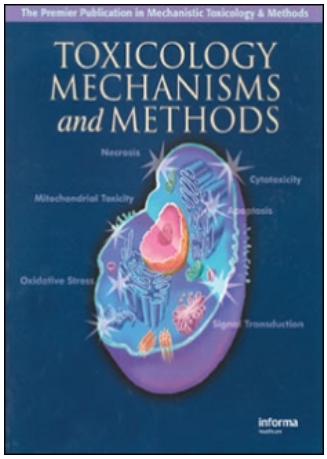


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# Metabolic Markers of Hypoxia: Systems Biology Application in Biomedicine

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# Metabolic Markers of Hypoxia: Systems Biology Application in Biomedicine

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**ABSTRACT** The overall goal of this review is to provide insight into methodologies for 'omic investigations and hypoxic biomarkers that have been identified using 'omic techniques. First, a detailed description of current metabolomic, proteomic, and genomic technologies is provided, followed by a basic introduction to biostatistics and how to interpret 'omic data. Metabolomic biomarkers of diseases in which hypoxia plays a role are then reviewed by those that involved *chronic* (pulmonary disease, cardiovascular disease, cancer) and *acute* (stroke, myocardial infarction, ischemia) hypoxia. Data are presented with consideration for the source of hypoxia, the severity of hypoxia, the length of hypoxia, and the cell or organ affected, all of which can have significant effects on biomarker profiles. Drugs that promote and antagonize hypoxia are discussed and important points to consider during tissue collection in hypoxia 'omic studies are then reviewed.

**KEYWORDS** Metabolomics; Proteomics; NMR Spectroscopy; Mass Spectrometry; Biomarkers; Chronic and Acute Hypoxia; Drug Toxicity

## INTRODUCTION

All eukaryotic cells rely on a supply of oxygen in order to be able to perform oxidative phosphorylation to generate high-energy phosphates (mostly adenosine triphosphate [ATP]). The energy-rich molecules are then utilized to maintain normal cellular functions such as proliferation, membrane transport, and biochemical reactions. A variety of pathophysiological conditions, such as cardiovascular disease, stroke, and cancer, lead to a significant (acute or chronic) decrease in the oxygen supply to the cell (Table 1). Furthermore, hypoxia, which can be caused, for example, by transplantation, bypass surgery, or exposure to high altitude, induces significant metabolic changes due to the decrease/lack of oxygen supply. Finally, a significant number of xenobiotics increases reactive oxygen species (ROS) formation in major metabolizing organs (such as liver, gut, and kidney), which will also lead to induction of chronic hypoxia by inhibition of mitochondrial enzymes. In general, metabolic and molecular mechanisms of acute hypoxia differ from those of chronic hypoxia. As a result, significant changes in metabolic response and adaptation can be observed. Besides the well-known depletion of ATP stores, a large variety of putative metabolic markers have been reported for hypoxia-induced conditions. Novel markers/metabolic pathways are currently under investigation, often using modern analytical technologies for global metabolic profiling in the cell/organ of interest or a surrogate biofluid.

Metabolomics, one of the 'omic sciences in systems biology, is a comprehensive and quantitative analysis of all metabolites in a biological system, or alternatively, the global analysis of small molecule metabolites and metabolic patterns (Fiehn 2001; Nicholson et al. 1999) (Fig. 1). Metabolism is a dynamic process that is influenced by several environmental (e.g., food, exercise, stress), exogenous (e.g., drug exposure), and endogenous (e.g., inborn metabolic defects) factors. Frequently, the term metabolic phenotype is used to describe the

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**TABLE 1** **Major pathophysiological conditions in humans associated with the exposure to acute and chronic hypoxia**

| Acute hypoxia | Chronic hypoxia |
|---|---|
| Hypoxia-induced pulmonary hypertension | Cardiovascular disease including congenital heart disease |
| Transient tumor hypoxia/reoxygenation | Pulmonary hypertension |
| Stroke | Cancer |
| Myocardial infarction | Fetal and newborn hypoxia |
| Surgery-induced ischemia/reperfusion including organ transplant and bypass procedures | Environmental hypoxia including chronic mountain sickness and hibernation |
| Environmental hypoxia including acute mountain sickness and diving | Treatment-induced oxidative stress including xenobiotics and radiation |
| Experimentally induced hypoxic artifacts* | |

*This condition usually occurs during tissue sampling procedure and represents a significant artifact in assessment of metabolic changes of disease of interest.

metabolic response in the cells to drug toxicity, organ injury, or cancer. The metabolic phenotype, also sometimes called metabotype, may help in gaining important insights into genomic and proteomic changes, either upstream or downstream (Serkova and Niemann 2006; Nicholson and Wilson 2003; Griffin 2004). An example of a "downstream" metabolic response is when lactate accumulates in cancer cells as a result of Akt-pathway activation (Elstrom et al. 2004). Conversely, metabolic changes can be the first or upstream step even before any genomic or proteomic changes have taken place. For example, ATP depletion during the early phase of organ ischemia, followed by up-regulation of glycolytic genes and hypoxia-inducible factor (HIF) expression, is an excellent metabolic "upstream" example (Niemann et al. 2005). Therefore, metabolic responses can represent either the first or the last step in the response cascade (see also Fig. 2). Therefore, acute ischemia (upstream) and chronic hypoxia (downstream) represent an ideal model for metabolic investigations.

A recent review (Storey 2006b) explores the use of 'omics technologies in comparative biochemistry and physiology studies, including hypoxia gene and protein expression. Similarly, Thongboonkerd et al. (2005) reviewed the use of systems biology techniques in hypertension research, including 'omics data on the pathophysiology of hypoxia-induced and renovascular hypertension. In an excellent review of protein expression and posttranslational modification, Kumar and Klein (2004) explored both targeted and discovery-based proteomics methods of examining hypoxia-induced changes. In the present article,

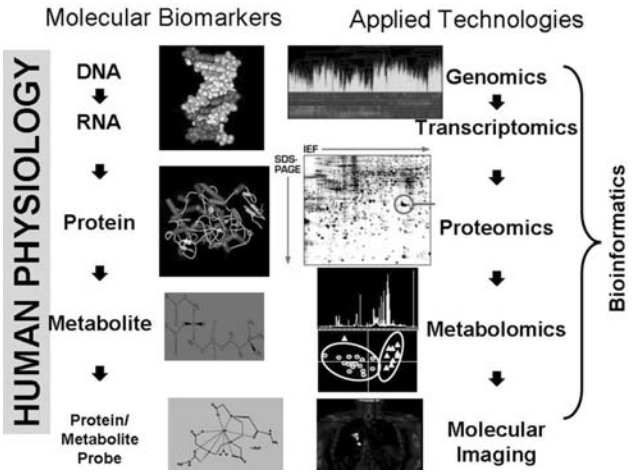


**FIGURE 1** **System biology and "omics" approach in biomedical translational research. Genomics is the study of genomes and the complete collection of genes that they contain. The first microarray analysis was described in the mid-1990s, followed by functional genomics (also known as transcriptomics). Proteomics (starting in 1997) examines the collection of proteins to determine their dynamic changes, localization, and biological meaning. Proteomics uses two-dimensional gel electrophoresis, mass spectrometry, and protein microarrays. Metabolomics (or metabonomics, the term was first introduced in 1999 by a group from the Imperial College) deals with global metabolic profiling and its dynamic changes (metabolic fluxes) by monitoring as many as possible endogenous low-weight biochemicals (metabolites) in a single analytical assay. Techniques applied to metabolic profiling include nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) and, to a lesser extent, near-infrared spectroscopy (NIR). Bioinformatics remains a key element in data management and analysis of collected data sets arising from genomics, transcriptomics, proteomics, and metabolomics. Bioinformatics uses techniques developed in the fields of computer science and statistics. Identified proteins and metabolites can be assessed by tracer-based molecular imaging using magnetic resonance imaging/spectroscopical imaging (MRI/MRSI) and positron emission tomography (PET).**

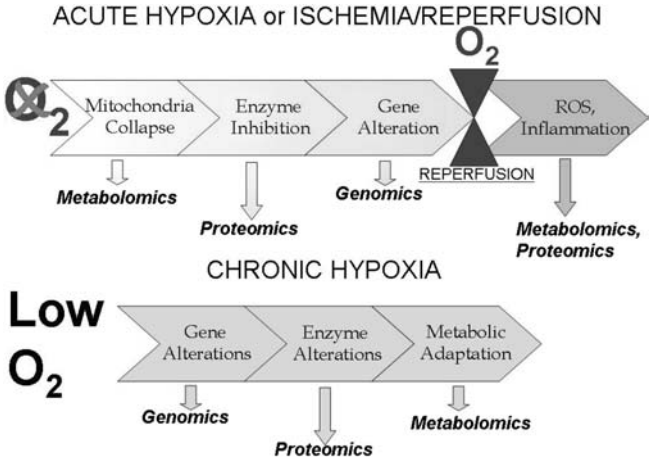


**FIGURE 2** **Pathophysiological cascade for acute hypoxia/ ischemia followed by reperfusion vs. chronic hypoxia.**

we will describe, in detail, metabolomics principles with brief reference to proteomics and genomics and their application for investigation of biochemical changes in hypoxic conditions.

## PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF HYPOXIA

Hypoxia is a complicated phenomenon, and its physiological and biochemical aspects are not easily defined. A pathophysiological cascade for acute hypoxia/ischemia followed by reperfusion is compared to chronic hypoxia in Figure 2. In the absence of oxygen, cells undergo mitochondrial dysfunction and eventual collapse followed by the inhibition of enzymes and gene alteration. Upon reperfusion with oxygen, there is an increase in reactive oxygen species and a general inflammatory response. Conversely, in a chronic hypoxic state, mitochondrial collapse and reperfusion injury are absent, but rather a new metabolic phenotype is established as an adaptive process to chronic low oxygen.

The response to hypoxia can vary widely depending on the source of hypoxia, the amount of oxygen available, the specific cell or organ studied, and the amount of time hypoxia is experienced. The source of hypoxia or low oxygen availability can result from the low oxygen tension ($pO_2$) (asthma), low oxygen content (anemia), and reduced or appended blood flow (stroke). The oxygen tension at which cellular responses to hypoxia are activated can be highly variable and likely based on what the cell/organ perceives as normoxia. Pulmonary endothelial cells show signs of hypoxic stress at 3% oxygen (Irwin et al. 2005), while placental trophoblast cells are hypoxic at <1% but not 5% oxygen (Hung et al. 2001). The specific function of the cell/organ will affect the response to hypoxia. A highly metabolic cell, such as a hepatocyte, would likely have a different metabolomic profile in response to a low oxygen environment in comparison to resting skeletal muscle. Currently, there is no consensus on the length of time defining an acute vs. chronic hypoxic exposure. Hypoxia can also be intermittent, such as that used to precondition hearts for a subsequent hypoxic event. An example of how the elements of hypoxia must be considered when interpreting 'omic results is illustrated by Farber and Barnett (1991) in a report indicating that cyclooxygenase metabolism in endothelial cells is dependent on the duration of hypoxia and the oxygen tension to which cells were exposed. Metabolomic, proteomic, and genomic methodologies applied to experiments in which hypoxia source, oxygen tension, cell/organ type, and duration of hypoxia are compared and contrasted could lead to a far greater understanding of physiologic and pathophysiologic responses to hypoxia.

## INSIGHTS AND CHALLENGES FOR SYSTEMS BIOLOGY TECHNOLOGIES

As shown in Figure 2, 'omics technologies can be used to elucidate individual components of the hypoxia cascades. In addition, these technologies can be used to discover novel information about hypoxia-induced pathways, including biomarker discovery studies. The National Institutes of Health (NIH) defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathologic processes or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). The Food and Drug Administration (FDA) definition for a known valid biomarker is "a biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is widespread agreement in the medical and scientific community about the physiologic, toxicologic, pharmacologic or clinical significance of the results" (http://www.fda.gov/cder/guidance/6400fnl.pdf). Biomarkers, including several classes of biomolecules such as genes, proteins, lipids, and other small molecules, can be used in several areas of clinical and nonclinical research and patient care including disease prediction, detection and diagnosis, staging, development of therapeutic targets, drug monitoring, and assessing drug efficacy. The importance of genes, proteins, and small molecules as biomarkers in medicine has been illustrated through applications in metabolic disorder screening in newborns, drug toxicity and efficacy, organ transplant, and monitoring disease and nutritional status (Serkova and Niemann 2006; Griffin and Shockcor 2004; Wishart 2005). While the usefulness of biomolecules in medicine cannot be disputed, the choice of methods used to discover new biomarkers is subject to intense discussion and debate. With the recent emergence of powerful technologies has come the potential for large-scale, widely applicable, inexpensive methods for discovery. The integration of these methods in the context of human physiology, technology, and biostatistics is shown in Figure 1. While significant advances toward a successful, easily adoptable paradigm has been made in the fields of genomics and nuclear magnetic resonance (NMR)-based metabolomics, comparatively few advances have been made in the area of liquid chromatography mass spectrometry (LC/MS)-based metabolomics and proteomics. In spite of these drawbacks, a number of biomarkers have been discovered for the hypoxic condition in several diseases. Specific examples will be explored in the areas of cardiovascular disease (CVD), pulmonary disease, cancer, and environmental stress.

### NMR-Based Metabolomics

The NMR-based metabolomics approach is one of the two most widely used techniques for metabolic profiling. The NMR methodology includes (1) high-resolution multinuclear NMR spectroscopy (mostly $^{1}H$ NMR and to a lesser extent $^{31}P$ NMR on high-energy/phospholipid metabolism and $^{13}C$ NMR for glucose fluxes) on cell extracts, biopsy extracts, fine-needle aspirates, and body fluids; (2) high-resolution magic angle spinning (HR-MAS) $^{1}H$ NMR on intact biopsies; and (3) magnetic resonance spectroscopy and magnetic resonance spectroscopic imaging (MRS and MRSI) in animals and humans to validate distinguished metabolic markers in vivo (see Fig. 1 for molecular imaging application). This translational component of the magnetic resonance technique is one of the most useful and unique among existing biomedical technologies.

The ability of proton $^{1}H$ NMR to resolve all small-molecule metabolites at a concentration of about 10 $\mu$M and above (based on the chemistry of their CH-, $CH_2$-, etc., chemical groups) in one conventional NMR experiment (with minimal sample preparation) is a unique advantage of NMR technology in the field of metabolic profiling (Serkova and Niemann 2006; Griffin and Shockcor 2004; Pelczer 2005). This concentration limit can be well bellow the 10 $\mu$M if a combination of cryogenic probe

and high field is used. The average analytical time for a high-resolution $^{1}$H NMR experiment is about 10 min for an NMR spectrometer range of 400 to 600 MHz (field strength 9.4 to 14.0 Tesla, respectively). However, an acquisition time longer than 10 min can always be utilized to further push the concentration limits. Body fluids, mostly urine and blood, as well as expressed prostatic secretions (EPS), bronchoalveolar lavage fluids (BALF), and cerebrospinal fluids (CSF) for specific diseases, can be directly analyzed by high-resolution $^{1}$H NMR. Most biofluids contain a "heterogenous" mixture of endogenous metabolites in the same molecular weight range, which require minimal sample preparation. The only requirement is the addition of deuterated solvents such as deuterium oxide for holding the lock source and phosphate buffers for pH adjustments. Sophisticated NMR experiments with novel pulse sequences allow for an excellent solvent suppression without the need of sample lyophilization (Serkova and Glunde 2007). Intact tissue specimens (e.g., biopsies, fine-needle aspirates) need to be extracted (usually using an acid or dual-phase extraction protocol) for conventional NMR analysis or are increasingly analyzed nondestructively by high-resolution magic angle spinning (HR-MAS) and developing slow MAS high-resolution $^{1}$H NMR (Serkova and Glunde 2007). The one-dimensional NMR technique is a quantitative approach (usually requiring addition of external concentration standards to the sample) and, in addition to the routinely used $^{1}$H NMR, phosphorous $^{31}$P, and carbon $^{13}$C NMR, can be applied for metabolomics. While $^{1}$H NMR provides quantitative information on, but not limited to, amino acids, carbohydrates, fatty acids, lipids, and phospholipids, $^{31}$P NMR yields quantitative information on high-energy phosphates (ATP, phosphocreatine), phospholipid precursors, and sugar phosphates. On the other hand, $^{13}$C NMR allows for assessment of metabolic fluxes of $^{13}$C-labeled precursors (e.g., $^{13}$C-labeled glucose fluxes through glycolysis), the tricarbocylic acid (TCA) cycle, the pentose-phosphate cycle, and de novo fatty acid synthesis. Finally, two-dimensional NMR approaches allow for precise metabolite identification with the aid of NMR metabolome databases (one of the best examples is the Human Metabolome Database from the University of Alberta, [Wishart et al. 2007]: http://www.hmdb.ca).

## MS-Based Metabolomics

Mass spectrometry (especially LC-MS and Fourier-transform [FT] ion cyclotron resonance FT-MS) is the second major spectroscopic technique applied for metabolic profiling analysis. The basic workflow follows that of NMR-based studies: quenching/extraction of metabolites → data collection → data processing/analysis. A major advantage of LC-based mass spectrometry is the high sensitivity (typically in pg/mL concentrations), which allows for more compounds to be screened. However, MS sensitivity depends on such features as metabolite pK, hydrophobicity, and ionization potential (Pan et al. 2005; Want et al. 2007). While polar molecules may be detected when electrospray ionization (ESI) is used, nonpolar molecules may require atmospheric pressure chemical ionization (APCI). Similarly, the methods of extraction, quenching, and sample storage conditions can affect and potentially modify metabolite structure, thereby confounding already complex data sets. In spite of a rich history of discovery- and targeted-based methods in small molecules using MS, a widely adopted and validated methodology for sensitive, high-throughput discovery-based LC/MS metabolomics is lacking. Although high-resolution profiling methods exist for gas chromatography mass spectrometry profiling (GCMS), detectable compounds are limited to those that can be derivatized, and derivatization can be time consuming and costly, and there is a risk of metabolite loss. Conversely, LC/MS has only recently begun to be applied to metabolic profiling due to recent major advances in chromatography, instrumentation, ionization capabilities, and software. To date, LC/MS-based metabolic profiling experiments have confirmed that data sets can be compared and that differences can be detected in human samples (Want et al. 2006); these methodologies are currently undergoing validation (Gika et al. 2007).

While high-throughput discovery methods are still currently in development, it should be noted that multiple methods exist for targeted analysis of small molecules, including fatty acids, amino acids, organic acids, steroids, etc. Arguments in favor of a more targeted approach to metabolic profiling using LC/MS include the fact that a single extraction method will presumably be biased toward certain metabolites and therefore result in an overall lower number of features when multiple extractions are used. In addition, specific technologies incorporate a bias toward certain classes of molecules, due to factors such as the aforementioned ionization, chromatography, and the type of mass spectrometer used. Finally, when extraction and analysis methods are tailored to specific metabolite chemistries, percent recovery will be increased and these simplified samples will have lower ion suppression and therefore a more thorough coverage of metabolites in the mixture.

## Proteomics Techniques

While metabolites can be classified into many different species, each of which may require different methodologies for analysis using mass spectrometry–based metabolic profiling, in proteomics a single class of molecule is analyzed (i.e., chains of amino acids). Proteomics profiling studies generally focus on one of two basic workflows: (1) two-dimensional gel electrophoresis (2DE) to resolve and relatively quantitate proteins followed by mass spectrometry, or (2) quantitative mass spectrometry approaches including metabolic and postmetabolic labeling and nonlabeling strategies (Bowler et al. 2006). In one type of postmetabolic labeling experiment, peptides from two samples are labeled with either a heavy (e.g., deuterium) or light (e.g., hydrogen) tag. The mass difference between these two peptides can be detected, and the relative peptide amount measured, using mass spectrometry while proteins are simultaneously identified. Similarly, in a typical metabolic labeling experiment cells are cultured in media containing variants of an amino acid, for example $^{13}$C/$^{15}$N-labeled arginine, which is incorporated into the entire cell proteome. Up to three different masses of arginine [Arg0 ($^{12}C_6{}^{14}N_4$), Arg6 ($^{13}C_6{}^{14}N_4$), and Arg10 ($^{13}C_6{}^{15}N_4$)] can be used, and therefore three states can be compared. While many variations of these workflows exist, including the incorporation of front-end or back-end separation/fractionation of peptides and proteins, proteins are ultimately identified through mass analysis of the peptides and a subsequent database search. The advantages of such techniques include the ability to analyze large numbers of proteins without relying on gel-based methods, which are generally known to underrepresent certain protein classes, such as proteins present in low abundance and membrane proteins. In addition, high-salt

and low-molecular-weight proteins, which are found in biofluids such as urine, may also make 2DE an undesirable method. However, in a review by Agarwal et al. (1995), a comparison between 2DE and a commercially available labeling method showed consistent results between the methods.

Although various techniques have been applied to a large number of clinical studies, to date no methods have been developed that successfully analyze an entire proteome, such as the human plasma or urine proteome, and no biomarkers for hypoxia have yet been validated for clinical assays. In fact, data from some large-scale proteomics studies could be interpreted as emphasizing the dynamic nature of human biofluid proteomes (Ommen 2005; Adachi et al. 2006), perhaps suggesting the need for more longitudinal studies. In proteomics, one major problem identified with such studies is an overall lack of sensitivity of the currently accepted assays. In contrast, targeted analyses, such as those relying on antibodies, are very sensitive but are difficult to develop into high-throughput screens when the antibodies are not available and the specificity of the assay may sometimes not be adequately tested (Anderson 2005). In addition, it has been noted that assays relying on single biomarkers may not be as effective as those that include panels of proteins or metabolites. Not only are panels potentially less prone to noise than single protein biomarkers, but they could also lessen the effect of normal variations between individuals.

One potential solution is the application of a mass spectrometry technique more commonly used in small-molecule analysis, termed multiple reaction monitoring (MRM). This directed approach consists of the quantitation of peptides, and therefore presumably proteins, using mass spectrometry and $^{15}N$- and $^{13}C$-stable isotope-labeled synthetic peptide as internal standards. MRM is performed on both the labeled and the endogenous peptides and the area under peaks of both are measured, providing quantitative information. This method is more precise than antibody-based assays, which are often not able to distinguish between protein isoforms. Previous studies using this method include Barr et al. (1996), who quantified apolipoprotein $A_1$ with CV <4%; Gygi et al. (1999), who applied the method to larger-scale proteomics studies; and Kuhn et al. (2004), who analyzed C-reactive protein in patients with rheumatoid arthritis. For a more complete review of the MRM technique, please refer to Wright et al. (2005).

Antibody arrays could also conceivably be used for such high-throughput screens; in general, immunoassays are highly sensitive and specific (Hultschig et al. 2006). However, the arrays are limited by the availability, quality, and quantity of antibodies. The cost of developing antibody-based assays for large numbers of proteins, especially when it is unknown if the proteins are potential biomarkers, is currently prohibitive in terms of time and money.

## Application of Genomics

Although seemingly static in comparison to the more obviously dynamic human metabolome and proteome, the genome is in fact proving to be more than just a fixed blueprint for cellular and organismal development, structure, and function. A variety of mechanisms act in concert to direct tissue- and response-specific gene expression including promoter methylation, histone modifications, microRNAs, alternate mRNA splicing, single nucleotide polymorphisms (SNPs), and modulation of transcription factor activity (Kirby et al. 2007). Technologies continue to improve that allow investigation of the genome at increasing levels of sophistication. In addition to expression analyses, bead-based and microarray platforms allow global and targeted analyses of DNA methylation patterns, microRNA activity, splice variants, SNPs, location analyses (to determine transcription factor binding activity), and high-resolution detection of chromosomal aberrations. Several studies have examined gene expression changes in response to hypoxia as outlined extensively by Storey (2006b). More recently, Kulshreshtha et al. (2007) showed that the expression of several microRNAs were up-regulated in response to hypoxia. Moreover, these microRNAs exerted effects on expression of genes from various functional classes including cell cycle regulation and apoptotic signaling. Using these techniques, in addition to examining traditionally hypoxic genes, several genes have been discovered that had not been previously implicated in the hypoxic pathways. Studies of this type illustrate a major strength of genomic methods (i.e., the capability of examining several related, or even nonrelated, molecules simultaneously across several different conditions). Biochemical pathways can thus be readily elucidated and key components identified. In spite of a predicted interplay between genes, proteins, and small molecules, significant differences between 'omics data sets have been noted. While this may be due in part to actual differences in mRNA and protein expression, turnover, modifications, sampling/experimental error, and functional status may also play roles. In addition, software packages have continued to evolve behind instrumentation, and no commonly accepted platform yet exists to combine data sets from multiple 'omics technologies. As these powerful technologies continue to mature, issues such as these will no doubt become satisfactorily resolved.

## Basic Principles of Biostatistics

In the last 5 years, the majority of metabolic profiling studies have been computer-aided statistical interpretation of spectroscopic data. Given the amount and complexity of spectroscopic data from NMR and MS studies, data reduction was usually undertaken followed by a computer-based pattern-recognition approach in order to classify a spectrum as "normal" or "abnormal." Pattern recognition techniques (mostly within SIMCA-P program, UMETRICS AB, Umea, Sweden) view each database as a whole, and the dimensionality of the spectral data can be reduced by principal component analysis (PCA), as well as some other statistical approaches (cluster analysis, linear discriminant analysis, Bayesian spectral decomposition (BSD), and several other chemometric methods) (Holmes and Antti 2002).

Principal component analysis has been used in the majority of metabolic profiling studies (Serkova and Niemann 2006). A principal component (latent variable) consists of two parts: score [$t_i$] and plot [$p_i$]. In the first step, PCA scores will cluster the samples based on their spectral information or total metabolic profile (Fig. 3). Therefore, scores [t] describe the variation in ***the sample*** direction (i.e., similarity/dissimilarity between samples), allowing for pattern recognition (normal/abnormal group assignment). Interpretation of scores gives information about relationships between samples (trends, groupings, outliers), but the metabolic nature of this group clustering remains unclear.

The next step is identification of markers in the biological sample (Fig. 3). The PCA loading plot will explain patterns

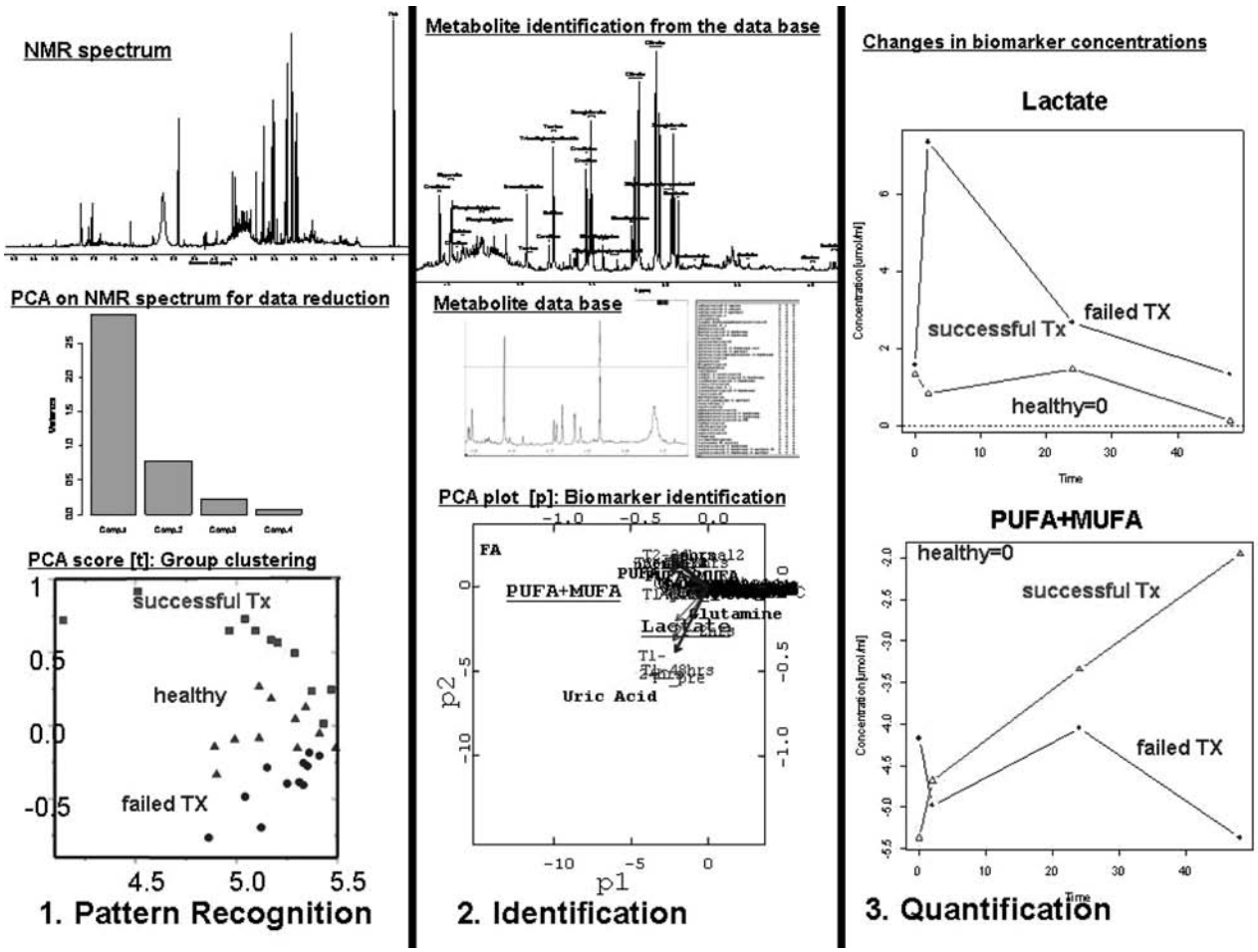


**FIGURE 3** **Three steps of NMR-based metabolomics analysis (as an example in the area of human transplantation, Tx) using principal component analysis (PCA): pattern recognition ("black box" using PCA scores) → metabolite identification ("biomarker" using PCA plot) → metabolite quantification ("validation"). Partly adapted from Serkova and Niemann (2006).**

in previous scores, since loadings [p] describe the variation in ***the variable*** direction (i.e., similarity/dissimilarity between variables). This step explains the variation in score and, therefore, identifies the spectral area (spectral analysis) or metabolites (quantitative analysis) responsible for the group clustering (Fig. 3).

Finally, the third and last step is the quantification and validation of the distinguished metabolic markers between distinguished study groups (Fig. 3). The statistical approach of this step can be a standard Student t-test or ANOVA, depending on the group number and size.

## METABOLIC MARKERS IN CHRONIC HYPOXIA

While potentially powerful for discovery-based studies, data from existing genomic, proteomic, and metabolomic hypoxia studies may provide sufficient information for new studies to perform *targeted* rather than comprehensive 'omic studies, with the goal of determining hypoxia-induced molecular responses important for the disease or condition in question. Table 2 provides a list of some identified biomarkers of hypoxia with consideration of the source of hypoxia, oxygen tension, cell/organ type, and length of hypoxia. In addition, several examples of such targeted studies are detailed below.

## Cardiovascular Disease

Stress proteins associated with hypoxia in endothelial cells are reviewed by Graven and Farber (1998). Using an $^{1}$H NMR platform, a sophisticated pattern recognition technique was able to distinguish patients with coronary artery disease from controls (Brindle et al. 2002). Application of supervised partial least squares-discriminate analysis allowed 90% specificity in distinguishing diseased patients from the control cohort. Overall differences were mainly attributed to the differences in lipoprotein particle composition between samples. However, since nonextracted blood samples were used in this study, lipoprotein produced the dominant signals compared to other low-concentrated (water-soluble) metabolites. As such, because of these technical limitations, this fast nontargeted approach was unlikely to achieve clinical acceptance in the current form and further clinical trails failed (Brindle et al. 2002).

In a recent review, White and Van Eyk (2007) discussed proteomics in cardiovascular disease, and protein markers discovered in dilated cardiomyopathy (DCM), atherosclerosis, and ischemia/reperfusion (I/R) injury. For example, the link

**TABLE 2** General metabolic biomarkers for acute and chronic hypoxia

| | Biomarker name(s) | Source of hypoxia | Oxygen tension | Cell/organ type | Time |
|---|---|---|---|---|---|
| Acute hypoxia in endothelial cells (Graven et al. 1998) | ↑ GAPDH<br>↑ NNE | ↓ $PO_2$ | $PO_2 = 40$ mmHg | Bovine pulmonary artery endothelial cells | 18 h |
| Congenital heart disease vs. sleep apnea of prematurity (Douglas et al. 2007) | ↓ NAC /creatine | ↓ $PO_2$ | 11% | CNS neurons | 4 wk, constant vs. intermittent (4-min cycles) |
| Coronary heart disease (Brindle et al. 2002) | ↑ Lipoproteins | ↓ $PO_2$ | Not reported | Plasma | Chronic |
| Myocardial infarction (Sabatine et al. 2005) | ↑ Lactate<br>↑ AMP catabolism<br>↓ TCA cycle | ↓ Blood flow | Not reported | Plasma | Acute ischemia induced by stress test |
| Melanoma cell line response to hypoxia (Stockwin et al. 2006) | ↑ CD13<br>↑ CA IX<br>↑ pt-ATPase<br>↑ MMP-9<br>↑ SDF-1 | ↓ $PO_2$ | 1% | Murine B16F10 melanoma cells | 24 h |
| Hypoxia-induced pulmonary vasoconstriction vs. femoral vasodilation (Leach et al. 1998) | ↓ PCr/ATP | ↓ $PO_2$ | Not reported | Plasma | 2 h |
| Cerebral malaria susceptibility (Parekh et al. 2006) | ↓ glucose use<br>↓ TCA cycle<br>↓ high-energy phosphates<br>↓ glutamine<br>↓ phosphorylation potential | Inflammation "cytopathic hypoxia" | Not reported | Brain | 6–7 days |
| Liver cirrhosis (Harvey et al. 2000) | ↓ ATP/ADP<br>↓ $P_i$/ATP<br>↑ Lactate/pyruvate | Diffusion limitation | Not reported | Liver | 3 wk |
| Adaptation to high-altitude residence (Gelfi et al. 2004) | ↑ GST-p1—1<br>↔ 4-HNE<br>↑ ECH<br>↓ GAPDH<br>↓ LDH<br>↑ PGA<br>↑ NUGM<br>↑ myoglobin | ↓ $PO_2$ | $PO_2 = 90$–$106$ mmHg | Skeletal muscle | Lifetime at altitude |
| Adaptation of high-altitude residence (Droma et al. 2006) | ↑ *Glu298Asp*<br>↑ eNOS4b/a polymorphisms of the *eNOS* gene | ↓ $PO_2$ | $PO_2 = 106$ mmHg | Leukocytes | Lifetime at altitude |
| Pulmonary hypertension (COPD) (Tanaseanu et al. 2007) | ↑ VEGF<br>↑ sP-selectin<br>↑ PLA2-LDL<br>↔ APLA | ↓ Blood flow | Not reported | Plasma | Chronic disease |
| Acute ascent to high altitude (Jefferson et al. 2004) | ↑ *$F_2$-isoprostane<br>↑ 8-iso PGF2$\alpha$<br>↑ Total GSH<br>↑ TBARS | ↓ $PO_2$ | $S_aO_2$—81% | *Urine plasma | 48 h |

PI3K, phosphoinositide 3 kinase; PLA2, lipoprotein-associated phospholipase $A_2$; NAC, N-acetyl cysteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NNE, nonneuronal enolase; GST-p1, glutathione-S-transferase p1; ECH, $\Delta^2$-enoyl-CoA-hydratase; LDH, lactate dehydrogenase; PGA, phosphoglycerate mutase; NUGM, NADH-ubiquinone oxidoreductase; 4-HNE, 4-hydroxynonenal protein adducts; *Glu298Asp and eNOS4b/a,* polymorphisms of the *eNOS* gene; CDE 13, aminopeptidase N; CA IX, carbonic anhydrase IX; pt-ATPase, potassium-transporting ATPase; MMP-9, matrix metalloproteinase 9; SDF-1, stromal cell–derived factor I; VEGF, vascular endothelial growth factor; sP-selectin, soluble P-selectin; PLA2-LDL, lipoprotein-associated phospholipase $A_2$; APLA, antiphospholipid antibodies; COPD, chronic obstructive pulmonary disease; GSH, glutathione; TBARS, thiobarbituric acid reactive substance.

between DCM and I/R and myofilament proteins troponin I and myosin light chain is described.

## Pulmonary Disease

Hoshikawa et al. (2003) used genomics to examine differential gene expression in rat vs. mouse lungs after hypoxia. Following exposure to 1 and 3 weeks of hypobaric hypoxia, both species had pulmonary hypertension, but mice had less pulmonary vascular remodeling. Following gene expression patterns enabled the authors to follow the balance between the up-regulation of genes encoding cell proliferation and the corresponding down-regulation of genes required for vascular tone. These studies support the concept that chronic hypoxia causes pulmonary hypertension in humans and most animals, but the degree of remodeling varies.

Proteomics in pulmonary disease has thus far seemed to focus on developing techniques to analyze biofluids such as bronchiolar lavage fluid (BALF), epithelial lining fluid (ELF), exhaled breath condensate, and even plasma and urine (Bowler et al. 2006). This could be due in part to the complex and dynamic nature of these samples; for example, the location of sampling, the high concentration of abundant proteins such as albumin, and even sample handling are major challenges in the field of pulmonary proteomics (Hirsch et al. 2004). Although pulmonary proteomics studies have been mostly descriptive in nature, some information regarding potential biomarkers has been obtained (Bowler et al. 2006; Hirsch et al. 2004). Much of these data is based on protein profiling experiments utilizing surface-enhanced laser desorption ionization (SELDI) techniques, which do not result in protein identification. Therefore, although classification of patients has been accomplished using SELDI, no clinical proteomics biomarker for pulmonary disease has yet been validated using this technique. Other studies have utilized a variety of the quantitative proteomics techniques outlined above (Bowler et al. 2006); for example, investigators report a protein panel in BAL representative of smokers with chronic obstructive pulmonary disease (Plymoth et al. 2007).

## Cancer

The physiology and the microenvironment of solid tumors are very different from those of normal tissues. Cancer cells also possess a unique metabolic phenotype, which was intensively characterized by conventional and NMR-based studies. In addition to metabolic changes that are associated with uncontrolled cell growth (mostly related to increased phospholipid turnover), human solid tumors frequently contain a substantial fraction of cells that are hypoxic. The efficacy of irradiation and chemotherapeutics relies on an adequate oxygen supply. Consequently, hypoxic regions in solid tumors often contain viable cells that are resistant to therapeutic interventions (Tomes et al. 2003). It is clear that oxygen deficiency strongly influences major intracellular pathways such as those involved in cell proliferation, cell cycle progression, apoptosis, cell adhesion, and angiogenesis (Fig. 4) (Nordsmark et al. 2001).

While the oxygen tension ($pO_2$) in normal tissue ranges, depending on tissue type, between 10 and 80 mmHg, tumors frequently contain regions with a $pO_2$ <5 mmHg (Fyles et al. 2002), which results in chronic hypoxia. Often chronic hypoxia is referred as "diffusion-limited" hypoxia, arising from large intervascular distances, beyond the diffusion limit of oxygen (>150 microns). However, in tumors, the origin of chronic hypoxia is more complex (including structural abnormalities in blood vessels). Moreover, it has been shown that a majority of cancer cells are "programmed" to be hypoxic in the very beginning of tumor development even before the oxygen supply is cut down (Fig. 4). Thus, even in the presence of oxygen, tumor cells develop a hypoxic phenotype with a highly activated HIF-1$\alpha$ pathway. The major metabolic hallmark of all tumors is increased aerobic glycolysis (the famous Warburg's effect); as such, tumors make themselves hypoxic even before hypoxic conditions actually exist (due to increased size of the tumor and decreased tumor perfusion in the later phase of tumor progression). Therefore, increased stabilization of HIF1-$\alpha$ followed by increased VEGF production leads to development of a glycolytic phenotype of the tumor (Fig. 4). This is characterized by increased glucose uptake, increased lactate, and decreased mitochondrial activity such as decreased TCA cycle activity and oxidative phosphorylation (Shaw 2006). Succinate is often increased when the tumors become severely hypoxic, serving as a marker of decreased mitochondrial TCA cycle activity. As a result, energy balance is reduced in tumor cells.

As further evidence of the role of the glycolytic pathway in hypoxia and cancer, Mikuriya et al. (2007) used a similar workflow to identify four proteins from pancreatic tissue, with possible roles in tumor invasion or metastasis. Using a combination of proteomic and genomic techniques, Stockwin et al. (2006) studied differential regulation of genes and proteins of the plasma membrane, which acts as an interface between the cell and the hypoxic environment. The authors first used differential $^{16}O/^{18}O$ stable isotope labeling, followed by the multidimensional protein identification technology (MuDPIT) to quantitatively analyze 2433 plasma membrane proteins from hypoxia-adapted murine B16F10 melanoma cells. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was then used to gain further insight into the genomic regulatory events underlying the response to hypoxia. The authors observed increases in both proteomic and transcriptomic levels for several proteins (see Table 2): amino peptidase N (CD13), carbonic anhydrase IX, potassium-transporting ATPase, matrix metalloproteinase 9, and stromal cell derived factor I (SDF-1). The up-regulation of CD13 and SDF-1 was further confirmed using an antibody-based analysis of a human melanoma cell panel.

## Chronic Hypoxia in Newborns

Douglas et al. (2007) recently reported a study designed to determine if chronic constant hypoxia of the newborn, such as that experienced with congenital heart disease, right to left shunts, or hypoventilation, created more or less neuronal damage than chronic intermittent hypoxia, such as that experienced in apnea of prematurity and obstructive sleep apnea. Neonatal mice were exposed to 11% oxygen (21% $O_2$, normoxia) for 4 weeks either constantly or in 4-min cycling intervals to mimic sleep apnea. Metabolomic results indicated that the N-acetyl aspartate/creatine ratio was depressed in the animals exposed to chronic intermittent hypoxia, suggesting greater neuronal metabolic stress as compared to chronic continual hypoxia.

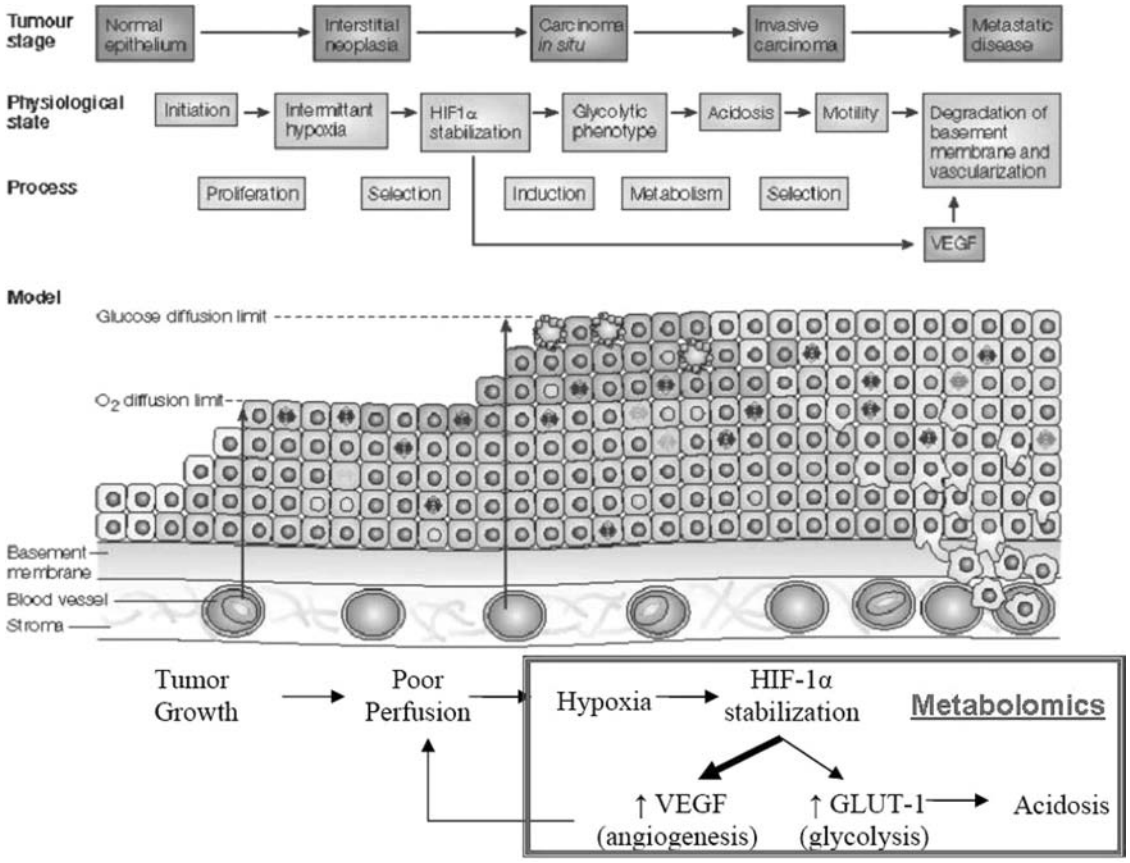


**FIGURE 4** **Induction of hypoxic phenotype in the cell during tumor development.**

## Environmental Hypoxic Stress

Metabolic responses to anoxia were first delineated in diving animals in the 1970s by Hochachka, Storey, and others (reviewed in Hochachka and Storey [1975]), using the targeted methods available at that time. More recently, Storey (2006a) has extended and examined these findings in greater depth using cutting-edge metabolomic, proteomic, and genomic methodologies. Specifically, they have found that glutathione-s-transferase (GST) and other antioxidants have greater constitutive expression in animals that dive and hibernate, and during low oxygen states there is greater induced antioxidant activity (reviewed in Storey [2006a]). In addition, Serkova et al. (2007) have recently used metabolomics to determine the metabolic profile of hibernating squirrels during different states of arousal. Data indicate that during the arousal state, when squirrels are hypoxic, betaine is accumulated and may be used to drive the S-adenosyl methionine cycle, providing for oxidative protection and normal liver function.

Altitude exposure is an excellent model for examining metabolomic, proteomic, and genomic differences between physiological and pathophysiological response to global hypoxic insult. Gelfi et al. (2004) used a proteomics approach to investigate the ability of native Tibetan highlanders to maintain muscle mass and strength at altitude in contrast to lowlanders ascending to altitude who lose muscle mass and strength with chronic hypoxic exposure. Glutathione-s-tranferase p (GST P1–1) protein and mRNA were two- to fourfold greater in Tibetan highlanders vs. Nepali lowlanders and 50% higher in Tibetan vs. Nepali lowlanders, while 4-hydroxynonenal (HNE) was equivalent between these populations. Furthermore, mitochondrial density was low in Tibetan highlanders and reduced in Caucasians after chronic exposure to altitude. Thus, adaptation to chronic hypoxia appears to involve both metabolic and genetic changes resulting in a greater ability to reduce reactive oxygen species generation and optimize metabolic control, very similar to findings in hibernating and diving animals (Storey 2006a).

# METABOLIC MARKERS OF ACUTE HYPOXIA

Acute hypoxia (often due to ischemia) usually comprises an immediate deprivation of oxygen. Such metabolic changes occur in the first minutes of ischemic/hypoxic insult in the form of mitochondrial collapse, total depletion of ATP production, inhibition of mitochondrial TCA cycle, and oxidative phosphorylation followed by activation of anaerobic glycolysis, increased glucose consumption, and depletion of glycogen stores.

## Hypoxia-Induced Pulmonary Hypertension

Prior to the widespread use of mass spectrometry in proteomics, Zimmerman et al. (1991) used a labeling approach to discover protein biomarkers of acute hypoxia in cultured bovine

aortic and pulmonary arterial endothelial cells. In these studies the investigators used $^{3}H$ amino acid synthesis to measure protein synthesis. Measuring proteins precipitated by TCA, the authors determined total proteins were decreased after 4 to 8 h of hypoxia. Labeling with $^{35}S$ methionine showed an induction of a specific set of proteins with specific molecular weights of 34, 36, 47, and 56 kDa, whose levels depended on the severity of hypoxia. Expression of these proteins corresponded with mRNA levels in most cases. The authors further observed that these proteins were maximally up-regulated only after total protein synthesis had fallen. Comparison of hypoxia-associated proteins to hyperthermia or with the addition of sodium arsenite showed these were distinct from heat shock proteins. Two of these proteins have since been identified as the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and nonneuronal enolase (NNE) (Graven et al. 1998). GAPDH and NNE are both up-regulated in the cytoplasmic fraction, although data suggest that GAPDH is also up-regulated in the nuclear fraction.

Leach et al. (1998) questioned whether energy state in response to hypoxia differed between the pulmonary and systemic arteries, a possible mechanism for acute hypoxia-induced pulmonary vasoconstriction vs. systemic vasodilation. Smooth muscle cells were isolated from porcine pulmonary and femoral arteries and then subjected to 467 or 23 mmHg $O_2$ for 2 h. These data indicated that energy state was reduced in femoral but not pulmonary artery smooth muscle cells.

## Transient Tumor Hypoxia/Reoxygenation

A recent article from Magagnin et al. (2007) not only highlights the changes in protein expression during reoxygenation of tumor cells, but also describes a now-typical discovery-based proteomics workflow. In these studies, HeLA cells were exposed to 4 h of hypoxia (<0.01%) followed by reoxygenation for 1 h. Proteins were extracted and resolved using 2DE followed by mass spectrometry to identify proteins of interest. Several proteins were identified, including ribosomal protein P0, VCP/p97, and FUSE binding protein. These proteins are responsible for regulating RNA metabolism and protein synthesis and degradation, suggesting they play a role in recovering after endoplasmic reticular stress and reinitiation of protein synthesis during reoxygenation.

## Stroke

A large variety of in vivo and in vitro metabolic data on stroke patients and animal models has been performed using MRS and NMR during cerebral infarction episodes. In the first minutes after vascular occlusion, tissue deoxygenation occurs followed by energy failure (depletion of ATP) and membrane failure (increase of phospholipid catabolism products), and increased succinate can be seen as a marker for inhibited TCA cycle (Rudin et al. 1999). A biomarker study has revealed that four biomarkers create a panel by which to diagnose acute cerebral ischemia, S100B, MMP-9, vascular cell adhesion molecule, and von Willebrand factor with 90% accuracy (Lynch et al. 2004). Biomarkers are also proving useful in determining which type of stroke has occurred; for example, the presence of glial fibrillary acidic protein (GFAP) distinguishes between intracranial hemorrhagic as compared to ischemic stroke (Foerch et al. 2006; Montaner 2006).

## Myocardial Infarction

Metabolic plasma markers (lactate, AMP catabolism products, TCA cycle) indicate myocardial ischemia as determined by a mass spectrometry–based quantitative metabolic profiling study (Sabatine et al. 2005) (see Table 2). Originally, the most commonly measured serum biomarkers measured for acute myocardial infarction (AMI) were lactate dehydrogenase, creatine kinase, and creatine kinase-cardiac muscle isoform (CK-MB). ELISA assays for CK-MB are considered the gold standard for diagnosing AMI, although other biomarkers are now recognized and tested such as cardiac troponin T, cardiac troponin I, and myoglobin. Myoglobin is the most rapidly detectable biomarker of AMI, while troponin can remain elevated for up to 10 days following an AMI. Micellular electrokinetic chromatography combined with cleavable tag immunoassay is currently being developed to simultaneously test for all of these biomarkers at the bedside, strengthening diagnostic capabilities and allowing appropriate treatment to be more rapidly administered (Caulum et al. 2007).

## Surgery-Induced Ischemia-Reperfusion Injury

A temporary interruption of blood supply during surgical intervention represents one of the most severe hypoxic injuries, when a prolonged episode of warm/cold ischemia is followed by warm reperfusion. Solid organ transplantation (especially kidney and liver) served as an ideal model (both clinically and preclinically) to investigate metabolic changes of induced ischemia/reperfusion. In addition to general hypoxic markers, such as decreased ATP and increased lactate levels (Niemann et al. 2005), there are some organ-specific markers for ischemic injury during organ preservation and transplantation. In the kidney, decreased ratios of polyunsaturated to monosaturated fatty acids (PUFA/MUFA) were markers for increased lipid peroxidation after kidney transplantation (Serkova et al. 2005). In addition, elevated levels of the renal osmolyte trimethylamine N-oxide (TMAO) and the end product of xanthine pathway, allantoin, were found in the blood of renal transplant animals after prolonged ischemia/reperfusion injury (Serkova et al. 2005). For liver, an increase in circulating level of betaine–one of the major osmolytes in hepatocytes–was found to correlate with the severity of hepatic ischemia/reperfusion injury (Niemann et al. 2006; Park et al. 2007).

## Acute Environmental Hypoxia

Certain species of seals and whales can dive as deep as 600 m and remain there for up to 2 h, while the percent of arterial oxygen drops below 10% within the first 15 min. These animals have hematocrit values as high as 60% and hemoglobin concentrations up to 25 g/dL. Their skeletal muscles have very high myoglobin concentrations (50–80 mg/g) and the myoglobin has an incredible affinity for oxygen ($P_{50} = 2.5$) (Ramirez et al. 2007). Comparing metabolomic biomarker panels between species that routinely experience acute hypoxia (seals, whales)

and those that do not (humans, mice) may provide insight into physiological vs. pathophysiological responses to acute hypoxia.

Complications arising from acute ascent to altitude include acute mountain sickness (AMS), high-altitude pulmonary edema (HAPE), and high-altitude cerebral edema (HACE), conditions quite similar to pulmonary edema induced by pulmonary hypertension and neurogenic pulmonary edema. Research studies on the effects of acute exposure to hypobaric hypoxia on human and animal models have led to considerable insights into the effect of hypoxia in human disease. In fact, the ability of hypoxia to cause pulmonary hypertension and vascular remodeling was first discovered in cows with altitude-induced brisket disease (Grover et al. 1963).

Using electron paramagnetic resonance spectroscopy (EPR), Bailey et al. (2001) have identified biomarkers of oxidative stress in subjects with acute mountain sickness. The biomarkers included lipid hydroperoxides, total phosphocreatine kinase activity, and myoglobin. Bailey expanded his findings by using magnetic resonance imaging (MRI) and other advanced technologies to investigate the relationship between the ROS biomarkers during hypoxia and hypoxia-induced pathologies. There was no relationship between oxidative stress markers and membrane permeability in neurons and brain, and there was no evidence for redox regulation of energy homeostasis in subjects acutely exposed to hypobaric hypoxia (Bailey et al. 2006, 2004a, 2004b). Oxidative stress markers are also elevated in healthy subjects exposed to acute altitude (Baillie et al. 2007; Pialoux et al. 2006; Maiti et al. 2006; Jefferson et al. 2004), suggesting that, to some extent, oxidative stress is part of the normal physiological response to altitude.

Because the liver is the largest metabolizing organ in the body and performs a number of tasks crucial for survival, Dolt et al. (2007) used cDNA microarray technology, RT-PCR, and gene mapping to elucidate the effect of acute hypobaric hypoxia on the liver transcriptome in mice. This is the first study in which the in vivo acute hypoxic response of the liver gene profile has been determined. There were significant variations in the expression of genes involved in various metabolic pathways that have yet to be explored. However, using software to functionally classify and map the genes and metabolic pathways of the genes identified, it became apparent that the sterol synthesis pathway was significantly inhibited by exposure to acute hypoxia. Specifically, the sterol regulatory element binding protein (SREBP) gene was reduced, as were its downstream genes, although there was no change in plasma cholesterol within the 10-h time frame studied. Clearly, the SREBP data represent only a portion of the information gained about the effect of acute hypoxia on liver metabolism in vivo, leaving the investigators with many future potential pathways, a valuable benefit of 'omic studies.

## PRO- AND ANTIHYPOXIA DRUGS

Two major classes of xenobiotics will be discussed: (1) the drugs that prevent hypoxia vs. (2) the drugs that induce hypoxia. As mentioned above in the Cancer section, drug tissue distribution and therefore drug efficacy directly depend on hypoxic conditions of the target tissue (e.g., blood and oxygen supply) and need to be taken into consideration for development of drug resistance.

A significant amount of drug discovery studies have been focused on targeting hypoxia and the HIF-1$\alpha$ signaling cascade, mostly in cancer (Lee et al. 2007). The goal of these therapies is to manipulate the HIF signaling pathway to inhibit tumor growth and to allow for delivery of other anticancer therapeutics. The best examples include (a) direct inhibitors of the HIF pathway (PX-478, echinomycin, siRNA, chetominin) as well as (2) downstream regulators of the mammalian target of rapamycin (mTOR) (CCI-779, rapamycin, everolimus), epidermal growth factor receptor (EGFR) (gefitinib, erlotinib), and vascular endothelial growth factor and receptor (VEGF and VEGFR) (bevacizumab, vatalanib, Zactima). Treatment with these drugs usually leads to decreased glucose uptake and significant inhibition of glycolytic activity and lactate production (Serkova et al. 1996; Harrigan et al. 2005).

On the other hand, a wide variety of drug-induced side effects are directly implicated in induction of hypoxic conditions in target organs (mostly liver and kidney). Some pharmaceutical agents cause respiratory depression (central nervous system depressants and convulsant chemicals) and tissue hypoxia (cocaine and ergot alkaloids). Other drugs lead to inhibition of mitochondrial metabolism, induction of ROS formation, and subsequent hypoxia (immunosuppressant cyclosporine, cisplatin, and others). These drugs can lead to specific metabolic changes in target organs, which can also be reflected in blood. For metabolomics assessment of drug toxicity, nephrotoxins, followed by hepatoxins, are the best examples (Yap et al. 2006; Williams et al. 2005; Serkova and Niemann 2007). For example, cyclosporine is known to interfere with mitochondrial metabolism by induction of oxidative stress and a subsequent increase of glucose, lactate, and alanine in the blood, while glutathione is significantly depleted (Fig. 5). Because the kidney is the primary organ in which cyclosporine toxicity occurs, hypoxic damage to the renal cortex and medulla result in the release of renal osmolytes, such as TMAO (Fig. 5).

Interestingly, some drugs that induce hypoxic conditions in normoxia can serve as potential pretreatment agents to ameliorate acute hypoxic insult in some organs—a phenomenon known as "pharmacological preconditioning." Cyclosporine is an example of this type of drug. While inducing hypoxic conditions by mitochondrial depression and decreasing energy production during normoxia, cyclosporine pretreatment prevents brain and heart damage during episodes of acute hypoxia (Fig. 6) (Serkova et al. 2002; Niemann et al. 2002). Prolyl hydroxylases (PHDs) play a significant role in HIF degradation during normoxia. Early evidence suggests that PHD inhibition and thus activation of HIF may protect against some pathologies. Recently, Kasiganesan et al. (2007) reported that mice pretreated with PHDs are protected from global hypoxia and show enhanced exercise performance during hypoxia. Also, PHD treatment of premature primate lungs promotes angiogenesis, potentially protecting from development of bronchopulmonary dysplasia (Asikainen et al. 2006).

## EXPERIMENTALLY-INDUCED HYPOXIC ARTIFACTS

When collecting tissue or cells that have been exposed to hypoxia, it is crucial to limit exposure to higher oxygen tension. Oxidative stress factors, hypoxia-inducible transcription factors, and metabolic byproducts can all change immediately upon exposure to even small increases in oxygen. Using metabolomics, Serkova et al. (2003) investigated the effect of delayed tissue

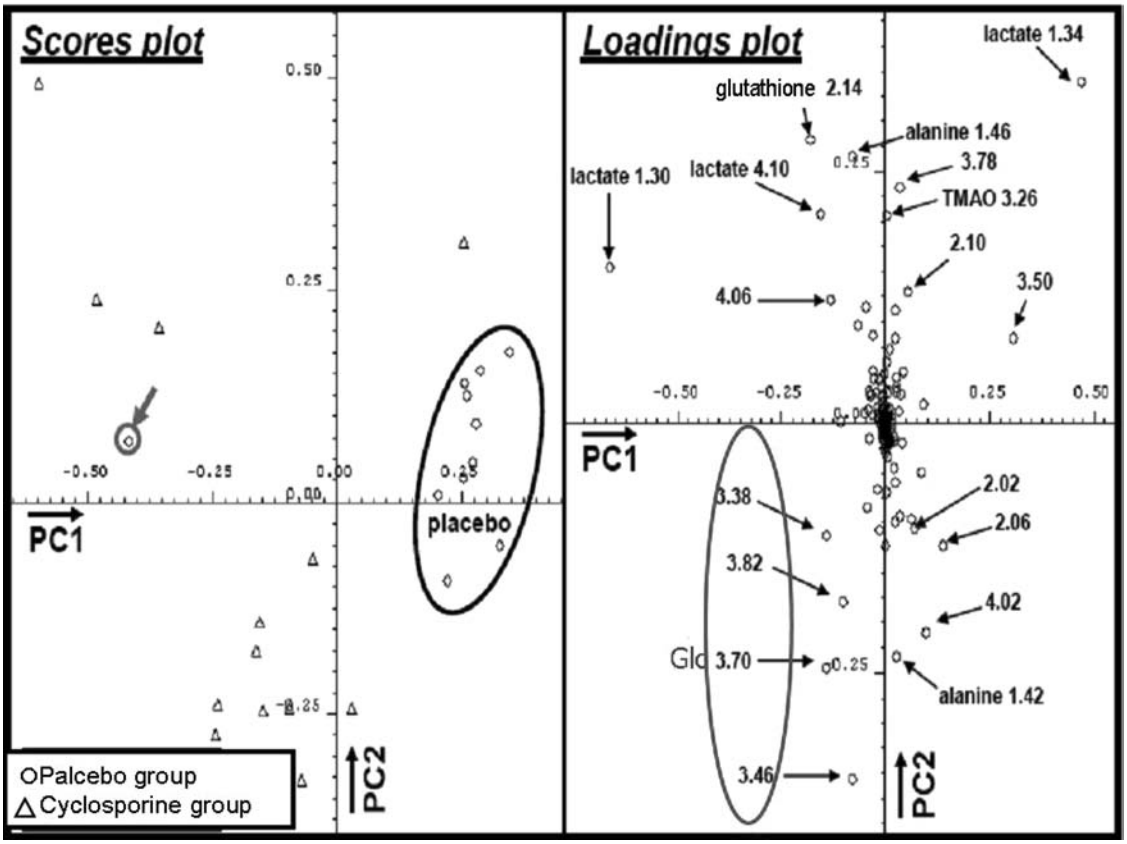


**FIGURE 5** **Partial least square-discriminant analysis on blood of healthy volunteers and stable kidney recipients under cyclosporine treatment based on $^{1}$H NMR metabolomics study. The group clustering is distinguished on the score plot. Changes in glucose, lactate, alanine, and glutathione, visible on the plot score, are indicative for increased oxidative stress and induced chronic hypoxia by cyclosporine. Trimethylamine-N-oxide (TMAO) was increased in cyclosporine-treated renal recipients and is indicator of hypoxic renal injury in the medulla (cyclosporine possesses nephrotoxic properties).**

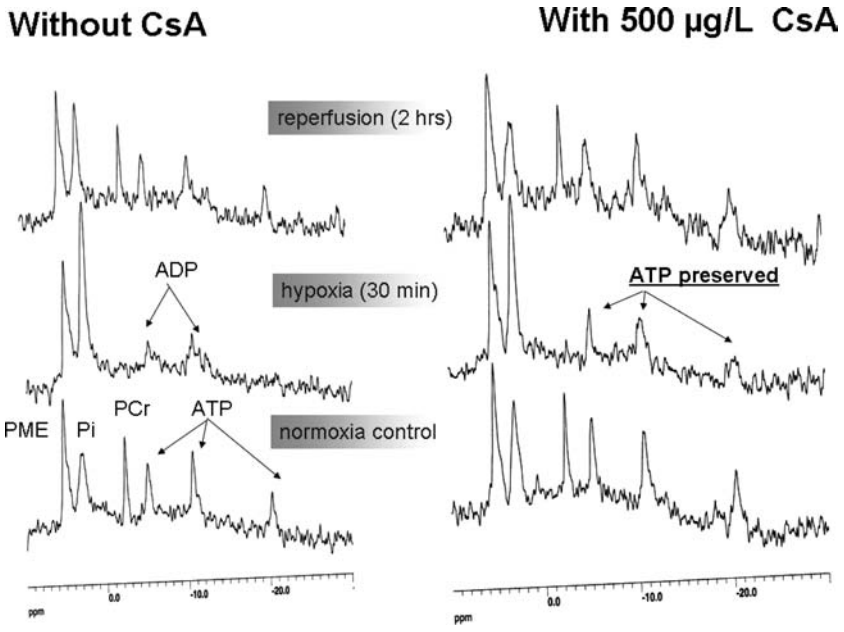


**FIGURE 6** **Representative in vivo $^{31}$P NMR spectra of perfused rat brain slices under hypoxia and reperfusion with and without 500 $\mu$g/L cyclosporine. Cyclosporine pretreatment ameliorate ATP depletion during 30 min of acute hypoxia. Adapted from Serkova et al. (2002).**

collection on placental samples, following placental delivery. Lactate was significantly elevated in tissue allowed to sit for longer than 9 min following placental delivery, and glucose and ATP were reduced. After 16 min, $NAD^+$ was lower, and after 25 min or longer, there was lipid peroxidation and changes in lipid metabolites. For the placenta, a relatively hypoxia-tolerant organ, artifacts of hypoxic/ischemic insult will be created if the tissue collection is delayed by 9 min or longer. Time to tissue collection varies depending on the organ and cell type; for example, brain and kidney must be collected within 30 sec of cessation of blood flow. When collecting cells from cell culture, it is best to stop cellular activity in a hypoxic environment, such as within the hypoxia culture chamber. Overall, it is best to collect all tissue from hypoxia experiments during or as soon as possible to avoid artifacts from either exposure to higher oxygen concentration or damage from ischemia when collecting tissue from in vivo experiments.

## CONCLUSIONS AND FUTURE DIRECTIONS

With the recent emergence of powerful technologies has come the potential for large-scale, widely applicable, inexpensive methods for discovery. In reality, the development and application of such methods are proving difficult in practice for several reasons, including cost, lack of comprehensive technical platforms, and the often extreme variability found in the metabolomes of human populations, which result in confounding data sets. Concerns over poor reproducibility, low sample numbers, lack of validation, and high cost associated with several cutting-edge techniques have lessened enthusiasm for these modern analysis methods. In addition, the effects of experimental variability have not been globally defined. Integrating and applying technologies to relevant disease states requires well-characterized samples from strictly stratified patient cohorts that, in turn, necessitate strong collaborations between clinicians and basic scientists. Even when all of these elements are in place, performing statistical and bioinformatics analyses is challenging, especially when multiple data sets must be examined simultaneously.

In summary, carefully designed and validated protocols for sample handling and sample extraction followed by appropriate NMR or MS techniques and statistical analyses will allow for establishing reliable and quantitative metabolic markers of hypoxic injury. Using 'omics technology to determine differences in hypoxia responses in regard to hypoxia source, length, severity, and cell/organ affected could greatly enhance research into the pathology of hypoxia-mediated disease. Ideally, these biomarkers will be noninvasively assessed in biofluids (blood, urine) using conventional biochemical and/or metabolomics assays or, alternatively, directly in the organ of interest using modern molecular imaging technologies.

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