OXYGEN SENSING IN THE ORCHESTRATION OF HYPOXIC METABOLIC ARREST .

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RESUMEN: La Prcepción del Oxígeno en la Organización del Paro Metabólico Hipóxico

La base metabólica del mecanismo sensor de oxígeno ha sido estudiada utilizando hepatocitos aislados de la tortuga pintada occidental, un vertebrado anaerobio facultativo. Como parte de una respuesta sistémica coordinada a la hipoxia, estas células suprimen activamente la síntesis de ATP en sincronía con la demanda de ATP por parte de todos los consumidores principales (Na^+ -K^+ ATPasa, Ca^{2+} ATPasa, recambio de proteínas, síntesis de urea, liberación de glucosa y gluconeogénesis). El resultado es una supresión en 10 veces de la tasa metabólica, impuesta sobre una redistribución de la demanda de ATP entre los procesos celulares. Esta reorganización metabólica dependiente de oxígeno está estrechamente controlada, es rápida en su inicio, ocurre sin perturbación de las concentraciones de adenilato o del potencial de membrana, implica la supresión y expresión de genes dependientes de oxígeno y mantiene una nueva tasa, inferior de flujo a través de vías bioquímicas específicas hasta la reoxigenación. El efecto neto ahorra dramáticamente sustrato fermentable, limita las tasas de acumulación de deshechos metabólicos y extiende profundamente el tiempo de supervivencia en anoxia. Se ha explorado roles directos de mecanismos receptivos de oxígeno en el control de las tasas de flujo para dos eventos celulares significativos luego de supresión metabólica hipoxica. El recambio proteico, que es el proceso celular energéticamente más costoso en normoxia mostró una modulación dependiente de oxígeno de bandas proteicas específicas cuya expresión podía ser predeciblemente manipulada por Co^{2+}, Ni^{2+} y CO. Esto apoya la teoría de un rol para el mecanismo receptor de oxígeno hem-proteína en el control de la expresión hipóxica de genews al ingresar en paro metabólico. Segundo, estudios no invasivos que usan electrodo de autoreferencia selectivo de Ca^{2+}demuestran que hay una supresión selectiva del 75% en el flujo transmembrana de Ca^{2+} que es oxígeno-concordante y que exhibe un KmO_2 aparente de 145 µM. La supresión del flujo de Ca^{2+} era dependiente de proteina kinasa y no era repetible bajo inhibición anaeróbica de la transferencia de electrones por KCN. Estos resultados sugieren que las respuestas hipóxicas de diferentes procesos confluyen para formar una reorquestación metabólica y molecular coordinada de la función celular que permite la supervivencia prolongada sin oxígeno. parte integral de ello es el potencial evidente de los mecanismos receptivos de oxígeno para señalar y coordinar los cambios de flujo a través de vías complejas, energéticamente costosas.

Palabras claves: Oxígeno, Paro metabólico, Respuesta hipóxica.

RÉSUMÉ: La perception de l''oxygène dans lórganization de lárrêt métabolique hypoxique

La base métabolique et moléculaire du mécanisme capteur d'oxygène a été étudié en utilisant des hépatocytes extraits de la tortue tachetée occidentale, un vertébré anaérobie facultatif. Comme élément d'une réponse systémique en relation avec l'hypoxie, ces cellules suppriment activement la synthèse de l'ATP en synchronisation avec la demande d'ATP de la part de tous les consommateurs principaux d'énergie (Na^+/K^+ ATPase, Ca^{2+} ATPase, remplacement de protéines, synthèse de l'urée, libération de glucose et néoglucogenèse). Le résultat est une suppression en 10 fois du taux métabolique, se surimposant à une redistribution de la demande d'ATP entre les processus cellulaires. Cette réorganisation métabolique dépendante de l'oxygène est étroitement contrôlée; elle est rapide à son début, se déroule sans perturbation des concentrations d'adénilate ou du potentiel de membrane; elle implique la suppression et l'expression de gènes dépendants de l'oxygène et soutient un nouveau taux, inférieur, de flux à travers des voies biochimiques spécifiques jusqu'à la réoxygénation. L'effet net économise considérablement le substrat fermentable, limite les taux d'accumulation des déchets métaboliques et allonge énormément le taux de survie en anoxie. Le rôle direct de mécanismes récepteurs d'oxygène dans le contrôle des taux de flux pour deux événements cellulaires significatifs après suppression métabolique hypoxique a été exploré. Le remplacement protéique, le processus cellulaire qui requiert le plus d'énergie en normoxie, a montré une modulation dépendante de l'oxygène de bandes protéiques spécifiques dont l'expression pouvait être manipulée de façon prévisible par Co^{2+}, Ni^{2+} et CO. Cela appuie la théorie du rôle du mécanisme récepteur d'oxygène heme-protéine dans le contrôle de l'expression hypoxique de gènes en entrant en arrêt métabolique. D'autre part, des études non invasives utilisant une électrode d'autoréférence sélective de Ca^{2+} démontrent qu'il y a une suppression de 75 % du flux transmembranaire de Ca^{2+}, qui est oxygéno-concordant et présente un KmO_2 de 145µM. La suppression du flux de Ca^{2+} était dépendant de la protéine kinase-c et ne pouvait être répétée sous inhibition aérobique du transfert d'électrons par KCN. Ces résultats suggèrent que les réponses hypoxiques des différents processus cellulaires se rejoignent pour former une réorchestration métabolique et moléculaire coordonnée de la fonction cellulaire, permettant la survie prolongée sans oxygène. Le potentiel évident des mécanismes récepteurs d'oxygène fait partie intégrante de ce processus pour signaler et coordonner les changements de flux à travers des voies complexes, très coûteuses du point de vue énergétique.

Mots-clés : Oxygène, Arrêt métabolique, Réponse hypoxique

SUMMARY: The metabolic and molecular basis of oxygen sensing has been probed using isolated hepatocytes from the vertebrate facultative anaerobe, the western painted turtle. As part of a coordinated systemic response to hypoxia, these cells actively suppress ATP synthesis in synchrony with ATP demand from all major energy sinks (Na^+/K^+ ATPase, Ca^{2+}ATPase, protein turnover, urea synthesis, glucose release and gluconeogenesis). The result is a 10-fold suppression in metabolic rate superimposed over a re-partitioning of ATP-demand among cellular processes. This oxygen-dependent metabolic re-organization is tightly controlled, being rapid in onset, occurs without perturbation to adenylate concentrations or membrane potential, it involves the oxygen-dependent suppression and expression of specific genes and it sustains a new, lower rate of flux through specific biochemical pathways until re-oxygenation. The net effect dramatically spares fermentable substrate, limits rates of metabolic waste accumulation and profoundly extends survival time in anoxia.

Direct roles for oxygen-receptive mechanisms in the control of flux rates have been explored for two energetically significant

cellular events during the onset of hypoxic metabolic suppression. Protein turnover, the most energetically eostly cell process in norrnoxia, exhibited oxygen-dependent modulation of specific protein bands whose expression could be predictably manipulated by Co^{2+}, Ni^{2+}, and CO. This supports a role for heme-protein based oxygen receptor mechanism in the control of hypoxic gene expression on entering metabolic arrest. Secondly, noninvasive studies using a Ca^{2+}-selective self-referencing electrode demonstrate that there is a 75% suppression in transmembrane Ca^{2+}-flux that is oxygen conforming, exhibiting an apparent KmO_2 of 145μM. The suppression of Ca2+-flux was protein kinase-c dependent, and was not repeatable under aerobic inhibition of electron transfer be KCN.

These results suggest that the hypoxic response of different cellular processes coalesce to forrn a coordinated metabolic and molecular re-orchestration of cell function that enables longterm survival without oxygen. Integral to this is the clear potential for oxygen-receptive mechanisms to signal, and coordinate, flux changes through complex, energetically expensive pathways.

Key Words: Oxygen, Metabolic arrest, Hypoxic response

INTRODUCTION.

Although emerging studies indicate that there is a genetically-linked tolerance to hypoxia among high altitude human populations, generally, we are not a species that tolerates any degree of oxygen limitation at all well. Chronic exposure to high altitude hypoxia is both debilitating and potentially lethal, so for these reasons, biomedical research is particularly driven to determine how the oxygen signal is perceived at the organ-to-cell level, how resulting physiological changes come about, and how clinical strategies can be developed for the prevention and treatment of hypoxic pathologies. The research base is therefore directed towards understanding and sustaining a system that is failing during mild-to-moderate oxygen lack.

This review addresses the question of hypoxic survival by examining how naturally evolved hypoxia-tolerant systems deal with chronic exposure to anoxia. Hypoxia tolerance is particularly well developed in the Western painted turtle, a vertebrate facultative anaerobe that is capable of surviving systemic anoxia for up to 2 weeks at 25ºC, extending upwards to 6 months at temperatures <10ºC. This extreme capacity for anoxic survival is based on an oxygen-sensitive re-organization of cellular metabolism, superirnposed upon a cell biology that is geared towards the long-term provision of energy via glycolysis, and a capacity to deal with the accumulation of toxic metabolic end-products (lactate and protons). In keeping with the questions central to high altitude biomedical research, this review presents studies conducted on hepatocytes isolated from this species which were used to understand how cellular metabolism is re-orchestrated in anoxia, at what advantage to the survival of the organism and the role of the oxygen signal in coordinating cellular events among complex energy-demanding processes.

Orchestrating Coordinated Adjustments Between Cellular Processes.

Four general elements comprise cellular metabolism: i) the generation of reducing power, that drives *ii*) ATP synthesis; *iii*) a selectively permeable membrane that generates the potential energy for driving metabolic processes and *iv*) lipid, protein and nucleic acid biosynthesis. Due to the inter-relatedness of each of these processes, physiological responses to environmental change requires coordination between all of these components; the failure of one link in the chain ultimately results in the failure of the loop.

From single cells to whole organisms, metabolic responses to hypoxia can be broadly categorized into one of two adaptive responses (1). The response associated with oxygen regulation maintains the provision of energy via a Pasteur effect (enhanced rate of glycolytic flux) to support cell processes at rates approaching those found during oxidatively supported metabolism. Finite stores of fermentable substrate, and the accumulation of metabolic wastes (lactate, H^+), together, set time-restrictions on the success of oxygen regulation as a useful means for surviving oxygen lack. In contrast, the oxygen conforming response minimizes rates of energy supply coordinately with energy demand to invoke a controlled suppression of metabolic rate below resting levels. Figure 1 compares the oxygen-regulating (generally anoxia intolerant) versus conforming strategies (anoxia tolerant) for dealing with hypoxia. The trade-offs are clear and define remarkably different time limits for tissue function and survival without oxygen. In oxygen regulators, depletion of available substrate, and the rapid accumulation of toxic metabolic endproducts, leads to failure of the Na^+/K^+ ATPase, membrane depolarization, Ca^{2+} release from internal stores followed by capacitative Ca^{2+} entry over the cell membrane, and cell death (2). Although survival time is dependent on the severity of hypoxia, oxygen regulating systems will typically cope with severe hypoxia over a time frame metered in minutes. The oxygen conforming response, on the other hand, favors cell survival at the expense of metabolic scope. ATP demand and supply are coordinately down-regulated requiring specialized adaptations in cell physiology to cope with attenuated energy turnover. Principle among these

are the capacity to maintain membrane polarity, stability of macromolecules, and remain capable of detecting changes in oxygen availability (reviewed in ref. 3). The combined effect results in a survival time in severe hypoxia that is extended from months, outwards to years in some cases.

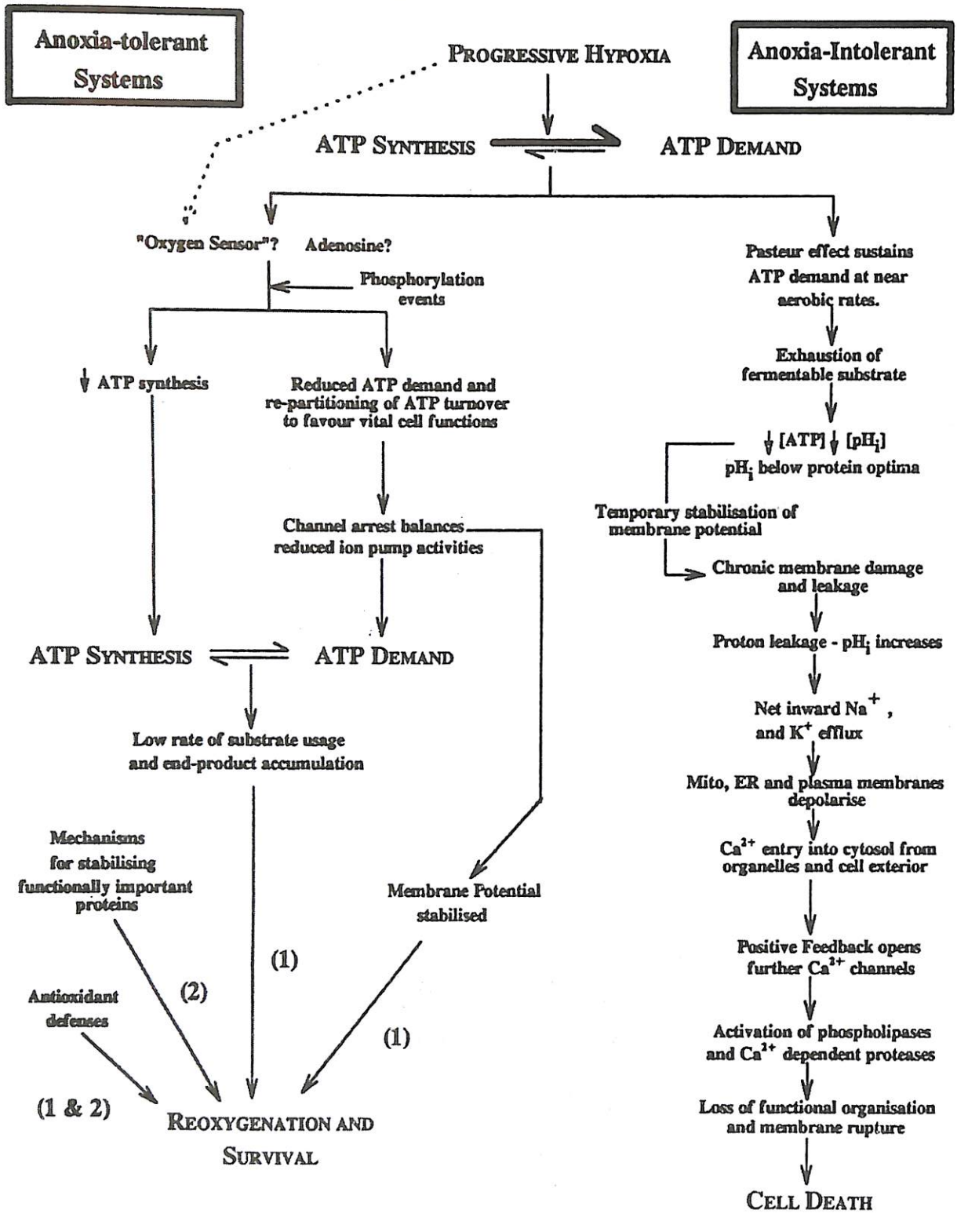

Figure 1. The pathway of acute cellular damage invoked by severe hypoxia in anoxia in-tolerant (typically oxygen-regulating) tissues, and the mechanism of avoidance in anoxia-tolerant (oxygen conforming), tissues. Anoxia-tolerant paths marked (1) denote adaptive metabolic readjustments that occur early in the transition towards hypoxia. Paths marked (2) denote probable genotypic features (ie these are physiologically inherent to the species). Figure derived from references 1-3, 10 and 13.

Oxygen regulation and oxygen conforrning responses deal with hypoxia in strikingly different ways, yet lack of oxygen is the primary signal for the induction, and presumably control, of each response. The question posed here asks: ***How does the oxygen signal coordinate all principle components of metabolism together towards an effective strategy of hypoxia survival?***

Metabolic Suppression in Response to Hypoxia, Studied in Cultured Isolated Turtle Hepatocytes.

Naturally evolved tolerance to hypoxia comprises a series of more-or-less conserved physiological characteristics that are common among species that occupy periodically hypoxic or anoxic habitats. The systemic response to hypoxia is typically oxygen conforming and involves a coordinated suppression of metabolism below standard metabolic rates. This suppression necessarily involves coordination between every aspect of cellular function residing on both ATP synthesis and demand sides of energy turnover. It therefore constitutes a useful system to probe the relationship between an oxygen signal and the metabolic, cellular, and molecular adjustments that are necessary for the periodic and long-term survival of anoxia.

We studied the cellular basis for anoxia-tolerance using hepatocytes isolated from the Western Painted Turtle (*Chrysemys picta bellfi*). As a component of the systemic metabolic suppression during hypoxia, these cells will actively suppress ATP synthesis synchronously with demand from major ATP sinks (ion pumping, protein turnover, urea synthesis, glucose release), even when isolated and maintained in a culture environment. This presented a unique opportunity to examine the cellular and molecular basis of metabolic suppression and in particular the relationship of the cessation in cellular function to the disappearance of oxygen itself. To address this issue fully, it was irnportant to understand which cellular processes constituted the major energetic drains, and to what extent their suppression contributed to the overall metabolic suppression in anoxia. The first step was therefore to tease apart the ATP synthesis and ATP demand sides of energy turnover to construct an energy budget for these cells in normoxia and after 10 hours of severe anoxia.

ATP synthesis in anoxia Figure (2) demonstrates the ATP synthesis component of ATP turnover during metabolic suppression in turtle hepatocyte primary cultures. Three important features are characteristic: i) there is a 90% fall in metabolic rate (mirrored by a 70% fall in rnicrocalorimetric heat flux), ii) there is little change in the adenylate energy charge, iii) the onset of metabolic suppression is rapid and is fully reversible on reinstatement of a normoxic environment (4,5). Flux through pathways of carbohydrate metabolism change profoundly. Rates of glucose release fall by 60%, remaining attenuated, but continuous, throughout anoxia. Gluconeogenic rates were practically immeasurable constituting an insignificant component of both ATP synthesis and demand in anoxia. Energy production in support of remaining anoxic ATP demand in anoxia relies solely on glycolysis and is fueled by near molar concentrations of on-board glycogen. Experimentally deplete glycogen stores or block glycolytic flux and ATP concentrations, remaining heat flux, and total cell viability all rapidly diminish. Given that optimally minimized, but *sustained*, ATP turnover is essential for maintaining cell viability, what is the response of the major ATP consuming components of ATP turnover?

ATP Demand in Anoxia. The maintenance of ATP concentrations throughout the period of anoxia suggests that the stoichiometry of ATP turnover never gets too far out of balance. Figure (3) demonstrates the re-partitioning of ATP demand among the most energetically expensive cell functions as oxygen availability dissipates. In normoxia, protein turnover (the compounded cost of protein synthesis and protein degradation) accounts for the majority of ATP demand (about 45%). Na^+/K^+ATPase accounts for the majority of the remainder at about 25%. In anoxia, there is a striking re-partitioning of ATP demand among the major energy consuming processes that is superimposed on a 70 to 90% suppression in energy demand for each individual pathway examined. As noted in figre 2, glycolysis supports an anaerobic ATP turnover of 6.5 µmol $ATP.g^{-1}.min^{-1}$, a 10-fold suppression in ATP turnover from normoxic standard metabolic rates. Within anoxic total ATP turnover, energy demand by Na^+/K^+ ATPase accownts for 75% (6). Protein synthesis and degradation are both coordinately suppressed (protein content of the cells does not change) and accownt for the majority of remaining anoxic metabolism (7,8,9). The emerging pattern suggests that normoxic cells expend the majority of energy metabolism on anabolic processes such as protein turnover whereas in anoxia, there is a re-organization of energy expenditive that favors the maintenance of the cellular membrane potential at a proportionate increase in the overall proportion of ion pumping activity. The postulated mechanism behind the maintenance of the membrane potential despite an overall suppression in ion pump activity

(The channel arrest theory) has been reviewed elsewhere (10).

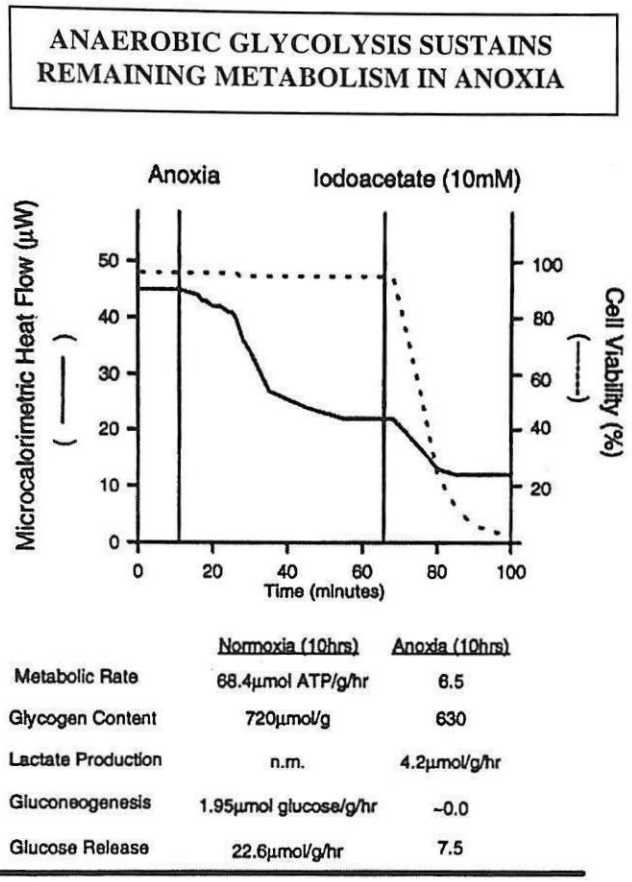

Figure 2. Scheme denoting characteristics of carbohydrate metabolism in support of ATP synthesis in normoxia and over 10h anoxia in isolated turtle hepatocytes. Microcalorimetric heatflow (solid line) falls 70% in anoxia with biochemical determinations of metabolic rate reflecting a 10-fold reduction in ATP turnover rates. Addition of the glycolytic inhibitor, iodoacetate, results in the death of the tissue emphasizing the significance of energy supply via glycolysis and the functional importance of remaining sources of ATP demand in anoxia. Note high internal stores of glycogen in support of glycolytic flux. Figure derived from references 4 & 5

Evidence that an Oxygen Signal Controls Complex ATP Consuming Processes.

The coordinated suppression of ATP turnover, and associated re-partitioning of ATP demand among cellular processes, incorporates nothing short of a complete biochemical and molecular re-arrangement to achieve a metabolically dormant state in hand with a stable cell structure. The crucial issue centers around the signal: do cells possess the capacity to sense changes in oxygen availability and respond in an adaptive manner? To investigate this issue we examined the oxygen-sensing characteristics of protein turnover and transmembrane Ca^{2+}-flux as examples of two complex, energetically expensive, metabolic pathways. As both processes represent the sum of multiple components, each tests the capacity of the oxygen signal to coordinate tightly coupled flux changes between a number of related cell functions.

I) Oxygen Sensing and Protein Turnover.

Protein turnover (protein expression and degradation) accounts for about half of normoxic and about one third of anoxic ATP demand. Aside from defining the functional and phenotypic characteristics of cells, it also maintains the relative health of the functional protein pool by ensuring the removal of damaged and dysfunctional molecules. Suppression of protein turnover is therefore perilous as it diminishes the capacity for cells to adaptively express new functional protein translates, and remove old ones (3). Through the induction of genes, appearance of mRNA, translation of proteins, modification of protein structure, proteolytic turnover of the protein, and the ATP synthesis that is required to support this process, the overall scheme of protein turnover offers a rigorous test of the oxygen signal to coordinate changes among cellular processes.

To tease out the oxygen signal and receptor mechanism, we employed a similar strategy to that employed in the erythropoietin field which aimed to modulate the responsiveness of oxygen dependent cell processes independently of oxygen availability. We could functionally achieve metabolic anoxia despite the presence of oxygen by out-competing oxygen at cytochrome-c oxidase using KCN. By assuming a transduction mechanism based ferro-heme binding of oxygen, we could modulate the conformational state of the putative heme protein by substitution of Co^{2+} or Ni^{2+} ions in the central Fe^{3+} position (induced de-oxygenated conformation) by incubation with carbon monoxide (induces a K_m-dependent oxygenated conformation) (11,12). With this approach, our hypothesis specifically tested the notion that the oxygen signal was transduced through the change in conformation of a heme protein oxygen receptor, linked through an unidentified intracellular signaling pathway.

Our results indicated that a heme protein was indeed involved in transducing an oxygen signal to elevate expression of 5 unidentified protein bands, and decreased expression of a further 5 distinct protein bands (9). This clearly suggests a role for oxygen in the modulation of protein expression at both levels of synthesis and degradation. Furthermore, it highlights a role for oxygen in the fine-tuning of cellular characteristics in the transition towards hypoxia: despite a 10-fold suppression in rates of ATP-dependent protein turnover, the phenotype of the protein pool was subject to oxygen-specific modulation via adaptive expression (or suppression) of sets of hypoxia-sensitive genes (9,13).

A broad body of literature now identifies a clear link between a ferro heme oxygen receptor mechanism and changes in the relative availability of oxygen to mediate expression of the up-stream transcriptional regulator, Hypoxia Inducible Factor-l. HIF-1 is a basic-helix-loop-helix PAS heterodimer consisting of a novel 120kd α-subunit and a 94kd , ß-subunit that is identical to the aryl hydrocarbon nuclear translocator (ARNT), a transcriptional regulator in the dioxin response (14). In hypoxia, HIF-1 regulates increased expression of the hepatic hematopoiesis factor, erythropoietin (12), vascular growth factors (VEGF and PDGF, (15)) and certain specific glycolytic enzyme isoforms in a wide variety of tissues, specifically, phosphofructokinase (PFK-L and C), phosphoglycerate kinase (PGK-A), aldolase (ALD-A and C) pyruvate kinase (PK-A), and lactate dehydrogenase (LDH-A) (15,16). Hypoxia also abrogates receptor-mediated control over the expression of the gluconeogenic rate limiting enzyme, phosphoenolpyruvate carboxykinase (PEPCK, (17)). Recent evidence further implicates a similar oxygen sensing mechanism in the control over glucose transporter expression for the isoforms GLUT 1 and 3 (expressed in hypoxia) and GLUT 2 (suppressed in hypoxia) (16,18). From a metabolic point of view, this list underscores a clear role for oxygen in the control of glucose metabolism and in particular, the expression of rate limiting isozymes for glucose transport, glycolysis and gluconeogenesis as significant control points. A most important feature of the oxygen signal transduction pathway is its capacity for modular control over defined regulatory pathways for the same gene. In liver for instance, glucagon regulates PEPCK expression through a cAMP-dependent protein kinase pathway, unless a significant transition towards hypoxia occurs where control passes to a ferroheme protein/HIF-l regulatory pathway (17). Clearly, the intracellular signaling pathway from ferro heme protein to the induction of hypoxia-sensitive genes must juxtapose tonic regulatory pathways but how might this occur? A recently proposed hypothesis based on work conducted on carotid body and hepatoma cell lines suggests that a candidate heme-protein/REDOX based oxygen sensor may be NAD(P)H oxidase (19,20). The signal is transduced by reduction of the ferro-heme NAD(P)H oxidase subunit resulting in production of hydrogen peroxide. H202 subsequently mediates the reduction of glutathione groups and the production of cGMP by reduction of the soluble guanylate cyclase heme-subunit. Although not necessarily a unifying theory of oxygen sensing among different tissue types, this pathway for oxygen transduction is intriguing because it unites the central principles and actions of oxygen sensing mechanisms in general: i) the oxygen sensor is located as far out in the oxygen gradient as possible (ie in the plasma membrane), ii) it possesses a high $K_m.O_2$, potentially signaling changes in oxygen availability early in the transition to, and from, hypoxia, iii) by initiating a REDOX potential it juxtaposes intracellular signaling mechanisms responsible for controlling multiple cell functions including channel opening probabilities and gene expression. As a candidate oxygen sensing pathway, this surely represents an ideal system on which to base further investigations into oxygen sensing mechanisms and consequences.

ii) Oxygen Sensing and Ca^{2+} Flux.

Ca^{2+} acts as a second messenger in a wide variety of cell processes stemming from fertilization, through

hormone responses, to cell death. As a result, alterations in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) form the basis of a signaling mechanism that can potentially coordinate a number of interdependent, Ca2+--sensitive cell processes together. This signaling mechanism must be tightly regulated as uncontrolled increases in cytosolic $[Ca^{2+}]i$ also lie at the root of events that lead to cell death (fig 1). Clearly, the success with which tissues survive hypoxia rests greatly on the ability to maintain the cell membrane potential and, in particular, control Ca^{2+}-fluxes between cellular compartments, the cytosol and the extracellular environment.

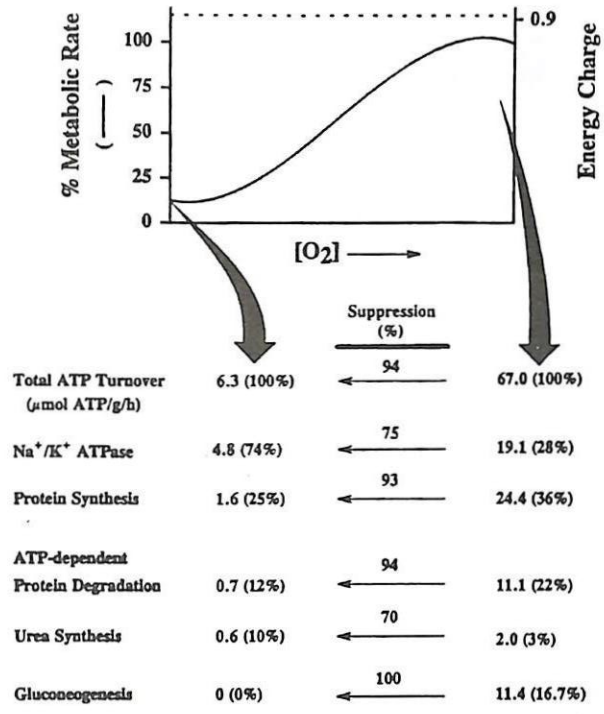

Figure 3. An energy budget for the major ATP demanding cell processes in normoxia and after 10h anoxia in isolated turtle hepatocytes. Graph at uppermost emphasizes an oxygen-dependent fall in metabolic rate (solid line) with little perceptible change in ATP concentrations or the cellular energy charge (dotted line). Normoxic ATP demand, demonstrated on the right side of the figure, largely sustains rates of protein turnover. After a 10-fold fall in metabolic rates, and energy demand by individual biochemical pathways, anoxic metabolism largely supports energy demand by ion pumping. Figure derived from references 4-9.

Continuing to use turtle hepatocytes as a model, we probed the relationship among oxygen availability, protein kinase-C activity, and adenosine purinoceptor activation in the control of turtle hepatocyte transmembrane Ca^{2+}-flux (the net balance between plasma membrane Ca^{2+} ATPase activitv. Na^{+}/Ca^{2+}-exchanger activity and Ca^{2+}-channel influx), measured with a non-invasive, Ca^{2+}-selective, self-referencing electrode (21). This technique enables non-invasive detection of extracellular Ca^{2+}-fluxes from single cultured cells by oscillating a Ca^{2+}-selective microelectrode (tip diameter of about 3 ,um) back and forth through the boundary layer next to the cell membrane. By referencing together the Ca^{2+}-specific signals obtained at each maximun of the arc described by the electrode movement, a $\Delta\mu V$ difference is obtained that can be applied to the Fick equation to determine directional Ca^{2+}-flux (figure 4). With this approach, we found that progressive hypoxia was associated with a reversible, oxygen-dependent suppression of Ca^{2+}efflux with an apparent KmO_2 of 145,μM (fig 5). As the technique only resolves slow-time course events (ie on the order of seconds) and the efflux was largely inhibited by Ca^{2+} ATPase blockers, the nature of the Ca^{2+}-efflux is likely to be Ca^{2+}ATPase activity (22). The high KmO_2 suggests that the suppression of Ca^{2+}-efflux is achieved through a signaling mechanism capable of detecting changes in oxygen availability over concentration ranges that are two orders of magnitude above the KmO_2 of the electron transfer system in liver [0.7μM 02 for tightly coupled isolated liver mitochondria (23)]. In support of this was the failure of KCN, administered over concentration ranges that achieve rapid inhibition of oxygen consumption in turtle hepatocytes, to diminish Ca^{2+}-flux when oxygen was present in the

medium (figure 6).

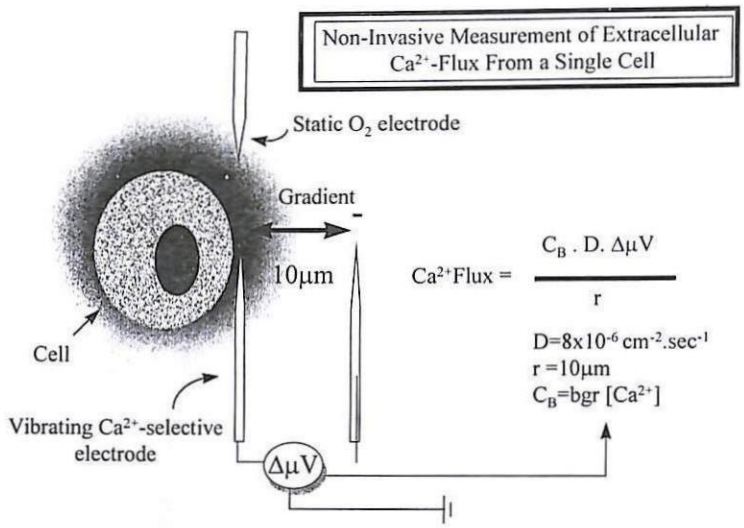

Figure 4. Operational principle of the Ca^{2+}-selective self-referencing microelectrode. Patch clamp style electrodes (tip diameter of 3 μm) were rendered selective for Ca^{2+} by placing a 25 ,μm column of a Ca^{2+}-selective ionophore in the tip. The electrode was then oscillated over a distance of 10pm, at a rate of 0.3 Hz, through the extracellular Ca^{2+} gradient at a point perpendicular to the equatorial centerline of the cell. The signals obtained at each maxima of the oscillation were referenced to one another to obtain a Ca^{2+}-specific ΔμV difference over the amplitude of oscillation. This could then be used in the Fick equation to calculate a directional value of Ca^{2+}flux where. D= diffusion coefficient for Ca^{2+} in physiological saline; r= amplitude of oscillation, C_B= background molar concentration of Ca^{2+} in the medium. As the tip of the electrode never contacts the cell surface, and there is no electrical field generated by the electrode, the technique is non-invasive. In the experiments described here, a static polarographic microelectrode was also maintained close to the cell to monitor local oxygen concentrations.

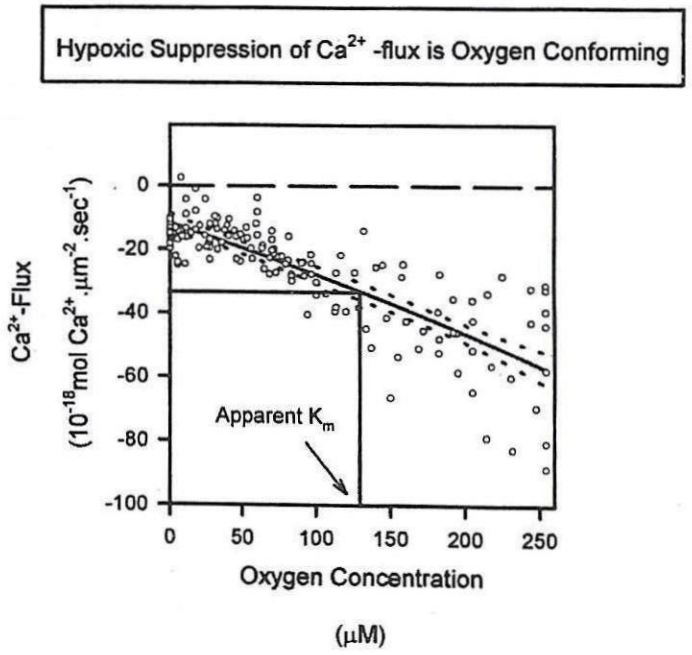

Figure 5. Oxygen conformity of transmembrane Ca^{2+}-flux during a transition towards hypoxia demonstrates an apparent K_mO2 of 145μM (sold line). 95% confidence limits in the fit of the line to the data points are also shown (broken line). The self-referencing Ca^{2+}-selective electrode was oscillated over a distance of 10, μm at a rate of 0.3Hz. Figure derived from reference 22.

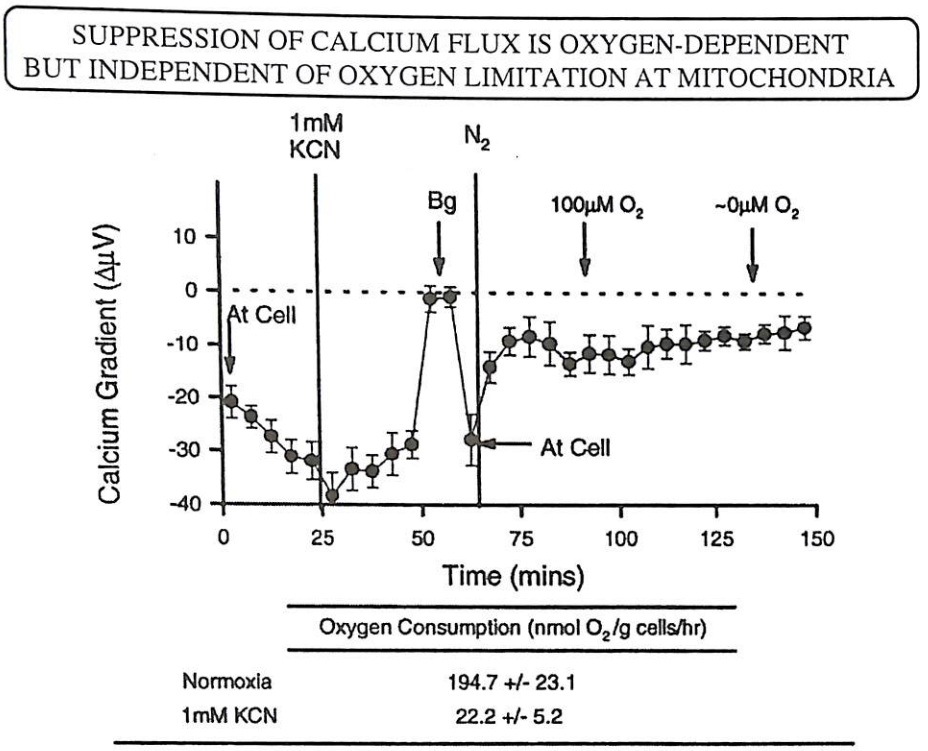

Figure 6. The suppression of Ca^{2+}-flux was oxygen dependent, but independent of inhibition of mitochondrial electron transfer. The electrode was moved to within a micron of the cell membrane ("At Cell") and a $\Delta\mu V$ signal output collected for 25 minutes. lmM KCN was then added to the medium and the signal output collected for a further 25 minutes. Note that aerobic addition of lmM KCN causes a 10-fold reduction in rates of oxygen consumption. The electrode was then moved out of the cellular Ca^{2+}-gradient to a background position (Bg) to ensure that the signal output was zero in the absence of a Ca^{2+}-gradient. Movement of the electrode back to the same measurement spot next to the cell retrieved the original signal magnitude, despite KCN blockage of cellular oxygen consumption. At 60 minutes into the experiment, dissolved oxygen was displaced by infusion of a N_2-pressure-head over the surface of the medium, causing the same, high apparent K_mO_2, oxygen-dependent suppression of Ca^{2+}-flux noted in figure 5. This occurred irrespective of the preceding inhibition of mitochondrial electron transfer by KCN. Figure depicts the change in the $\Delta\mu V$ signal difference in normoxia, with each plotted point being the mean +/-standard deviation of 100 raw data points collected

The oxygen signal was transduced by a pathway that involved the activation of PK-C as anaerobic inhibition of PK-C eliminated the controlled suppression of Ca^{2+}-efflux, causing a marked Ca^{2+}-influx followed by cell swelling and rupture. From the literature, the role PK-C might play in coordinating the suppression of Ca^{2+}-efflux during hypoxia, is vague. Aerobic experiments have detailed a role for PK-C in the opening of capacitatively coupled Ca^{2+}-channels in the plasma membrane after release of internal Ca^{2+} pools by thapsigargin (24,25). This fits with the case in rat hepatocytes where PK-C activation by TPA has been observed to prolong the time course of cytosolic Ca^{2+} re-sequestration into cellular compartments during glucagon-induced Ca^{2+} cycling (26). In glioma cells, PK-C activation by TPA results in an increase in $[Ca^{2+}]i$ that stems, in part, from an activation of plasma membrane Ca^{2+} channels (25). In normoxia, there is clearly a precedent to suggest PK-C activation opens capacitatively coupled Ca^{2+}-channels.

In our studies with turtle hepatocytes, we identified both an aerobic, TPA-induced, reduction in Ca^{2+}-efflux and also an absolute requirement for PK-C activation in the controlled suppression of Ca^{2+}-efflux towards a new steady-state during the hypoxic transition (22). Addition of the PK-C inhibitor, sphingosine, during anoxia led to a rapid Ca^{2+}-influx followed by cell rupture (figure 7A). Given the clear role of PK-C activation in the opening of Ca^{2+} channels, we cannot exclude the possibility that the observed suppression of Ca^{2+}-efflux was associated with an elevated, inwardly directed Ca^{2+}-flux component. When compounded, the absolute requirement for PK-C activation, the ensuing sustained pattern of hypoxic Ca^{2+}-flux, and the prolonged survivability of these cells during

anoxia, all serve to underscore this as a regulatory event rather than a precursor to the uncontrolled capacitative increase in $[Ca^{2+}]i$, as would be associated with hypoxic cell death.

Receptor-mediated Ca^{2+} entry in hepatocytes is under the control of two distinct, agonist activated pathways that exhibit different cation selectivities and modes of control (27). The first operates through an agonist-sensitive (vasopressin or thapsigargin), inositol $(1,4,5)P_3$-generating pathway to mobilize the intracellular Ca^{2+}-pool followed by a Ca^{2+}-specific influx over the plasma membrane by capacitative coupling. This pathway is distinct as it will not conduct Mn^{2+}. The second mechanism is less selective for Ca^{2+} over Mn^{2+} and requires continuous hormonereceptor binding to a G-protein complex with the subsequent activation of Ca^{2+} channel conductance (27,28). In turtle hepatocytes, it was important to assess the capacity for the oxygen signal to interact with existing receptor-mediated pathways of Ca^{2+}-mobilisation. We examined the adenosine-purinoceptor-mediated pathway because the production of extracellular adenosine during periods of metabolic stress has the general effect of reducing energy demand and increasing energy supply, making this an important regulator of pathways associated with the survival of anoxia (29). In hepatocytes, specific aerobic effects of adenosine include a suppression of protein synthesis (30) and gluconeogenesis (31), activation of glycogen phosphorylase and enhanced rates of glycogenolysis (32) and urea synthesis (33). All events are associated with increases in $[Ca^{2+}]i$ with activation of glycogenolysis and urea synthesis demonstrating an absolute dependence for the Ca^{2+} signal. Adenosine binding to the purinoceptor signals increases in $[Ca^{2+}]i$ by activating the $Ins(1,4,5)P_3$-mobilised Ca^{2+} pool with subsequent capacitative entry of Ca^{2+} over the plasma membrane.

In turtle hepatocytes, adenosine activated Ca^{2+}-effflux almost two-fold over normoxic controls (Figure 7B). The response was abrogated in the presence of 10,uM of the specific A, subclass-purinoceptor antagonist, 8-PT. As adenosine is well characterized to transiently increase $[Ca^{2+}]i$ in hepatocytes, we interpret the increase in apparent Ca^{2+} effflux as an elevation in outwardly directed Ca^{2+}-ATPase activity to compensate capacitative Ca^{2+} entry. When repeated under anoxic conditions, neither adenosine nor 8-PT administration altered the characteristic suppression of Ca^{2+}-effflux, suggesting a capacity for the oxygen signaling pathway to behave as a modulator of existing regulatory pathways of Ca^{2+}-efflux during severe oxygen lack. Although far from pin-pointing a clear-cut oxygen sensing mechanism, the oxygen-dependent suppression of transmembrane Ca^{2+}-flux in turtle hepatocytes (the net effect of channel, pump and transporter activity) indicates that processes associated with Ca^{2+} homeostasis are under tight control as oxygen availability diminishes.

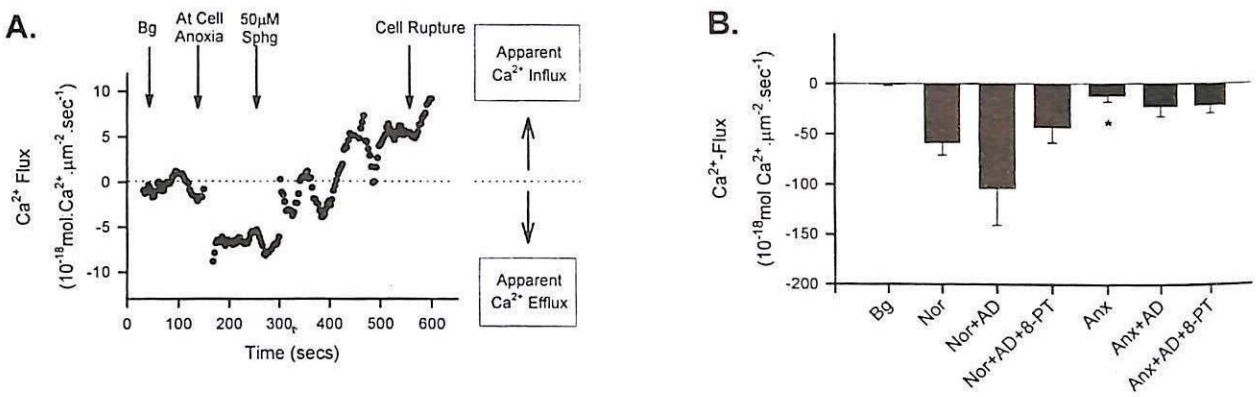

Figure 7(A) Effect of protein kinase-C inhibition by 50>M sphingosine during the anoxic suppression of transmembrane Ca^{2+}-flux. Normoxic PK-C activation using a phorbol ester leads to a reduction in the magnitude of transmembrane Ca^{2+}-flux, which is readily reversible with sphingosine. In anoxia, however, inhibition of PK-C leads to a rapid Ca^{2+} influx followed by cell rupture. **(B)** Normoxic administration of 100>M adenosine doubles transmembrane Ca^{2+}-flux which is readily inhibited by 10µM 8-PT, antagonist to the adenosine Al receptor. In anoxia, neither adenosine nor 8-PT abrogate the oxygen-dependent suppression in Ca^{2+}flux suggesting that the oxygen-dependent pathway possesses dominant control in hypoxia. Abbreviations: Bg, background recording position 200µm from cell surface: Sphg, sphingosine; Nor, normoxia; Ax, anoxia; AD, adenosine; 8-PT, 8-phenyltheophylline.

CONCLUSION.

How effective is the oxygen signal as a coordinator of metabolism? From the work described above, we now have an emergent picture of anoxic survival in turtle hepatocytes that centers around the shut-down of individual processes associated with ATP synthesis and demand, an oxygen-regulated change in protein expression profiles, preservation of the cellular membrane potential, and tight control over Ca^{2+} by oxygen and second messenger modulation of transmembrane Ca^{2+}-fluxes. This presents a clear demonstration of the interaction of the oxygen signal with each aspect of metabolism namely, reducing power (increased glycolytic flux being associated with elevated NAD^+/NADH, for example), ATP synthesis, biosynthesis and ion-flux. Perhaps most significantly, the studies described here, and elsewhere (13), suggest that critical, ATP-demanding cell processes can be regulated by oxygen before oxygen availability becomes limiting to aerobic function. Intrinsic to this is a capacity for the oxygen signal to over-ride or modulate other mechanisms involved in the regulation of the same cell process during the hypoxic transition.

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