Summary of Collaborative Studies

Intracellular Monitoring of Experimental Respiratory Failure[1-3]

COLLABORATIVE GROUP ON INTRACELLULAR MONITORING

SUMMARY The view that intracellular changes during oxygen depletion are the primary cause of abnormal function and altered physiology was originally proposed by Paul Bert (1). From that time it remains a basic assumption that hypoxia in intact animals produces alterations of cell and organ function, and that by measuring the intensity of these disturbances or the intensity of the functional impairment produced by these disturbances, a clearer understanding of the impact and consequences of oxygen depletion should emerge. At present, intracellular changes are inferred from the measurement of extracellular signals such as blood pressure, arterial oxygen tension and pH, or hemoglobin saturation, which provide mean values of changes occurring over the entire body (2). However, cells and organs in different parts of the body respond differently to a given degree of hypoxia or ischemia, and measurements of extracellular variables cannot provide precise information about abnormalities in any specific organ.

Extracellular variables also do not reflect adaptive responses of a specific organ such as autoregulation of its blood flow and the ability to alter energy demand in response to changes in energy production. Other factors include differences in metabolic rates and dependence upon oxidative and glycolytic reactions, cell heterogeneities within a tissue or organ, redistribution of blood flow to various organs during hypoxia, or other insults, and other, yet unknown, cell-specific changes that result in a range of survival capabilities among organs.

These considerations suggest the importance of direct monitoring of intracellular changes produced by cardiovascular or respiratory diseases. However, until recently, direct measurement was confined to the laboratory. In recent years, new technologies have developed that allow noninvasive measurements of intracellular events (3).

During the past three years, a collaborative effort was pursued that investigated intracellular monitoring using two experimental models of oxygen depletion, arterial hypoxemia, and blood loss hypotension. The perspective that emerged from these studies raised the possibility that these methods, several of which are currently being employed to study and monitor hypoxia in human neonates, may be applied generally to the evaluation of human patients with hypoxic or ischemic disorders.

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Introduction

These studies concentrated on two models of physiologic insults: (*1*) graded hypoxia produced by progressive decreases in the fraction of inspired oxygen (F_{IO_2}) and recovery, and (*2*) hypovolemic shock produced by progressive bleeding to reduce mean arterial blood pressure and then recovery by reinfusion of blood. Studies were carried out in dogs, cats, rats, and pigs as necessary to best suit the measurement techniques. Emphasis was placed upon intact animals because the impact of hypoxia and similar insults may be different in intact organisms than in isolated organ or cell preparations, where tissue and vascular compensatory activities may not apply. Acute rather than chronic changes were studied to emphasize the early warning of impending dysfunction and potential irreversibility. Most measurements were made in brain and heart since these usually determine survival in patients with respiratory failure, although some comparisons were made between the responses of heart and brain and the responses of liver and skeletal muscle.

The techniques* and intracellular variables discussed are summarized in table 1.

Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is an evolving technique that holds great potential for measuring (and imaging) metabolic activity by recording key intracellular variables such as ATP, creatine phosphate (PCr), inorganic phosphate (P_i), PCr/P_i, and intracellular pH (pH_i). Advantages of ^{31}P MRS include that it is noninvasive, measurements can be made repeatedly from the same organ, and the identity and concentrations of high energy phosphate compounds can be measured as a function of time. ATP is the major source of energy in mammalian cells; PCr is an important and readily accessible buffer for ATP, while ATP and PCr breakdown produces P_i. PCr breaks down into creatine plus phosphate, whereas ATP breaks down into adenosine diphosphate (ADP) and P_i. The phosphate potential (PP) is:

$$ATP/(ADP + P_i) \qquad (1)$$

Both these reactions are reversible, but it is the synthesis of ATP that drives the PCr reaction, as follows:

$$ATP + Cr = ADP + PCr + H^+. \quad (2)$$

By solving Eq. (1) for ADP and substituting the value in PP, we find that the thermodynamically determined index is equivalent to $PCr/(P_i)^2$ (4). Since ^{31}P MRS measures PCr and P_i, the energy state of cells and tissues proportional to $PCr/(P_i)^2$ is directly determined (4). The increase in the relative velocity of oxidative metabolism (V/V_m) and the effect of low oxygen concentrations are measured by decreases of PCr/P_i. In hypoxia, intracellular acidosis occurs and can be conveniently quantitated by MRS using any of three approaches: (*1*) the shift in the frequency of the P_i peak relative to the PCr peak as

* Three techniques that require invasion and/or radioisotopes that are not treated here include surface fluorescence, various intracellular and transcutaneous electrode techniques, and positron emission tomography, which affords high resolution images of glucose and oxygen metabolism.

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TABLE 1

SUMMARY OF THE INTRACELLULAR VARIABLES STUDIED DURING HYPOXIA

	Brain	Heart	Liver	Skeletal Muscle
Bioenergetic variables				
^{31}P magnetic resonance spectroscopy, MRS				
ATP	+*	+	+	0†
creatine phosphate, PCr	+	+	+	0
inorganic phosphate, P_i	+	+	+	0
PCr/P_i	+	+	+	0
intracellular pH, pH_i	+	+	+	0
^{1}H MRS				
intracellular lactate, $lactate_i$	+			0
Optical measurements of redox state of hemoglobin, cytochrome aa_3, and other mitochondrial electron chain components	+			+
Neural electrical activity				
evoked potentials	+	NA	NA	NA
EEG	+	NA	NA	NA

* + indicates that variable was studied.
† 0 indicates that variable was not studied.

$H_2PO_4^{2-}$ is converted to HPO_4^- (the principal components of the P_i peak); (*2*) measurement of lactate concentration via its methyl hydrogen directly by ^{1}H MRS; and (*3*) the PCr/P_i ratio, which decreases logarithmically with a decrease of pH.

Optical Techniques

The reduction/oxidation (redox) status of the mitochondrial reactants provides a sensitive index of the functional condition of oxidative metabolic processes because this status is determined by the availability of reducing equivalents from glycolysis, oxygen availability, oxygen consumption, and the rate of ATP utilization (5). Thus, the measurement of mitochondrial redox shifts offers a direct signal from many of the activities that comprise the intracellular energy conservation system. Spectroscopic monitoring of cytochromes became possible in the 1970s with reflectance techniques (6) and much research has been forthcoming in recent years to increase the penetration of light to allow for transcranial measurements in brain (7) or to increase wavelength resolution and monitor light absorption at multiple wavelengths (8). These technical developments have proven worthwhile since, as with MRS, optical monitoring can be accomplished "on line," with good temporal resolution and without interfering with cell physiology.

Transcranial optical measurements were made of the oxygen saturation of hemoglobin and the redox state of cytochrome aa_3. The transcranial measurement of hemoglobin saturation is a rough average of arterial venous and capillary levels. The redox state of cytochrome aa_3 provides information about this key intracellular oxygen receptor.

Evoked Potentials

Sensory-evoked potentials (EPs) reflect changing states of neuronal membrane potentials and the integrated activity of spiking neurons. Since neuronal function is dependent upon the metabolic maintenance of ionic imbalance across neuronal membranes, EPs were expected to be altered by metabolic disturbances and, so, indirectly indicate intracellular metabolic status. Questions of interest included the extent of metabolic disturbance required to alter EPs, whether multisensory EPs would indicate differential metabolic sensitivities of the several sensory systems, and if early and late components of the waveforms, which are elicited, by pathways with only a few or many synaptic contacts, respectively, are differentially sensitive to metabolic disturbance. EPs elicited by various sensory stimuli (light flashes, reversing checkerboard patterns, clicks, and electric shock to the tail) were recorded (9). EEG and auditory EPs were also recorded in some experiments in association with MRS.

Arterial Hypoxemia

Brain

In a previously normal but anesthetized animal, brain cytochrome aa_3 is approximately 25% reduced based upon its "total labile signal" (i.e., the difference between "maximal" oxidation produced by inspiration of 100% O_2 and full reduction produced by terminal inspiration of 100% N_2). When the brain is provoked to increase activity by direct cortical stimulation (neuronal activation), NAD and cytochrome aa_3 become transiently more oxidized (10). As $F_{I_{O_2}}$ is lowered from 21%, there is progressive desaturation of hemoglobin and reduction of cytochrome aa_3, beginning with even modest decreases of Pa_{O_2}. These changes are recordable, both in the visible and infrared regions of the spectrum. Neuronal activation, however, continues to provoke transient shifts toward oxidation of cytochrome aa_3.

Only when Pa_{O_2} is lowered to approximately 40 mm Hg do decreases in PCr/P_i become evident. This is accompanied by cytochrome reduction, and hemoglobin becomes highly desaturated. Perhaps not coincidentally, it is also at this Pa_{O_2} that neuronal activation is accompanied by transient shifts toward reduction (rather than oxidation) of cytochrome aa_3. As Pa_{O_2} is lowered further, cytochrome aa_3 approaches complete reduction, and no optical response to neuronal activation can be defined. However, PCr/P_i continues to decrease and this ratio approaches unity at Pa_{O_2} values between 18 and 25 mm Hg. At this level of arterial hypoxemia, pH_i decreases, but no further than 6.9. As Pa_{O_2} is decreased below 18 to 20 mm Hg, there are further decreases in PCr/P_i to 0.2 to 0.5 and pH_i and lactate values rise. ATP levels remain stable until PCr/P_i falls to 0.1, and at this point ATP values decrease. Decreases in ATP are often irreversible.

Evoked potential activity is largely unchanged until Pa_{O_2} decreases to approximately 30 mm Hg. As the Pa_{O_2} reaches approximately 25 mm Hg, there are significant decreases in amplitude and increases in latency of most components of the evoked potentials. The somatosensory EP (SEP) seems especially useful as a monitor of cerebral hypoxia. The later components of the SEP develop abnormalities when Pa_{O_2} is 28 to 32 mm Hg, with effects on increasingly early components as hypoxia is made more severe. Reduction or loss of the first negative wave indicates a state of metabolic crisis, whereas changes in later components provide graded and reversible indices of metabolic compromise. The EEG begins to change at the same point as the evoked potential. EEG changes include loss of high frequency, low amplitude activity, and often an increase in low frequency, high amplitude activity. The changes in EP and EEG occur coincident with, or just before, the changes in PCr/P_i.

After an initial, severe episode of hypoxemia lasting at least one hour, restoration of normoxia usually still produces recovery of baseline values of PCr/P_i, pH_i, hemoglobin saturation, cytochrome aa_3 redox status and evoked potentials. As expected, however, chances for recovery are dependent upon the degree and duration of the insult. While the same pattern of changes occurs during a second hypoxic insult, these abnormalities usually occur at higher Pa_{O_2} values, and restoration of normal Pa_{O_2} is less likely to be accompanied by restoration of the control state. A third exposure to severe hypoxia may result in death.

Comparisons among different organs provide early indications of the usefulness of the technical approaches to the study of intracellular activity. With MRS, for example, it is possible to demonstrate that the effects of arterial hypoxemia follow a different pattern in neonatal pig heart than in brain. Small decreases in PCr/P_i are observed in the heart at higher Pa_{O_2} values than in brain. These changes, which take place slowly over a 30 to 45 min period, appear to be early indicators of small, localized myocardial metabolic deficits. In adult dog heart, increased activity produces little change in ATP, PCr, or P_i

(and ADP), suggesting that metabolism is regulated by other factors such as calcium activation of critical dehydrogenases and a tightly controlled microcirculation. During hypoxia, oxygen delivery is regulated until there is an abrupt transition of cardiac performance with decreases in PCr/P_i that are heterogeneously distributed in heart according to oxygen delivery (11). In liver, rapid and extensive decreases in P_i, ATP, and pH_i occur also at relatively high Pa_{O_2} values, suggesting that liver is less buffered against hypoxia than brain or heart. With infrared optical spectroscopy, it is possible to monitor simultaneously the effects of hypoxemia or hypovolemia in brain and skeletal muscle. During both of these insults, cerebral blood vessels dilate while those in skeletal muscle constrict. Thus, redistribution of blood flow, with augmented brain-blood flow and reduced skeletal muscle blood flow, is found. Although redistribution of blood flow has been reported previously, the current technology is noninvasive and allows multiple, simultaneous measurement of both organs.

Hypovolemic Shock

Brain ischemia caused by hypovolemic shock produces changes that differ somewhat from those produced by arterial hypoxemia. Although cytochrome aa_3 becomes reduced as hypovolemia is induced and blood pressure declines, this effect is buffered at first by compensatory changes in blood flow that favor maintenance of brain perfusion. The fall in brain PCr/P_i is accompanied by a decrease in ATP, but severe decreases in blood pressure (to 40 mm Hg or less) are necessary to produce these changes in metabolites or evoked potentials. There is frequent failure to recover from this insult following reinfusion of blood. The metabolic changes noted during hypovolemic shock are presumably related to the heterogeneous response of brain cells to ischemia, in contrast to the hypoxemic insult described above. Other studies show that with ischemia, some portions of the brain maintain PCr/P_i greater than 0.1, while in others PCr/P_i falls below 0.1 and ATP levels decline. The latter brain areas are more likely to suffer irreversible damage as: (*1*) ADP may be degraded to hypoxanthine and lost for subsequent ATP synthesis; (*2*) reflow into these regions may cause free-radical or other damage, exacerbating that already caused by hypoxia; (*3*) neurotransmitter levels (exocitoxins) may rise, causing degeneration; and (*4*) loss of sodium and potassium ion homeostasis, water entry, osmotic swelling, and rupture of cell membranes may be more severe.

Clinical Applicability

From these studies, new and confirming experimental findings have been derived but, perhaps most importantly, key information concerning the potential clinical applicability of these techniques has been obtained. Of experimental significance is the demonstration that arterial hypoxemia is divisible into three levels of severity on the basis of these intracellular measurements.

In mild hypoxemia (Pa_{O_2} values above approximately 40 mm Hg), the concentrations of high energy intermediates are unchanged, consistent with earlier findings using freeze trapping and analytical measurements of brain extracts (12). It is well known that ATP stores in brain are maintained during arterial hypoxemia by microcirculatory regulation that preferentially increases brain blood flow to enhance its oxygen tension, by the continuance of oxidative phosphorylation, by utilization of PCr stores, by decreased ATP utilization, and by increased glycolysis. Since hemoglobin becomes highly deoxygenated (80 to 90%) and cytochrome aa_3 becomes markedly reduced (70 to 80%), there is no doubt that microcirculatory regulation is insufficient to maintain tissue oxygen tension. Since PCr/P_i is not decreased, and EP and EEG activity are unchanged, it appears that ATP utilization is still ongoing and that electron transport and oxidative phosphorylation are continuing despite the high level of mitochondrial reduction. This supports the concept that the critical tissue oxygen tension for oxidative phosphorylation *in vivo* is low, confirming *in vitro* findings (13).

Since psychomotor and other functional disturbances of brain are known to occur at these Pa_{O_2} values of mild hypoxemia, despite stability of bioenergetic values, definition of the mechanism of these disturbances should yield important information about brain regulatory activities. Possible explanations are that differences in metabolism among brain regions, or hypoxia-induced alterations in neurotransmission, may be involved (14). Whatever their basis, the psychomotor changes produced by mild hypoxia appear to reflect compensation for energy failure based upon decreasing energy demand. However, one cannot eliminate the possibility that global measurements of bioenergetic activities, such as those derived by MRS, may mask local heterogeneities that change certain physiological activities and, over prolonged periods, threaten tissue survival.

During moderate hypoxemia, the level of insult is characterized by the fall of PCr/P_i from the normal value of 3 to a critical value of 0.1, by transition from oxidation of NAD or cytochrome aa_3 to reduction in response to neuronal activation and by slowing and suppression of EP and EEG activity. In severe hypoxemia, ATP falls and the capability for recovery of brain metabolic and electrophysiological activities upon restoration of normoxia is threatened.

Very severe hypoxia is necessary to preclude a rapid recovery of metabolic status upon restoration of normoxia. For example, PCr/P_i values of 1 are tolerated for at least one, and usually several, hours but values below this level appear to induce metabolic overloads in recovery that caused progressively poorer performance in response to successive episodes of hypoxemia. When brain PCr/P_i falls below 0.5, ATP is decreased and rapid initiation of aggressive therapeutic procedures are required to avoid irreversible injury.

Also of interest are findings of increased vulnerability of PCr/P_i, ATP, and evoked potentials with repeated episodes of hypoxemia. This residual effect of hypoxemia may be explained by: (*1*) progressive membrane injury that becomes irreversible (15); (*2*) progressive depletion of a small high-energy pool not resolvable by present techniques; (*3*) the possibility that metabolic recovery, as expressed by PCr/P_i or mitochondrial redox status, is not indicative of full restoration of ion transport or neurotransmitter activities, so that the metabolic load may be higher in successive hypoxemia insults; or (*4*) there is failure of acute adaptive mechanisms leading to irreversible brain injury. Of clinical significance is the possibility that a "hysteresis" in response and recovery from successive hypoxemic insults indicate that an initial finding of low PCr/P_i values demonstrates advanced injury. This idea is derived from present results and also from studies in skeletal muscle in which increased P_i or decreased PCr/P_i occur in response to muscle overload (relaxation during work). The latter changes persist for the interval of recovery (16). Similarly depressed PCr/P_i values are observed in muscular dystrophies. Recovery from therapeutic interventions of vascular surgery also causes lowered PCr/P_i.

The existence of hysteresis suggests the use of intracellular monitoring to provide new approaches to the solution of common clinical problems.

For example, a common therapeutic dilemma is the problem of pulmonary oxygen toxicity, on one hand, and, on the other, the problem of normalizing tissue oxygen supply by supplying enriched oxygen mixtures. Currently, this dilemma is met by selecting an oxygen mixture that results in the lowest acceptable Pa_{O_2}. The Pa_{O_2} value of 50 mm Hg is a common choice based on the shape of the oxygen hemoglobin dissociation curve.

The results of intracellular monitoring suggest that two patients with a Pa_{O_2} value of 50 mm Hg might show different values for various intracellular parameters, depending on the previous history of oxygen supply in the patient (severity, duration, etc.). One patient might show a brain PCr value of 3 (or a normal somatosensory EP or a transient oxidation of the redox state of cytochrome aa_3 during cortical activation) and it could be concluded that a Pa_{O_2} of 50 mm Hg is an acceptable compromise. The other patient with the same Pa_{O_2} might show a brain PCr/P_i value of less than 1 (or an abnormal somatosensory EP or a reduction of the redox state of cytochrome aa_3 after cortical activation), and it could be concluded that an increase in oxygen supply above that provided by a Pa_{O_2} value of 50 mm Hg is required.

Finally, it would be necessary to demonstrate by appropriate clinical trials that improving the therapeutic decision-making process by using intracellular monitoring actually resulted in a statistical improvement in

the outcome of patients maintained at a Pao_2 of 50 mm Hg.

It appears that each degree of hypoxia is associated with intracellular events that can be monitored by noninvasive technologies. Optical and MRS techniques are complementary and provide continuous monitoring of the intracellular metabolic effects of changes in brain oxygenation from normoxia to the severest hypoxia.

With mild hypoxia, there is reduction of cytochrome aa_3 and deoxygenation of hemoglobin even without changes in PCr/P_i. The cytochrome and hemoglobin changes, while not coincident, offer good signals when tissue oxygenation is changing. Under clinical circumstances, the observation of increases in reduction of cytochrome aa_3, and desaturation of hemoglobin, could offer an "early warning" that oxygen delivery is declining. Recent work indicates that near infrared absorption spectroscopy of capillary bed hemoglobin can be used transcranially in neonates and perhaps in adults as well (17).

A limitation to clinical application of such optical measurements, however, is that calibration requires relationships to the extremes of oxygenation and deoxygenation. Such calibration to extreme hypoxia (or anoxia) in patients is not feasible. Unfortunately, without such calibration, measurements of cytochrome aa_3 redox status or of oxyhemoglobin can indicate trends but only when changes in oxygenation are occurring. There is no feasible way, as yet, to examine an unchanging optical signal in a steady state and determine the metabolic conditions of an organ (18).

In moderate hypoxia, PCr/P_i falls to levels such that MRS could be useful in the determination of whether a critical point, such as the transition to severe hypoxia, has been reached. The advantage of MRS measurements at these levels of hypoxic insult is that they are not limited to trends, but indicate actual tissue concentrations of critical cell metabolites. MRS measurements, in particular, can sensitively define whether a critical point is reached as a hypoxic insult becomes severe. Similar statements can be made for the evaluation of EEG and EP information when the hypoxic insult has reached a threshold level. The turnover (from oxidation to reduction) of cytochrome aa_3 after neuronal activation indicates that such measurements can also define the point at which the severity of hypoxia has reached a critical stage for such responses.

While the usefulness of these technologies in monitoring the metabolic status of the brain is evident, further work is needed to evaluate their usefulness in other organs. In addition, consideration must be given to clinical application of these technologies. It is unlikely, for example, that MRS monitoring will be available in the acute phase of a clinical situation. Since optical monitoring is rapid, easily accomplished, and less expensive, it is conceivable that this technology will be useful during surgical, acute and chronic care situations for assurance that there is no ongoing trend toward decreased oxygenation. While MRS measurements would provide a useful means to calibrate the optical signals, the greatest advantage that MRS and physiological monitoring (such as EP) have may be as key signals for diagnosis and quantification, and for evaluation of prognosis during or after severe insults. Certainly, such monitoring provides no cure in itself and, at least in the context of normal patient care, does not provide insights into physiological or biochemical mechanisms, although these technologies have already been shown to be very useful in experimental studies to define such mechanisms.

Intracellular monitoring, using the technologies described in this summary, provide exciting possibilities for the quantitation of mild, severe, and very severe hypoxia and ischemia in the brain. Examples of the consequences of these degrees of ischemia have been observed in studies of hypoxically stressed human neonates (17, 19). The question is not one of whether or not the observations can be made in neonates at the present time and, with frontier technologies, in adults. The question is whether or not these technologies will lead to therapeutic interventions that will result in an improved outcome for the patient. Therefore, controlled clinical trials, in a prospective randomized manner, seem of great potential importance in order to confirm these observations in an adult population and, at the same time, to evaluate therapeutic interventions.

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