed to occur during long term adaptation to severe hypoxia in vertebrate animals, including humans. Some of the major steps involved in mitochondrial biogenesis are based on recent reviews (Ceurzva et al, 1993); Nagley, 1991). Net expression effects are reported frequently in studies of adaptation to chronic hypoxia (see Cerretelli et al (1990), Hochachka and Randall, 1978; Hochachka and Somero, 1984; and Hochachka (1985)). Although no direct information is available on O2 sensing and transduction pathways, some possibilities are included based on hypoxia acclimation studies. The purpose of this kind of sketch is to outline as efficiently as possible the areas in greatest need of further research.

metabolism (Hochachka, 1985). In more extreme hypoxia exposure, in humans under hypobaric hypoxia for generations, the oxidative capacities at least of skeletal muscles seem to be down regulated (Kayser et al, 1991; Hochachka et al, 1992). Even lactate dehydrogenase (LDH) capacity, often used on an interspecies basis as an approximate indication of absolute anaerobic glycolytic capacity, is on the lower end of the spectrum for humans (Hochachka et al, 1992). However, the ratio of anaerobic/aerobic metabolic pathway capacity is up regulated in muscle of altitude natives such as Sherpas from the Himalayas or Quechuas from the Andes; for example, the ratio of muscle LDH/CS differs by a factor of 2-3 compared to lowland athletes. It is possible that the difference in response direction in moderate vs extreme chronic hypoxia is due to the inordinate hypoxia sensitivity of protein biosynthesis (Buc-Calderon et al, 1993; Land and Hochachka, 1993; Land and Hochachka, 1995)), although this concept remains to be more firmly established.

In addition to the above metabolic adjustments to chronic hypoxia, the regulation of fuel selection may be adjusted to maximize the amount of ATP conserved per O_2 utilized, an adaptation which would be particularly advantageous under chronic hypoxia.

This stoichiometric efficiency adjustment (achieved by preferential carbohydrate rather than free fatty acid - FFA - utilization and thus by avoidance of the 'FFA oxygen wasting effect') is evident in animals and in humans exposed to extreme chronic hypoxia over generational time (Hochachka, 1993). A consequence of this metabolic organization is that the glycolytic path influences the concentrations of the adenylates (the ADP-dependent pyruvate and phosphoglycerate) kinases of the glycolytic at are high activity, high Km(ADP) enzymes. As a result, the creatine phosphokinase near-equilibrium reaction shifts to lower phosphocreatine concentrations (Figure 8). These adjustments are clearly visible in whole body 4T magnetic resonance spectroscopy of the Sherpa heart *as* lower than expected PCr/ATP ratios - telling signatures of a heart biochemically organized for preferential aerobic carbohydrate metabolism (see Hochachka et al, 1995, for further literature and discussion in this area).

Although these kinds of empirical observations on adaptation to chronic hypoxia are widespread in the literature, the mechanism sunderlying them are not well understood. Diagrammatic summaries of the kinds of processes that may be operative under long term exposure to severe hypoxia are given in Figures 8). These kinds of summaries are based upon current conceptsof mitochondrial biogenesis (Cuezva et al, 1993; Nagley, 1991) and upon phenomenological observations onphylogenetic adaptations to

phylogenetic adaptations to relatively severe chronic hypoxia; to our knowledge, there is no information whatever on what the O_2 sensing mechanismsand signal transduction pathways here may actually be. The rough sketches given in Figures 9 are thus to be considered speculative and are prompted by studies of O_2 signalling systems in intermediate time courses of exposure. This clearly is an area in dire need of much more research.

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