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Subcellular Energetics and Metabolism: Potential Therapeutic Applications

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Abstract

Part I of this review discussed the similarities between embryogenesis, mammalian adaptations to hypoxia (primarily driven by Hypoxia Inducible Factor-1 [HIF-1]), ischemia-reperfusion injury (and its relationship with reactive oxygen species), hibernation, diving animals, cancer, and sepsis, and focused on the common characteristics that allow cells and organisms to survive in these states. Part II of this review describes techniques by which researchers gain insight into subcellular energetics and identify potential future tools for clinicians. In particular, P^{31} nuclear magnetic resonance to measure high energy phosphates, serum lactate measurements, the use of near infrared spectroscopy to measure the oxidation state of cytochrome aa₃, and the ability of the protoporphyrin IX-triplet state lifetime technique to measure mitochondrial oxygen tension are discussed. Additionally, this review will discuss novel treatment strategies such as hyperbaric oxygen, preconditioning, exercise training, therapeutic gases, as well as inhibitors of HIF-1, HIF prolyl hydroxylase (PHD), and Peroxisome proliferator-activated receptors (PPARs).

Introduction

The purpose of this two-part review is to explore the ability of cells and organisms to survive, and in some cases thrive, in hostile hypoxic environments, and identify common themes that may have therapeutic applications for humans subjected to hypoxic or oxidative stress. The first part of this review outlined the similarities between embryogenesis, mammalian adaptation to hypoxia (including hibernation and diving adaptations), the role of reactive oxygen species in ischemia-reperfusion injury, the ability of cancer cells to survive in oxygen-poor environments, and the energetic derangements that occur during uncontrolled infection and inflammation. The second part of this review will focus on technology that may allow investigators and clinicians to gain real-time insight into subcellular energetics, as well as describe potential treatment modalities that, based on this information, could be used to specifically target and modify the subcellular energetic machinery.

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1. Assessment of Subcellular Energetic State

Given the central role of the mitochondria in survival under “normal” conditions as well as during adaptation to stress, one logical approach to evaluating subcellular energetics would be to assess the individual mitochondrial pathways involved in energy production. Several techniques have been proposed for this purpose – P^{31} nuclear magnetic resonance spectroscopy (NMR) to measure high-energy phosphates, near infrared spectroscopy (NIRS) to measure the oxidation state of cytochrome aa3, the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) approach for measurement of mitochondrial pO_2 , and serum lactate measurements to gauge anaerobic energy production. (Figure 1)

P^{31} Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy takes advantage of the fact that magnetic nuclei exist in a finite number of energy states, also referred to as “spin” states, that application of electromagnetic radiation (EMR) in the radio frequency (RF) range can impart energy to nuclei and move them between spin states, and that transition from a higher to lower energy spin state leads to emission of detectable radio waves.¹ Performance of NMR thus involves aligning spin states through the application of a magnetic field, increasing the energy state of specific magnetic nuclei using RF-EMR, and detecting the resulting RF-EMR emitted when the magnetic nuclei return to their baseline state. The resulting EMR is referred to as free induction decay (FID), which has four components – amplitude or intensity, frequency, phase, and half-life.¹ These four spectroscopic features can then be utilized to identify and quantify specific compounds which have a unique NMR “fingerprint.” Additionally, because various environmental factors affect the behavior of magnetic nuclei, information about the surroundings and activity of certain compounds may also be derived.¹

While H^1 , C^{13} , Na^{23} are biologically relevant and can all be interrogated, P^{31} NMR is particularly useful to biomedical researchers as it can measure the concentration of phosphocreatine, adenosine triphosphate (ATP), and inorganic phosphate (Pi), in addition to measuring intracellular pH based on the frequency shift associated with Pi that is caused by alterations in pH.² Advantages of P^{31} NMR include the non-invasive nature of the technique and the wealth of information that can be derived. The primary disadvantages of P^{31} NMR are the requirement for large, magnetically isolated, expensive equipment, as well as the relatively long acquisition time, the latter of which precludes acquisition of continuous data. Furthermore, these traits have limited P^{31} NMR to research use exclusively, and clinical applications are not forthcoming. As described in Part I of this review, P^{31} NMR has been used extensively to understand the impact of sepsis and uncontrolled inflammation on subcellular energetics by analyzing both ATP levels and intracellular pH.^{3–8}

Serum Lactate Measurements

In the setting of inadequate mitochondrial oxygen supply, hypoxia-inducible factor 1 (HIF-1) activates lactate dehydrogenase-A (LDHA), converting pyruvate to lactate and depriving the TCA cycle of Acetyl-CoA.^{9,10} This response is part of a complex “metabolic switch” designed to transition cells away from oxidative phosphorylation so that energy production can continue in a relatively hypoxic environment.¹¹ Thus, lactate production

may be a sign of inadequate oxygen supply. Traditionally, lactate elevation has been almost universally ascribed to inadequate oxygen delivery.¹² Lactate production can also be upregulated by a variety of non-hypoxic stimuli including mitochondrial reactive oxygen species (which also upregulate HIF-1)^{13–16} as well as endogenous β 2-adrenergic stimulation.¹² Lactate is an alternative fuel source and can improve cardiovascular function in various pathological states.^{17–19}

Elevated serum lactate has been shown to be a marker for increased mortality in patients undergoing both cardiac and non-cardiac surgery^{20,21} and normalization of lactate has been associated with improved mortality in sepsis.²² A multicenter, randomized controlled trial (RCT) comparing lactate-guided resuscitation to standard of care in septic shock showed a significantly lower mortality in the lactate-guided group, although there were no significant differences in lactate levels between groups.²³ A similarly-sized trial comparing ScvO₂ to lactate-guided treatment in sepsis found no difference between groups and concluded that lactate was non-inferior to ScvO₂ guided therapy.²⁴ Interestingly, combination of lactate and ScvO₂ in a large, single center RCT of patients undergoing cardiac surgery demonstrated decreased length of stay and reduced organ failure in the treatment group but, as in the Jansen trial, found no differences in lactate or ScvO₂ between groups.²⁵ Combined, these data suggest that lactate may be a useful guide for therapy although larger trials in which serum lactate levels differ between groups are needed to rigorously assess the utility of this diagnostic tool.

In the absence of definitive clinical data, the understanding that lactate production can be due to hypoxia, ROS production, or β 2-adrenergic stimulation, the fact that serum lactate is a global marker and provides no regional or organ-specific information, and the fact that lactate is an energy source that may improve cardiac function make the rational use of this incompletely understood global marker difficult. While elevated serum lactate *may* signify a problem, additional information is needed to determine if and where such a problem exists.

Near Infrared Spectroscopy

The NIRS approach is based on the Beer-Lambert principle - because biological compounds (“chromophores”) have unique absorption profiles, tissue absorption of light can be used to calculate the relative concentration of each chromophore. This calculation is performed by inverting a matrix of the absorption coefficients for each species, and generating a series of linear equations describing concentration in terms of absorbance at each wavelength.²⁶ A clinical example of chromophore analysis is pulse oximetry, in which two wavelengths (red, infrared) are utilized to measure the concentrations of oxyhemoglobin and deoxyhemoglobin. Because one wavelength of light is required for each chromophore, a two wavelength pulse oximeter cannot measure chromophores such as carboxyhemoglobin, or methemoglobin. [Figure 2]

NIRS was originally developed to assess the oxidation state of cytochrome aa₃, the terminal component of the electron transport chain (ETC).²⁶ When oxygen is supplied to the cell, it enters the mitochondria, and accepts electrons from the ETC in a fashion that produces energy. Cytochrome aa₃ mostly exists in an oxidized state. When oxygen is not available, does not enter the mitochondria, or electron transfer is interrupted, cytochrome aa₃ is

reduced and its change in absorbance spectrum is detectable using NIRS. Thus, unlike estimates of “tissue oxygen saturation” based on hemoglobin the oxidation state of cytochrome aa₃ provides information about the redox state of the mitochondria themselves.

Measuring the oxidation state of cytochrome aa₃ (Cyt_{ox}) is challenging for several reasons. Overall, cytochromes are present in lower concentrations than hemoglobin and absorb less near infrared (NIR) radiation, effectively lowering the signal-to-noise ratio (SNR). To increase the accuracy of cytochrome measurements (overall, the cytochromes are responsible for less than 10% of NIR absorbance), “broadband” techniques that use a “band” of red and infrared light incorporating over 100 wavelengths have been developed.^{27–30} The ability of broadband NIRS to accurately quantify changes in Cyt_{ox} *in vivo* was convincingly demonstrated by Cooper et al. who used sodium cyanide to manipulate the redox state of cytochrome_{aa3} independent of hemoglobin³¹ and Lee et al., who found a simultaneous increase in StO₂ and reduction in of cytochrome_{aa3} in a rabbit model after NaCN injection.³² [Figure 3] Using a rat model, my laboratory has reported similar results. [Thiele RH. Ikeda K. Bartz RR. Wang Y. Zuo Z. *Broadband Near Infrared Spectroscopy Can Detect Cyanide-Induced Cytochrome aa3 Inhibition in Rats: A Proof of Concept Study* (Accepted to *Canadian Journal of Anesthesia* on October 31, 2016), in press]

The clinical utility of Cyt_{ox} is predicated on the belief that oxygen may not always diffuse out of the blood stream, into cells, and into the mitochondria for use in oxidative phosphorylation. Animal experiments, for instance, have demonstrated discrepancies between StO₂ and Cyt_{ox} during cooling in preparation for deep hypothermic circulatory arrest.³³ Furthermore, broadband NIRS has demonstrated reductions in Cyt_{ox} with normal StO₂ levels up to ten hours after application of experimental hypoxia and ischemia,³⁴ consistent with magnetic resonance spectroscopy studies suggesting that injury occurs hours after an ischemic insult.³⁵

Protoporphyrin IX-Triplet State Lifetime Technique

An alternative strategy called the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) has been developed to measure mitochondrial oxygen tension (pO_{2,mito}).^{36,37} Porphyrins are common biological macromolecules made of 4 pyrrole subunits, the best known of which are hemoglobin and chlorophyll. The cyclic shape of porphyrins allows them to conjugate with metallic ions, e.g. iron (hemoglobin) or magnesium (chlorophyll), and these ions give them their unique properties. Most porphyrins absorb light in the visible range of the spectrum, leading to excitation of the porphyrin-metal complex.¹²³

Understanding how PpIX-TSLT can be used to measure oxygen concentrations requires a brief detour into quantum mechanics. Elementary particles can be described by classical angular momentum (an example is an electron orbiting around a proton) but also by intrinsic angular momentum, or rotation around an internal axis. Most molecules exist in a “paired-spin” arrangement, in which parallel and antiparallel (paired) spins cancel each other out. This state is also referred to as a “singlet state,” and the total spin is 0. When the angular momenta of two parallel (i.e. not paired) spins are added, the result is non-zero total spin, and this is described as a “triplet state.”³⁸ Molecular oxygen is unique in that it exists primarily in a triplet state at room temperature.

Interestingly, while most porphyrins remain in singlet states even when excited, they can transition to a triplet state (e.g. T_1) through either internal conversion or intersystem crossing. A porphyrin molecule in a triplet state can then revert back to a singlet state through spontaneous relaxation or collision with an oxygen molecule, in which oxygen absorbs the porphyrin's extra energy and itself transitions from the normal, triplet state to a higher energy singlet state. The latter process is referred to as "triplet state quenching" and is a function of oxygen concentration. Triplet state quenching leads to both phosphorescence and delayed fluorescence, thus measurement of phosphorescence and/or delayed fluorescence can inform the triplet state lifetime (τ), the rate constant describing triplet state quenching (k), and thus oxygen concentrations.¹²³

Practically speaking, τ , k , and $pO_{2,mito}$ are measured by introducing 5-aminolevulinic acid (ALA, a porphyrin precursor that increases mitochondrial protoporphyrin IX concentrations), pulsing a green (510 nm) light into tissue, and detecting the delayed fluorescent signal (630–700 nm). In mitochondria with high levels of oxygen, triplet state quenching will occur quickly and fluorescence will decline rapidly [Figure 4]. Using a rodent lipopolysaccharide (LPS) injection model, Harms et al. have demonstrated reduced mitochondrial pO_2 in the setting of sepsis and improvements in mitochondrial $pO_{2,mito}$ with fluid resuscitation.¹²⁴ More recently, Romers et al. found that during progressive hemodilution in pigs, decreases in mitochondrial pO_2 preceded alterations in global hemodynamics, lactate, and StO_2 .³⁹

2. Therapeutic Applications Targeting Subcellular Energetics

A more comprehensive (albeit still incomplete) understanding of how cells adapt to oxygen-poor environments and of the crucial role of reactive oxygen species (ROS) / reactive nitrogenous species (RNS) in various pathological states may allow researchers and clinicians to design novel therapeutic modalities that move beyond the first order macroscopic approaches of maintaining adequate cardiac output, oxygen carrying capacity, and hemoglobin saturation. In particular, the measurement techniques described above may alert clinicians to previously invisible life-threatening perturbations in subcellular energetics that may, in some cases, be modifiable using the strategies that follow.

Hyperbaric Oxygen (HBO) Therapy

An early trial of HBO in a polymicrobial rodent sepsis model found a reduction in mortality despite higher levels of bacteria in the bloodstream in the HBO group, suggesting that the mortality reduction was not related to improved antimicrobial efficacy.⁴⁰ Lin et al. tested HBO and aminoguanidine (AG, a nitric oxide synthase inhibitor) in septic rats using the LPS infusion model. They found that while HBO did not affect nitric oxide production, superoxide production, or TNF- α levels in healthy animals, HBO decreased plasma nitric oxide, aortic superoxide, and TNF- α levels in animals exposed to LPS, in addition to improving mortality. Notably, septic animals treated with AG had similar plasma NO and TNF- α levels as those treated with HBO, but had higher levels of aortic superoxide anion and experienced no mortality benefit.⁴¹ This suggests that superoxide production in the setting of uncontrolled infection may be particularly harmful.

The results of more recent trials using higher amounts of HBO have been mixed. A mouse model of sepsis found a survival benefit at 2.5 ATM (every 12 hours) but worsened outcomes with 3.0 ATM (every 24 hours).⁴² In this case, HBO did show an antibacterial effect, with fewer colony forming units (CFUs) found in the spleen (but not the peritoneal fluid) of animals subjected to 2.5 ATM. The authors hypothesized that higher levels of HBO may have led to oxygen toxicity, as signs of injury have previously been shown at 3 ATM.⁴³ Other recent trials in a variety of animal models (rodents, equine) have also not found benefit.^{44,45}

HBO has also been proposed as a treatment to improve neurologic function after stroke. Pre-clinical work suggests that HBO may attenuate inflammation, stimulate neurogenesis, and inhibit apoptosis following stroke.^{46,47} An analysis of delayed HBO therapy (2.5 ATM) on neurologic recovery after middle cerebral artery occlusion showed improved neurologic function at 42 days. An increase in ROS and HIF-1 were observed in the HBO group, suggesting both that HBO can increase ROS and that elevated ROS levels can upregulate HIF-1 in the absence of hypoxia (as was described above^{13–16,48–50}).

Repeated, high doses of HBO (5 ATM) are neurotoxic, likely due to upregulation of neuronal NOS and the presence of increased ROS/RNS.⁵¹ HBO levels as low as 3 ATM increase NO formation in rat brains.⁵² In an effort to determine which ROS were responsible for the neurotoxicity associated with HBO, Oury et al. subjected animals to 6 ATM HBO. Using a combination of transgenic mice able to overexpress extracellular superoxide dismutase, ECSOD and inhibitors of both ECSOD and NO synthase, they found that NO overexpression was the most likely cause of HBO-mediated cellular toxicity, and that O_2^- may have a protective role by inactivating NO.⁵³ This finding conflicts with Lin et al.'s sepsis model which suggested that O_2^- was primarily responsible for the injury that accompanies sepsis, although the mechanisms of organ injury accompanying LPS infusion and 6 ATM HBO may be quite different.⁴¹

The role of HBO as a cardioprotectant has also been explored, primarily because of its potential to interact with NOS. In a rodent model of cardiac ischemia (25 mins) and reperfusion (180 mins), HBO was found to be cardioprotective. Interestingly, HBO increased endogenous nitrate/nitrite and the cardioprotective effect of HBO was reversed by pre-treatment with the NOS inhibitor L-NAME, strongly suggesting that NO production due to HBO had protective effects.⁵⁴ Small human studies using HBO to precondition the myocardium prior to CABG surgery have consistently demonstrated reduced perioperative troponin release in addition to clinical benefits.^{55,56}

Effective use of HBO may thus require identifying a “sweet spot” where HBO increases oxygen delivery to the ETC, upregulates HIF-1 through ROS-mediated mechanisms, and attenuates inflammation, but beyond which ROS production may cause oxygen toxicity. Real-time techniques designed to interrogate the subcellular redox state (described above) may be useful in the titration of this both helpful and harmful treatment modality.

Preconditioning

In 1986, Murray et al. demonstrated that multiple, brief ischemic episodes could “prepare” a tissue bed (in this case, canine myocardium) for a more significant ischemic insult. This observation gave birth to the concept of “ischemic preconditioning,” which was followed by pharmacologic (e.g. anesthetic) and other forms of preconditioning.^{57,58} Multiple groups have explored the genomic and proteomic changes that occur following ischemic and anesthetic preconditioning and have found significant overlap, suggesting that the underlying mechanisms may be similar.^{59,60} Common elements include up regulation in genes/proteins that control glycolysis, mitochondrial respiration, and the stress response. More specifically, preconditioning is mediated by nitric oxide synthase,^{61–63} activity of ATP-sensitive potassium (K_{ATP}) channels,^{64–66} opening of the mitochondrial permeability transition pore (MPTP),^{67,68} and maintenance of calcium homeostasis / avoidance of calcium overload,^{69–71} among other mechanisms.

Growing evidence suggests that HIF-1 plays an important, and in some cases, essential role in the development of ischemic or hypoxic preconditioning.^{72–78} HIF-1 may even play a role in the induction of pharmacologic preconditioning (at least with isoflurane).^{79–81} In addition to the roles for HIF-1 described in Part 1 of this review, HIF-1 may modulate both apoptosis and the inflammatory response to ischemia and in doing so induce organ protection after ischemia.⁸²

NO produced in response to HIF-1 mediated iNOS upregulation⁷⁸ also plays a prominent role in preconditioning. During ischemia, NO begins to accumulate (despite the relative lack of oxygen), eventually leading to an oversupply of ROS which, when coupled with a sudden increase in oxygen during reperfusion, cause peroxynitrate production and tissue injury.⁸³ However, brief, repetitious periods of ischemia modulate subsequent NO production and peroxynitrate formation, attenuating injury after more prolonged ischemic periods. This protective process can be reversed with NOS inhibition, suggesting a direct causal link between NO production and preconditioning.^{78,84} Additionally, studies indicate that while NO inhibition can completely block preconditioning, addition of L-arginine (a NOS substrate) can reverse this inhibition, cementing the critical role of NO in the development of preconditioning.⁸⁵ NOS can thus be both helpful (inducing preconditioning after exposure to transient periods of ischemia) and harmful (leading to NO and peroxynitrate production).

Preconditioning appears also be dependent on stimulation of NMDA receptors by glutamate - in cells pre-treated with NMDA-antagonists, hypoxic preconditioning is abolished, and exposure of preconditioned cells to excitotoxins still results in cell death. These data suggest that hypoxic preconditioning, through glutamate-mediated NMDA-agonism, may attenuate the release of excitotoxic agents following subsequent cellular stress.⁸⁶ The suggestion that NMDA receptors are implicated in the development of preconditioning is also supported by demonstrations that preconditioning produces moderate increases intracellular calcium concentrations and multiple demonstrations that treatment with NMDA antagonists can suppress the effects of preconditioning.^{85,87,88} Furthermore, IPC can be prevented through attenuation of ϵ -PKC, and re-activated through administration of DAG analogs, suggesting that while calcium accumulation likely plays a role in development of IPC, calcium-independent mechanisms are also involved.⁸⁸

Preconditioning and postconditioning (in which ischemia is induced during reperfusion) have been primarily utilized to protect against ischemic insults. An early, small study of postconditioning showed a reduction in infarct size following angioplasty in humans.⁸⁹ Multiple investigators attempted to utilize pre and post-conditioning to attenuate myocardial injury in the setting of myocardial infarction and while the data are mixed, an overall benefit is likely.⁹⁰ Outside the catheterization laboratory, preconditioning the myocardium directly is a challenge. Interest in *remote* ischemic preconditioning (RIPC), in which ischemia of another tissue bed (e.g. muscle) confers protective benefits on tissues not subjected to ischemia was generated by a demonstration of reduced myocardial injury and all-cause mortality in patients undergoing coronary artery bypass grafting (CABG) surgery and a similar trial finding less kidney injury in patients undergoing cardiac surgery.^{91,92} However, enthusiasm for RIPC was subsequently tempered by two large, multi-centered randomized controlled trials showing no benefit in patients undergoing CABG surgery.^{93,94}

The underlying mechanistic similarities between cellular injury that accompanies ischemia and inflammation have prompted investigators to study preconditioning in sepsis. Olguner et al. subjected rats with and without ischemic preconditioning to experimental sepsis and found that ischemic preconditioning led to less kidney injury, lower lung macrophage counts, and decreased lung and kidney injury scores.⁹⁵ Two subsequent preclinical trials found decreased markers of inflammation (TNF- α), reduced abscess formation,⁹⁶ and increased survival (possibly heme oxygenase-1 mediated) in animals subjected to preconditioning prior to sepsis.⁹⁷

Exercise Training

Physicians have long appreciated that patients who are “physically fit” are more likely to survive anesthesia and surgery than those who are not. This observation is based on a relatively large body of work suggesting that quantitative measures of fitness (e.g. maximal VO₂, aerobic threshold) predict morbidity and mortality following a variety of major surgical procedures.^{98–106} [Figure 5] This phenomena is not limited to surgical insults as studies suggest that patients who report recent physical activity are more likely to survive myocardial infarction than those who do not.¹⁰⁷ Of the available cardiopulmonary exercise testing (CPET) variables available, aerobic threshold (AT) and maximal VO₂ are the best validated.¹⁰⁸ Despite the great promise of CPET for discriminating between survivors and non-survivors and major surgery, two large questions remain. First, why does exercise tolerance confer a benefit in patients undergoing surgery, and second, can exercise be used as a therapeutic modality in patients who anticipate exposure to surgery or other forms of stress?

The epidemiological connection between vigorous exercise and reduced cardiac morbidity in humans has led several investigators to elucidate the molecular impact of exercise training on specific organ systems, in particular the cardiovascular system.^{109–111} Multiple groups have demonstrated that exercise training protects animal hearts from ischemia-reperfusion injury, both by attenuating the reduction in function that accompanies ischemia and by improving post-reperfusion function.^{112–114} Interestingly, trained animals exhibit increased concentrations of non-protein thiols and glutathione, increased superoxide dismutase activity

and increased levels of lactate dehydrogenase, suggesting a role of antioxidants as well as anaerobic metabolism in organ protection.^{112,114} Both superoxide dismutase and glutathione are correlated with ventricular function after injury, strongly implicating a role for antioxidant activity in preservation of function.¹¹⁴ Of all the potential mechanisms by which exercise training has been shown to induce organ protection, enhanced anti-oxidant activity is best supported by evidence.^{112,115–121}

Both age and obesity attenuate the beneficial effects of preconditioning. Interestingly, exercise therapy preserves the preconditioning effect of both ischemia and anesthetic administration in senescent and obese animals.^{113,122} The relationship between exercise training and preconditioning has led investigators to seek out shared mechanisms of organ protection. For instance - NOS expression and ROS are known to play prominent roles in PC. Although not all studies show an effect on NOS expression following exercise training,^{123–125} most studies suggest that exercise leads to endothelial, neuronal, or inducible NOS upregulation.^{126–134} However, because of differences in the animal model, type of exercise, and tissue bed(s) examined, it is not yet possible to make definitive conclusions about the impact of exercise training on NOS production in humans. Similarly, some (but not all¹³⁵) studies focused on ROS production in animal myocardium (as opposed to antioxidant expression) have suggested that exercise training attenuates ROS production during injury.^{136,137}

Exercise training can lead to local hypoxia, with tissue PaO₂ levels as low as 3 mm Hg (similar to those seen in hypoxia or ischemia).¹³⁸ Thus, one might expect HIF-1 activation during exercise training and indeed both exercise and muscle stimulation stabilize HIF-1 and increase VEGF expression.^{139–141} After longer periods of training (e.g. four to eight weeks), the increase in HIF-1 α and VEGF expression following acute exercise are attenuated, suggesting that chronic exercise reduces local hypoxia and/or the production of ROS (both of which can lead to HIF-1 α stabilization and subsequent VEGF expression).^{142,143}

Recognition that exercise training leads to mechanical, metabolic, and oxidative stress that produces downstream adaptations similar to those seen in classical ischemic preconditioning eventually led to the term “exercise preconditioning.”¹⁴⁴ In addition to antioxidant protection, HIF-1 α stabilization, VEGF expression, and possibly NOS expression, exercise may also lead to angiogenesis, enhanced expression of neurotrophin and thromboxane, and increased production of myocardial anti-apoptotic proteins and a reduction in ROS-induced cytochrome c release from mitochondria.^{145–147}

Unsurprisingly, attempts have been made to utilize exercise therapy for the prevention of organ injury following a variety of insults. Multiple studies have demonstrated reduced infarct volumes and in some cases improved survival in exercise trained animals subjected to stroke.^{146,148,149} Exercise therapy has also been tested in animals subjected to experimental sepsis, with trained animals demonstrating higher blood pressure, lower heart rate, and less pulmonary injury than untrained animals.¹⁵⁰ Recent work has suggested that quantifiable measures of fitness can be improved in the weeks leading up to elective surgery. Exercise therapy may thus be a viable pre-emptive treatment option for patients expecting to be subjected to significant stress in the near future (e.g. elective surgical procedures).^{151,152}

Therapeutic Gases

Nitric oxide (NO), hydrogen sulphide (H₂S), and carbon monoxide (CO) are gasotransmitter molecules that have specific biological functions, some of which are adaptive and some of which are potentially harmful.⁸³

NO is a well-established regulator of both endothelial function and vascular tone. It has multiple known biological targets, including soluble guanylate cyclase (GC), hemoglobin, and several cytochromes. Additionally, NO leads to the production of nitrite, nitrate, peroxynitrite, and S-nitrosothiols. Beyond its effects on the vascular smooth muscle and endothelium, NO is known to attenuate ischemia-reperfusion injury.¹⁵³ These effects are thought to be mediated by actions on the mitochondria. For instance – nitrite can nitrosate complex I of the ETC, limiting mitochondrial ROS production during hypoxia, and potentially decreasing cytochrome c release.¹⁵⁴ NO also directly inhibits the ETC, reducing energy production and ROS formation, the latter of which may limit apoptotic signaling.¹⁵⁵ In a small human trial, inhaled NO (80 ppm) was shown to attenuate hepatic ischemia-reperfusion injury during liver transplantation.¹⁵⁶

H₂S is produced endogenously by several enzyme systems, including 3-mercaptopyruvate sulphurtransferase (3-MST), cystathionine γ -lyase (CSE), and cystathionine β -synthase (CBS).⁸³ H₂S acts on both ion channels and several signaling proteins.⁸³ Like NO, H₂S appears to be required for preconditioning, as CSE inhibition (and thus attenuation of endogenous H₂S production) reduces the protective effects of preconditioning.^{157,158} H₂S appears to protect cells from increased intracellular Ca²⁺ overload by accelerating sarcoplasmic reticulum uptake and Na⁺/Ca²⁺ exchanger, both of which are protein kinase C dependent.⁷¹ Interestingly, administration of exogenous H₂S may protect against ischemia-reperfusion via a mTOR-dependent pathway.¹⁵⁹

Additionally, H₂S binds to cytochrome c oxidase, linearly reducing the metabolic rate (as measured by O₂ consumption and CO₂ production) and core body temperature of animals exposed to 0–80 ppm.¹⁶⁰ Notably, in mice subject to up to 80 ppm H₂S, complete reversal of this “suspended animation–like state” is possible with no observed deficits in function or behavior.¹⁶⁰ Furthermore, administration of H₂S (via Na₂S, an H₂S donor) prior to reperfusion leads to a reduction in inflammation, preservation of mitochondrial structure, and reduced infarct size following myocardial ischemia.¹⁶¹

While gaseous administration of H₂S is challenging, development of H₂S generating agents is underway (e.g. NaHS). Multiple authors have demonstrated a significant reduction in myocardial infarct size when NaHS is administered prior to the onset of ischemia.^{162,163} The mechanisms of preconditioning are multiple but may include antioxidant (including nuclear localization of Nrf2), anti-apoptotic (related to opening of mitochondrial K_{ATP} channels), and anti-inflammatory activity.⁸³

Carbon monoxide (CO), long known for its potential toxicity due to its inhibitory effects on the cytochrome c oxidase, is also produced endogenously by the heme oxygenase family of enzymes. Heme oxygenase 1 and 2 (HO-1, HO-2) degrade hemoglobin into CO and biliverdin, the latter of which is rapidly converted to bilirubin, an important

antioxidant.^{83,164} HO-1 has been shown to protect myocardial tissues from ischemia-reperfusion injury, and it is likely that CO production plays a prominent role in this phenomena.^{165–167} While bilirubin can readily scavenge ROS, CO can also impact ROS levels, primarily through its interaction with cytochrome c oxidase (which leads to increased ROS levels)¹⁶⁴ as well as activating anti-oxidant enzymes including superoxide dismutase, HO-1, and glutamate-cysteine ligase (which leads to GSH biosynthesis), referred to as a “pro/anti-oxidant duality.”¹⁶⁸ Interestingly, the cardioprotective effect of HIF-1 in the setting of ROS exposure may be dependent on HO-1 function.¹⁶⁹ Carbon monoxide’s HO-mediated antioxidant properties have also been demonstrated in a porcine model of CNS injury (seizure).¹⁷⁰ [Figure 6, adapted from Eltzschig and Eckle¹⁷¹]

Despite the fact that it inhibits cytochrome c oxidase, CO may, in some circumstances, actually *increase* ATP levels.^{167,172–174} While the mechanism for this counterintuitive phenomena has not been clarified, it is interesting to note that CO can lead to increases in HIF-1 α expression.¹⁷⁵ Also worth noting is that while CO binds to cytochrome c oxidase, it has much greater affinity for hemoglobin and myoglobin.¹⁷⁶ This raises the possibility that low dose CO may lead to a “pseudohypoxic” state, in which cells sense and respond to “hypoxia” when oxygen supplies are adequate.¹⁶⁷ In addition to potential protection from ROS-mediated injury as well as increased ATP production (at low levels of exposure), CO exerts anti-inflammatory effects primarily through MAP kinase pathways (in particular, p38 MAPK),¹⁶⁴ modulates apoptosis,¹⁶⁷ and induces mitochondrial biogenesis.¹⁷⁷

Exposure to CO either directly (gaseous form) or via a CO releasing molecule (e.g. CORM-3) has been shown to reduce inflammation and improve mortality in a variety of sepsis models.^{172,173,177–181} Similarly, in pigs exposed to cardiopulmonary bypass (which elicits a profound pro-inflammatory response), exposure to CO improves myocardial energetics, increasing ATP availability and decreasing apoptosis following cardioplegic arrest.¹⁷⁴

H₂ has been shown to protect cultured cells against hydroxide exposure, and inhalation of H₂ has been shown to attenuate ischemia-reperfusion injury in rats subjected to middle cerebral artery occlusion as well as reduce oxidative stress in rats subjected to experimental subarachnoid hemorrhage.^{182,183} Unlike other potentially therapeutic gases, H₂ is not produced endogenously.

HIF and PHD Inhibitors

A variety of anti-neoplastic agents have been developed to inhibit HIF-1 α -related transcription, protein translation (and degradation), or DNA binding. Particular promising include YC-1 (which inhibits expression of HIF-1 α by interfering in a variety of HIF-1 α mediated activities and has been demonstrated to arrest cell cycling and lead to apoptosis in cancer cells), and ENMD-1198 and PX-478 (both of which also inhibit HIF-1 α at multiple levels and are part of the 2-methoxyestradiol analog class of agents that have moved to clinical trials).¹⁸⁴ In general these agents show great anti-neoplastic potential with less cytotoxicity than traditional agents.

HIF inhibitors seek to prevent cancerous cells from adapting to hypoxia. HIF prolyl hydroxylases (PHDs) degrade HIF-1 α during normoxia, preventing expression of HIF-1 α -regulated genes (which improve tolerance to hypoxia and ROS, in addition to mediating preconditioning). The goal of PHD inhibition is to prevent the development of a hypoxic environment conducive to cancer cell survival and metastasis. From an anti-neoplastic perspective, the main interest in PHD inhibition is in the potential to alter the vascular endothelium by inducing “vascular normalization” and angiogenesis, both of which prevent the development of a hypoxic environment that encourages tumor growth.^{185–188}

Outside of the oncologic environment, expression of HIF-1 α without exposure to hypoxia or ischemia could potentially produce the beneficial effects of HIF-1 α upregulation on cell survival without incurring the risks associated with hypoxia or ischemia. Inhibition of PHDs has, for instance, been shown to attenuate ischemia-reperfusion injury in both renal and myocardial tissue in mice.^{77,189} PHD inhibitors are now under development for humans, and they appear to be well tolerated.^{190,191} Injection of the HIF-stabilizing agent cobalt chloride (CoCl₂) has also been shown to decrease tissue injury in an animal model of ischemia and reperfusion.⁸⁴

PPAR Activation

As described above, PPAR α mediates the metabolic switch that accompanies the HIF-mediated response to hypoxia. Administration of the PPAR α agonist fenofibrate to mice improved their tolerance to hypoxia.¹⁹² Similarly, administration of the PPAR α / δ agonist T33 reduces infarct size following middle cerebral artery occlusion in rats.¹⁹³ Administration of the PPAR γ agonists, rosiglitazone and pioglitazone (but not WY-14643, a PPAR α agonist) have been shown to reduce mortality in rodents subjected to sepsis.^{194,195} Like PPAR α , PPAR γ modulates metabolism and is upregulated by HIF-1 in the setting of hypoxia.¹⁹⁶

3. Next Steps and Clinical Application

More widespread use of broadband NIRS and the protoporphyrin IX-triplet state lifetime technique in humans studies could clarify much of the confusion about the role of lactate and subcellular alterations in a variety of critically ill or perioperative patients populations. The mortality rate of sepsis remains disturbingly high,^{197–200} and advanced diagnostic techniques combined with targeted therapeutics may offer the ability to identify and correct subcellular derangements that were previously misunderstood, undiagnosed, and untreated. Given their favorable risk-benefit profile and relatively low cost, preconditioning and exercise training deserve additional investigation in prospective, clinical trials. Therapeutic gases, HIF-1 α and PHD inhibitors, and PPAR α activators will likely need additional translational work before being tested in humans.

While the 30-day mortality for perioperative patients is considerably lower,²⁰¹ for specific patient populations the ability to better monitor and manage subcellular energetics might still be useful. Spinal cord paralysis after thoracic aortic surgery, kidney injury after cardiac surgery, and cerebral dysfunction after deep hypothermic circulatory arrest all pose substantial risks in patients undergoing these high risk surgical procedures.^{202–205} The

ability to prepare for, detect, and actively manage organ injury could be lifesaving in these and other at risk populations.

4. Conclusions

P^{31} NMR has provided researchers with significant insight into intracellular pH and high energy phosphate levels during a variety of pathologies including sepsis but is clinically impractical. Measurement of serum lactate can provide insight into activity of anaerobic metabolic pathways but is not organ-specific and larger trials are needed to assess the utility of this simple, non-invasive measurement technique. Near infrared spectroscopy allows researchers to measure the oxidation state of cytochrome aa₃, a critical component of the electron transport chain, in real time. The protoporphyrin IX-triplet state lifetime technique allows researchers to measure mitochondrial oxygen tension. These latter two techniques may yield additional insight into the mechanisms leading to subcellular energetic disarray, in addition to one day providing a useful clinical monitor, and have significant clinical potential. Recent findings in subcellular energetics implicate hyperbaric oxygen therapy, preconditioning, exercise training, therapeutic gases, HIF and PHD inhibitors, and PPAR inhibition as potential treatment modalities in a variety of pathological conditions for which current treatment strategies are only marginally successful (e.g. sepsis, ischemia, cancer).

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Figure 1.

Four techniques for assessing the state of subcellular energetics (in yellow). ³¹P NMR can measure the concentration of ATP (and also intracellular pH) in both intact animals and tissue, but requires expensive, bulky equipment and cannot provide continuous measurements. Broadband NIRS can assess the oxidation state of cytochrome aa₃, the terminal component of the electron transport chain and the enzyme which adds protons from the mitochondrial matrix and electrons from the electron chain to molecular oxygen, producing water. This technique requires advanced spectroscopy equipment but is non-invasive and can be performed at the bedside. Lactate is a byproduct of anaerobic metabolism and, while produced by the mitochondria when oxygen supplies diminish, can be easily measured in the serum. The Protoporphyrin IX-Triplet State Lifetime Technique can measure *mitochondrial* oxygen levels and can also be performed at the bedside.

1) Each Chromophore Produces A Unique Spectrum

2) These Can Be Mathematically Combined Using Matrix Algebra

$$\begin{pmatrix} [\text{Compound X}] \\ [\text{Compound Y}] \\ [\text{Compound Z}] \end{pmatrix} = \begin{pmatrix} c_{11} & c_{12} & c_{13} & c_{14} & c_{15} & c_{16} \\ c_{21} & c_{22} & c_{23} & c_{24} & c_{25} & c_{26} \\ c_{31} & c_{32} & c_{33} & c_{34} & c_{35} & c_{36} \end{pmatrix} \times \begin{pmatrix} OD1 \\ OD2 \\ OD3 \\ OD4 \\ OD5 \\ OD6 \end{pmatrix}$$

3) By Transmitting and Recording Light Into Tissue, Absorbance / Optical Density Can Be Measured

4) Concentrations Are Then Estimated Based On Absorbance / Optical Density At Various Wavelengths

$$[\text{Compound X}] = c_{11} \cdot OD1 + c_{12} \cdot OD2 + c_{13} \cdot OD3 + c_{14} \cdot OD4 + c_{15} \cdot OD5 + c_{16} \cdot OD6$$

Figure 2.

Graphical depiction of how near infrared radiation can be utilized to calculate chromophore concentrations using the Beer-Lambert principle. In this example, a six wavelength device is used to measure three chromophore concentrations (X, Y, and Z). A light source (A) produced light at various wavelengths, which enters tissue (a). Transmitted light (b) is carried to a spectrometer (B), which sends the information to a laptop computer (C). Based on measurements of the emitted and detected light, absorbance (and optical density) can be calculated, which, when combined with known spectral data for compounds X, Y, and Z, can estimate the concentration of each.

Figure 3.

Impact of sodium cyanide infusion on they oxygenation of tissue hemoglobin (right, deoxyhemoglobin [blue], oxyhemoglobin [red], and total hemoglobin [black] are shown) and the oxidation state of cytochrome aa₃ (right, reduced cytochrome aa₃ [blue], oxidized cytochrome aa₃ [red], and tissue hemoglobin saturation [black] shown). Note that while cytochrome aa₃ transitions from and oxidized to reduced state, StO₂ *increases*.

Figure 4.

A. Graphical depiction of the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT). 5-aminolevulinic acid (ALA), a porphyrin precursor which increases mitochondrial protoporphyrin IX concentrations, is applied topically. A green (510 nm) excitation pulse passes into tissue, exciting the porphyrin-metal complex. Delayed fluorescence is a function of oxygen concentrations and is detected at 630–700 nm wavelength. **B.** A collision between oxygen and a porphyrin in a triplet state will sometimes transfer energy to the oxygen, which then transitions into a triplet state. This is referred to as “triplet state quenching” and leads to both phosphorescence and delayed fluorescence, both of which are detectable and allow for the estimation of pO_2 in tissue.

Figure 5.
In patients subjected to cardiopulmonary exercise testing (CPET) prior to major surgery, those who fail to meet population-based activity thresholds (e.g. VO_2 at aerobic threshold, peak VO_2 , and the ventilatory equivalent ratio for carbon dioxide) are less likely to survive.

Figure 6.

Ischemia leads to inflammation, tissue injury, and ROS production, which, when coupled with reperfusion can lead to injury. Gaseous transmitters now known to be produced endogenously (e.g. CO, NO) may have the ability to modify the response to IR injury at various points along the pathway to injury.

Table 1

Endogenous Gases and their Effects

Endogenous Gas		Site of Action(s)	Effect(s)
Nitric Oxide	NO	Guanylate cyclase, hemoglobin, cytochromes	Regulates vascular tone, inhibits electron transport chain
Hydrogen Sulphide	H ₂ S	Ion channels, signalling proteins	Prevents intracellular Ca ⁺⁺ overload
Carbon Monoxide	CO	Cytochrome c oxidase; L-type Ca ⁺⁺ channel	Activation of anti-oxidants, anti-inflammatory activity