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The Role of Nitric Oxide in Low Level Light Therapy.

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ABSTRACT

The use of low levels of visible or near infrared light for reducing pain, inflammation and edema, promoting healing of wounds, deeper tissues and nerves, and preventing tissue damage by reducing cellular apoptosis has been known for almost forty years since the invention of lasers. Despite many reports of positive findings from experiments conducted in vitro, in animal models and in randomized controlled clinical trials, LLLT remains controversial. Firstly the biochemical mechanisms underlying the positive effects are incompletely understood, and secondly the complexity of choosing amongst a large number of illumination parameters has led to the publication of a number of negative studies as well as many positive ones. This review will focus on the role of nitric oxide in the cellular and tissue effects of LLLT. Red and near-IR light is primarily absorbed by cytochrome c oxidase (unit four in the mitochondrial respiratory chain). Nitric oxide produced in the mitochondria can inhibit respiration by binding to cytochrome c oxidase and competitively displacing oxygen, especially in stressed or hypoxic cells. If light absorption displaced the nitric oxide and thus allowed the cytochrome c oxidase to recover and cellular respiration to resume, this would explain many of the observations made in LLLT. Why the effect is only seen in hypoxic, stressed or damaged cells or tissues? How the effects can keep working for some time (hours or days) post-illumination? Why increased NO concentrations are sometimes measured in cell culture or in animals? How blood flow can be increased? Why angiogenesis is sometimes increased after LLLT in vivo?

Keywords: biostimulation, low level laser therapy, mitochondria, cytochrome c oxidase, nitric oxide

1. LOW LEVEL LIGHT THERAPY

Although low level light therapy (LLLT) has been known and increasingly widely practiced for over forty years, it is still regarded with some skepticism by laymen and medical professionals alike, and has not reached acceptance by mainstream medicine. The single most important reason for this lack of acceptance is likely to be the inability of most practitioners of LLLT to satisfactorily explain how it works on a molecular, cellular and tissue level. There is a need for more fundamental research on identifying photoacceptor molecules, elucidating cell and signaling pathways that are engaged after cells absorb visible photons. Furthermore it is necessary to investigate relationships between the optical parameters of the light such as wavelength, total delivered energy, rate at which energy is delivered, coherence, polarization state and pulse structure

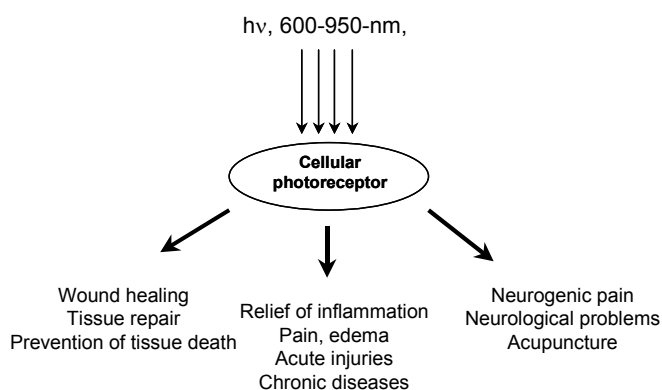


Figure 1. Schematic representation of the main areas of application of LLLT

2. MITOCHONDRIA CONTAIN CELLULAR PHOTOACCEPTORS

2.1 Mitochondria

Mitochondria are distinct organelles with two membranes and are usually rod-shaped. Mitochondria are sometimes described as "cellular power plants," because they convert food molecules into energy in the form of ATP via the process of oxidative phosphorylation. A typical eukaryotic cell contains about 2,000 mitochondria, which occupy roughly one fifth of its total volume. Mitochondria contain DNA that is independent of the DNA located in the cell nucleus. Mitochondrial DNA is circular and lies in the matrix in punctate structures called "nucleoids" each containing 4-5 copies of the mitochondrial DNA (mtDNA). Mitochondria have their own ribosomes, and can make many of their own proteins. The outer membrane limits the organelle, while the inner membrane is thrown into folds or shelves that project inward called "cristae mitochondriales". A mitochondrion contains inner and outer membranes composed of phospholipid bilayers and proteins, and consequently there are 5 distinct compartments within mitochondria. There is the outer membrane, the intermembrane space (the space between the outer and inner membranes), the inner membrane, the cristae space (formed by infoldings of the inner membrane), and the matrix (space within the inner membrane). Mitochondria range from 1 to 10 μm in size.

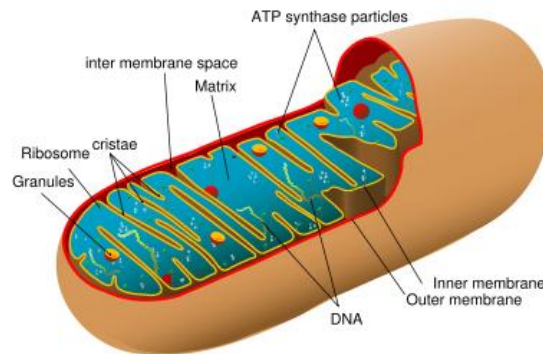


Figure 2. Schematic representation of the structure of a mitochondrion in a mammalian cell

A dominant role for the mitochondria is the production of ATP as reflected by the large number of proteins in the inner membrane needed for this task. This is done by oxidizing the major products of glycolysis, pyruvate and NADH that are produced in the cytosol. This process of cellular respiration, also known as aerobic respiration, is dependent on the presence of oxygen. When oxygen is limited the glycolytic products will be metabolized by anaerobic respiration, a process that is independent of the mitochondria. The production of ATP from glucose has an approximately 15-fold higher yield during aerobic respiration compared to anaerobic respiration. In addition to their role in producing cellular energy in the form of ATP, mitochondria play an important role in many other metabolic tasks, such as, apoptosis (programmed cell death), glutamate-mediated excitotoxic neuronal injury, cellular proliferation, regulation of the cellular redox state, heme synthesis and steroid synthesis.

Each pyruvate molecule produced by glycolysis is actively transported across the inner mitochondrial membrane, and into the matrix where it is oxidized and combined with coenzyme A to form CO_2 , acetyl-CoA and NADH. The acetyl-CoA is the primary substrate to enter the citric acid cycle, also known as the tricarboxylic acid (TCA) cycle or Krebs cycle. The enzymes of the citric acid cycle are located in the mitochondrial matrix with the exception of succinate dehydrogenase, which is bound to the inner mitochondrial membrane. The citric acid cycle oxidizes the acetyl-CoA to carbon dioxide and in the process produces reduced cofactors (three molecules of NADH and one molecule of FADH_2), that are a source of electrons for the electron transport chain, and a molecule of GTP (that is readily converted to ATP).

2.2 Mitochondrial Respiratory Chain

Four membrane-bound complexes have been identified in mitochondria. Each is an extremely complex transmembrane structure that is embedded in the inner membrane. Three of them are proton pumps. The structures are electrically connected by lipid-soluble electron carriers and water-soluble electron carriers.

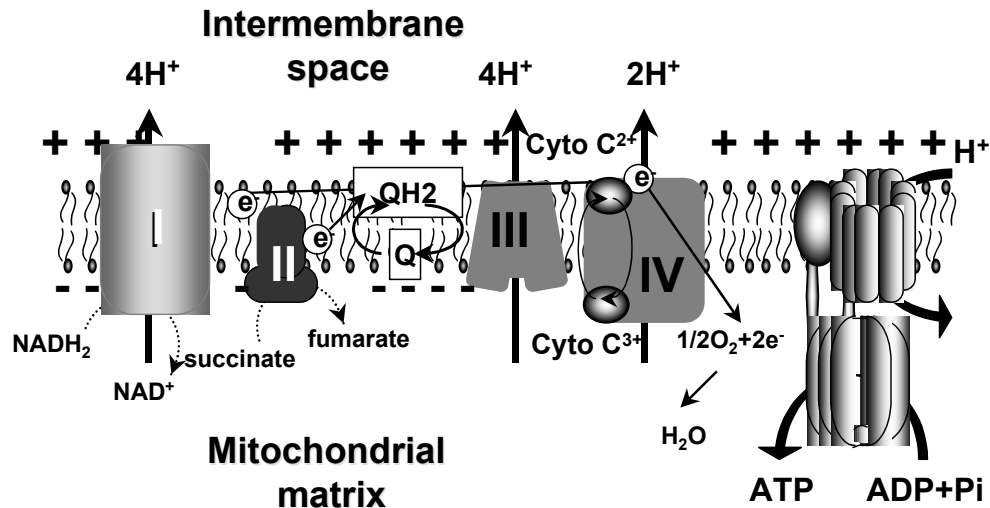


Figure 3. Structure of the electron transport chain in the mitochondrial inner membrane

2.2.1 Complex I

Complex I (NADH dehydrogenase, also called NADH:ubiquinone oxidoreductase; EC 1.6.5.3) removes two electrons from NADH and transfers them to a lipid-soluble carrier, ubiquinone (Q). The reduced product, ubiquinol (QH₂) is free to diffuse within the membrane. At the same time, Complex I moves four protons (H⁺) across the membrane, producing a proton gradient. Complex I is one of the main sites at which premature electron leakage to oxygen occurs, thus being one of main sites of production of a harmful free radical called superoxide. NADH is oxidized to NAD⁺, reducing Flavin mononucleotide to FMNH₂ in one two-electron step. The next electron carrier is a Fe-S cluster, which can only accept one electron at a time to reduce the ferric ion into a ferrous ion. In a convenient manner, FMNH₂ can be oxidized in only two one-electron steps, through a semiquinone intermediate. The electron thus travels from the FMNH₂ to the Fe-S cluster, then from the Fe-S cluster to the oxidized Q to give the free-radical (semiquinone) form of Q. This happens again to reduce the semiquinone form to the ubiquinol form, QH₂. During this process, four protons are translocated across the inner mitochondrial membrane, from the matrix to the intermembrane space. This creates a proton gradient that will be later used to generate ATP through oxidative phosphorylation.

2.2.2 Complex II

Complex II (succinate dehydrogenase; EC 1.3.5.1) is not a proton pump. It serves to funnel additional electrons into the quinone pool (Q) by removing electrons from succinate and transferring them (via FAD) to Q. Complex II consists of four protein subunits: SDHA, SDHB, SDHC, and SDHD. Other electron donors (e.g., fatty acids and glycerol 3-phosphate) also funnel electrons into Q (via FAD), again without producing a proton gradient.

2.2.3 Complex III

Complex III (cytochrome bc₁ complex; EC 1.10.2.2) removes in a stepwise fashion two electrons from QH₂ and transfers them to two molecules of cytochrome c, a water-soluble electron carrier located within the intermembrane space. At the same time, it moves two protons across the membrane, producing a proton gradient (in total 4 protons: 2 protons are translocated and 2 protons are released from ubiquinol). When electron transfer is hindered (by a high

membrane potential, point mutations or respiratory inhibitors such as antimycin A), Complex III may leak electrons to oxygen resulting in the formation of superoxide, a highly-toxic species, which is thought to contribute to the pathology of a number of diseases, including aging.

2.2.4 Complex IV

Complex IV (cytochrome c oxidase; EC 1.9.3.1) removes four electrons from four molecules of cytochrome c and transfers them to molecular oxygen (O₂), producing two molecules of water (H₂O). At the same time, it moves four protons across the membrane, producing a proton gradient.

2.3 *Mitochondria absorb visible light.*

Several pieces of evidence suggest that mitochondria are responsible for the cellular response to red visible and NIR light. The most popular system to study is the effects of HeNe laser illumination of mitochondria isolated from rat liver. Increased proton electrochemical potential and ATP synthesis was found [1]. Increased RNA and protein synthesis was demonstrated after 5 J/cm² of HeNe laser light [2]. Pastore et al [3] found increased activity of cytochrome c oxidase and an increase in polarographically measured oxygen uptake after 2 J/cm² of HeNe laser. A major stimulation in the proton pumping activity, about 55% increase of $\Delta\psi$ ratio was found in illuminated mitochondria. Yu et al [4] used 660 nm laser at a power density of 10 mW/cm² and showed increased oxygen consumption (0.6 J/cm² and 1.2 J/cm²), increased phosphate potential, and energy charge (1.8 J/cm² and 2.4 J/cm²) and enhanced activities of NADH: ubiquinone oxidoreductase, ubiquinol: ferricytochrome c oxidoreductase and ferrocyanide: oxygen oxidoreductase (between 0.6 J/cm², and 4.8 J/cm²).

Irradiation of mitochondria with light at wavelengths of 650, and 725 nm [5] enhanced ATP synthesis. Light at wavelengths of 477 and 554 nm did not influence the rate of this process. Oxygen consumption was increased by illuminating with light at 365 and 436 nm, but not at 313, 546, and 577 nm [6]. Irradiation with light at 633 nm increased the mitochondrial membrane potential and proton gradient, caused changes in mitochondrial optical properties, modified some NADH-linked dehydrogenase reactions (NADH is a reduced form of nicotinamide adenine dinucleotide), and increased the rate of ADP/ATP exchange (ADP is adenosine diphosphate) [7], as well as RNA and protein synthesis in the mitochondria. In the case of state 4 respiration, 351 and 458 nm laser irradiations accelerated the oxygen consumption of rat liver mitochondria; such an acceleration was not observed with 514.5 nm irradiation. In the case of state 4 respiration (slower rate after all the ADP has been phosphorylated to form ATP), 351 and 458 nm laser irradiations accelerated the oxygen consumption of rat liver mitochondria; such an acceleration was not observed with 514.5 nm irradiation. On the contrary, in the case of state 3 respiration (active rate in presence of sufficient substrate, O₂ and ADP), 514.5 nm argon laser irradiation activated the oxygen consumption of mitochondria. Activation did not occur with 458 nm irradiation and 351 nm irradiation reduced the oxygen consumption in state 3 [8]. 660 nm irradiation increased state 3 oxygen consumption, as well as increasing the respiratory control ratio [4]. It is also believed that mitochondria are the primary targets when the whole cells are irradiated with light at 630, 632.8 [9-11], or 820 nm. Irradiation with light at 812 [12] or 632.8 nm altered the rhodamine 123 uptake by fibroblasts. These results were interpreted by the authors as inducing the perturbation of mitochondrial energy production and membrane potential.

2.3 *Cytochrome c oxidase is a photoacceptor.*

In 1995, Karu defined the action spectra for mammalian cells of several processes stimulated by LLLT such as DNA and RNA synthesis, and cellular adhesion [13]. The action spectra for all of these secondary markers were very similar suggesting a common photoacceptor that can transduce light energy to accelerate all these processes. Karu then compared these action spectra with visible and NIR absorption spectra of the copper centers of cytochrome c oxidase in both reduced and oxidized states. Cytochrome c oxidase contains four redox active metal centers and has a strong absorbance in the near infrared spectral range. The spectral absorbance of cytochrome c oxidase and the action spectra were very similar. Based on this, Karu suggested that the primary photoacceptors are mixed valence copper centers within cytochrome c oxidase [14]. Cytochrome c oxidase is the terminal enzyme of the mitochondrial electron transport chain of all eukaryotes and is required for the proper function of almost all cells especially those of highly metabolically active organs, such as the brain and heart. Recently, work from the Whelan

group from Medical College of Wisconsin has also suggested that cytochrome c is the critical chromophore responsible for stimulatory effects of irradiation with infrared light [15-17]. Wong-Riley et al. [18] demonstrated that infrared irradiation reversed the reduction in cytochrome c oxidase activity produced by the blockade of voltage dependent sodium channels with tetrodotoxin and up regulated cytochrome c activity in primary neuronal cells. In vivo Eells et al demonstrated that rat retinal neurons are protected from damage induced by methanol intoxication [19]. The actual toxic metabolite formed from methanol is formic acid which inhibits cytochrome c.

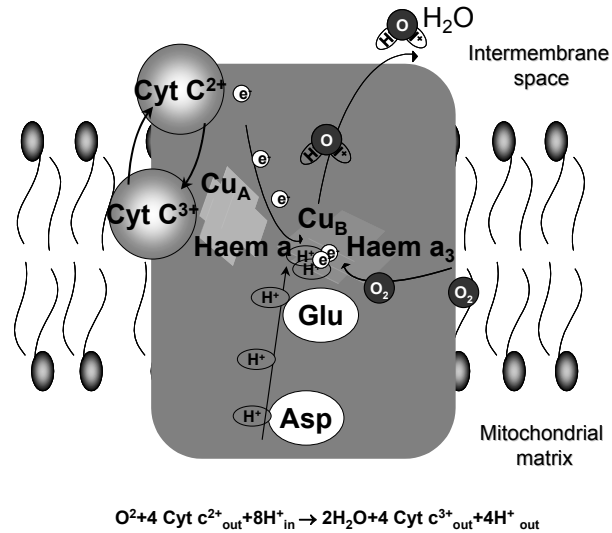


Figure 4. Structure and mode of action of cytochrome c oxidase

Absorption spectra obtained for cytochrome c oxidase in different oxidation states were recorded and found to be very similar to the action spectra for biological responses to light. Therefore it was proposed that cytochrome c oxidase is the primary photoacceptor for the red-NIR range in mammalian cells [13]. Cytochrome c oxidase (Structure is shown in Figure 4) contains two iron centers, haem *a* and haem *a*₃ (also referred to as cytochromes *a* and *a*₃), and two copper centers, Cu_A and Cu_B [20]. Fully oxidized cytochrome c oxidase has both iron atoms in the Fe(III) oxidation state and both copper atoms in the Cu(II) oxidation state, while fully reduced cytochrome c oxidase has the iron in Fe(II) and copper in Cu(I) oxidation states. There are many intermediate mixed-valence forms of the enzyme and other coordinate ligands such as CO, CN, and formate can be involved. All the many individual oxidation states of the enzyme have different absorption spectra [21], thus probably accounting for slight differences in action spectra of LLLT that have been reported. A recent paper from Karu's group [14] gave the following wavelength ranges for four peaks in the LLLT action spectrum: 1) 613.5 - 623.5 nm, 2) 667.5 - 683.7 nm, 3) 750.7 - 772.3 nm, 4) 812.5 - 846.0 nm.

A study from Pastore et al [22] examined the effect of He-Ne laser illumination on the purified cytochrome c oxidase enzyme and found increased oxidation of cytochrome c and increased electron transfer. Artyukhov and colleagues found [23] increased enzyme activity of a different enzyme catalase after He-Ne illumination. Absorption of photons by molecules leads to electronically excited states and consequently can lead to acceleration of electron transfer reactions [4]. More electron transport necessarily leads to increased production of ATP [24].

3. NITRIC OXIDE

3.1 Formation and action of NO.

Nitric oxide (NO), a free radical gas that is a powerful regulator of circulation (it is an endogenous vasodilator) and a neurotransmitter (it helps in the processing of nerve signals as they cross synapses). L-arginine, one of 20 amino acids that make up proteins, is the only amino acid that generates significant amounts of NO. The Nobel Prize was awarded to three Americans in 1998 for their work on discovering NO and clarifying its role in health [25]. Their most important contributions lay in describing the effect of NO on the circulation. The blood flow and nerve responses are rapid. Small increases in NO lead to both vasodilation and to better sensory perception. NO metabolism is necessary for normal circulation (venous, arterial, and lymph flows) and for the ability to sense pain, temperature, and pressure.

The amino acid L-arginine, that is the main source of NO is released from proteins and small peptides in the small intestine and is then absorbed, along with other amino acids into the circulation from which it is delivered to every cell in the body. Some L-arginine is metabolized for NO synthesis and some is used for protein synthesis. In endothelial cells, the small cells that make up capillaries and line every blood vessel and lymph duct in the body, L-arginine can be converted to NO. This occurs only if the enzyme that makes NO and its co-factors are available in adequate amounts. In diabetic patients and those with atherosclerotic disease plaques often occludes a portion of a vessel so that the endothelial cells are not able to properly absorb NO. If the endothelial cell cannot take up L-arginine, then NO synthesis will be impaired. Moreover, if atherosclerotic disease is present, oxygen delivery to all cells is impaired and molecular oxygen is one of the cofactors needed by the enzyme to generate NO from L-arginine. The NO diffuses into the smooth muscle cells that surround the endothelial lining of blood vessels cells causing a biologic chain of events that lead to smooth muscle cell relaxation. This results in more blood flow to the tissues. Tissues that are hypoxic (deprived of good, normal circulation) can not produce as much NO as do normal, well oxygenated tissues. Thus an initial period of hypoxia leads to declines in NO production and less and less blood flow over time.

Nitric oxide synthase (NOS) is the enzyme that generates NO from L-arginine. There are three different type of NOS: neuronal nitric oxide synthase (NOS1), inducible nitric oxide synthase (NOS2) and endothelial nitric oxide synthase (NOS3) [26]. Each of them have different tissue distributions and located on different human chromosomes. They may related to many human diseases, such as Alzheimers dieeseases, Parkinsons. diabetes, asthma, heart disease, infection diseases. Often all three isoforms will be found in the same cell but occasionally one cell will contain only one of the isoforms.

NOS1 is the neuronal (or brain) isoform. It helps in synaptic transmission, the processing of nervous information from nerve to nerve, across gaps between the nerves called synapses, and from peripheral nerves to the brain.

NOS2 is called inducible or iNOS. This enzyme generates extraordinarily high concentrations of NO, in part to kill bacteria. NOS2 (iNOS) takes several hours to be mobilized and the response is due to an injury or infectious process. NOS2 produced by macrophages is responsible, in part, for their effects to repair injury and to ward off infections. In other words, when the body mounts an inflammatory response to injury, macrophages are attracted to the site of injury where they produce large amounts of NO. Extraordinarily high concentrations of NO (100 to 1000 times normal) are produced very locally by this isoform. In fact, reports suggest that wound (ulcer) fluid may contain levels of NO that are very high and can only be attributed to iNOS. Unlike NOS1, which is part of normal neurotransmission, there must be something very abnormal (a wound, tissue damage, hypoxia, bacterial infection, etc.) to induce this enzyme.

The third isoform is eNOS (or NOS3) which stands for “endothelial” NOS. This isoform is active at all times (it does not need to be induced as does iNOS) and is found in endothelial cells which are the cells that line the inner surface of all blood vessels and lymph ducts. eNOS is activated by the pulsatile flow of blood through vessels. This leads to a “shear stress” on the membrane of the endothelial cells as the column of blood in the vessel moves forward and then stops. This NO, produced by eNOS, maintains the diameter of blood vessel so that perfusion of tissues (skin, muscle, nerves, and bone) is maintained at optimal levels. In addition, eNOS mediated NO causes angiogenesis, which is the growth of new blood vessels. This is especially important in healing an ulcer or wound on the skin.

One interesting interplay of iNOS and eNOS is in tissue repair. Initially, NO is generated from iNOS in order to ward off infection and to destroy and remove the irreversibly damaged, necrotic tissue. This is often referred to as the inflammatory stage of wound repair. This phase lasts only a short time (a few days with an acute wound) and then eNOS is (or should be) mobilized to cause vasodilation and angiogenesis to induce the healing response. NO will relax smooth muscle cells and thus dilate veins, arteries, and lymphatics. This increases blood supply both to the repairing tissues and from the damaged region. The latter removes metabolic waste products, reduces edema, and prevents swelling that would otherwise compress capillaries. In the absence of adequate blood supply tissue will remain hypoxic and heal only slowly, if at all. Moreover, since iNOS is produced in large part by white blood cells (WBC), vasodilation permits delivery of additional WBC to the area that needs to be defended from infection. There are wounds that do become infected and often only marginal reduction of the infection is seen even with high dose and high potency antibiotics. If the vascular bed (arteries, veins, and lymphatics) were dilated, more of the antibiotic would get to site of infection. Thus it is essential that eNOS be activated to produce NO. Clearly both eNOS and iNOS play a role in wound healing; neither alone is sufficient to achieve full recovery. In diabetic patients, however, eNOS activity is often well below normal so these patients cannot produce NO at normal levels.

3.2 Nitric oxide in mitochondria

Over the past decade it has been discovered that cells often use NO to block respiration. Nitric oxide is emitted by nerve endings and can act on an enzyme called guanylate cyclase to relax blood vessels. For a long time, scientists thought that guanylate cyclase was the only target of NO, but in the mid-1990s, they found that the molecule could also bind to cytochrome oxidase and hinder respiration [27]. The finding that the body could poison one of its own enzymes was initially shrugged off as an imperfection, but a few years later, several groups reported that mitochondria contained a particular isoform of nitric oxide synthase [28]. This mitochondrial NOS was identified as the neuronal isoform by Kanai et al [29]. Moncada proposed that evolution really crafted cytochrome oxidase to bind not only oxygen but also NO [27]. One effect of slowing respiration in some locations would be to divert oxygen elsewhere in cells and tissues. This prevents oxygen levels sinking dangerously low. NO blocks respiration in the cells lining blood vessels and that this helps to transfer oxygen into smooth muscle cells in these vessels. Respiration does not just generate energy, but it also generates feedback that allows a cell to monitor and respond to its environment. Blocking respiration generates chemical signals, in the form of reactive oxygen species (ROS) such as superoxide. ROS are normally associated with cell damage, but now it is thought they can interact with the proteins that control gene activity and adapt cells to changing circumstances. In the past few years, researchers have compiled a list of these proteins, or transcription factors, the activity of which depends, at least in part, on interactions with ROS [30]. These include many proteins known to be linked to cellular life and death, such as p53. The whole system is thought to be controlled by cytochrome c oxidase, which catalyses the final step of respiration, in which electrons and protons are transferred onto oxygen to form water. The cell can suppress the number of free radicals coming from these respiratory chains by allowing protons to leak back through the membrane without driving the synthesis of ATP, a process known as uncoupling [31]. But if uncoupling does not bring free-radical leak under control, the signal may be amplified. Cells that depend on mitochondria for energy, such as neurons, may be pushed to apoptosis by NO binding, making degenerative disease more likely. The activity of cytochrome c oxidase is inhibited by nitric oxide (NO). This inhibition of mitochondrial respiration by NO can be explained by a direct competition between NO and O₂ for the reduced binuclear center CuB/a₃ of cytochrome c oxidase and is reversible [32].

3.3 Interaction of Cytochrome C Oxidase With Nitric Oxide

The interaction of NO with cytochrome C oxidase in different types of cells is associated with the resistance to apoptosis induced by various kinds of stressors, including growth factor deprivation [33], treatment with staurosporine [34], O₂ limitation [34], or intracellular calcium overload [35]. Depending on the system under study, protection was shown to be associated with an increase in mitochondrial membrane potential ($\Delta\Psi_m$) [33], with an increase in glycolytic output linked to upregulation of AMP-activated protein kinase (AMPK) [36], or with changes in calcium efflux leading to the induction of the cytoprotective chaperone protein Grp78.[35]. Further studies also showed that competition between NO and O₂ at the level of cytochrome C oxidase is responsible for the inhibition of hypoxia-inducible factor (HIF) 1- α stabilization observed in the presence of NO under otherwise limiting O₂ concentrations [37], suggesting that mitochondria under the influence of NO may also be involved in the attenuation of adaptive responses to low O₂. In addition, there is evidence that NO promotes mitochondrial biogenesis by a mechanism that is independent of cytochrome C oxidase but involves activation of the soluble guanylate cyclase.

Excessive production of NO and mitochondrial dysfunction have for many years been independently associated with pathophysiological mechanisms. However, the fact that NO inhibits mitochondrial respiration suggests that there may be instances in which NO production, mitochondrial dysfunction, and pathology could be intimately related [38]. This may depend on the biochemical actions of NO on mitochondria, their signaling consequences, and their possible relationship to cellular homeostasis and pathophysiology.

Cytochrome C oxidase is situated on the inner membrane of the mitochondrion, where it catalyzes the oxidation of cytochrome C and the reduction of O₂ to water in a process linked to the pumping of protons out of the mitochondrial matrix. The enzyme contains 2 heme (a and a₃) and 2 copper centers (CuA and CuB), of which the heme iron of cytochrome a₃ together with CuB, in their reduced form, form the O₂-binding site. NO closely resembles O₂ and therefore can also bind to this site. In the mid 1990s it was demonstrated that NO inhibits the activity of cytochrome C oxidase [39-41]. This inhibitory effect was shown to be reversible, in competition with O₂, and to occur at concentrations of NO likely to be present physiologically. Thus for example at 30 μM O₂ (approximately the tissue concentration of O₂) the IC₅₀ of NO for cytochrome C oxidase is 60 nM, whereas at 10 μM (a possible intracellular concentration of O₂) [42] the IC₅₀ of NO for the enzyme would be predicted to be approximately 20 nM. In addition, it has recently been reported [43] that the K_i of NO for the O₂-binding site of cytochrome C oxidase is 0.2 nM, confirming that concentrations of NO that have been detected in tissues (10 to 450 nM) [44, 45] would be sufficient to compete with intracellular O₂. The potential biological relevance of the NO-cytochrome C oxidase interaction has been further highlighted by a number of studies demonstrating inhibition of respiration by endogenously-generated NO, or its enhancement by inhibitors of NOS in a number of cells, isolated tissues, and whole animals [46-49]. In studies with vascular endothelial cells in culture it was found that endogenous concentrations of NO modulate cell respiration in an oxygen-dependent manner [48]. Furthermore, treatment with the neuropeptide bradykinin, which activates the endothelial isoform of NO synthase (eNOS), generated concentrations of NO that inhibited respiration further. Conversely, treatment with an inhibitor of NOS resulted in an immediate increase in O₂ consumption, suggesting that endogenous NO interacts with cytochrome C oxidase and modulates O₂ consumption under basal and stimulated conditions. Consistent with studies with isolated cytochrome C oxidase [50, 51], further work using intact cells suggests that NO interacts with the enzyme in two ways [52]. In the first case, which occurs at high O₂ and low electron turnover in the enzyme, NO interacts primarily with the prevailing oxidized species of the catalytic cytochrome C oxidase cycle, resulting in an increase in the reduced fraction of cytochromes c₁ and consequently a rise in the reductive pressure on the NO-free fraction of the enzyme. This situation, in turn, causes an increase in the electron turnover of the uninhibited fraction of the enzyme, thus allowing for steady state respiration to be maintained. The second case takes place at low O₂, and possibly also at high O₂, if NO levels rise above the physiological nM range. Under these conditions, which favor a high electron turnover, the high affinity interaction of NO with the reduced species of the catalytic cycle will result in inhibition of respiration.

3.4 Interaction Between Nitric Oxide and Cytochrome C Oxidase: Generation of Reactive Oxygen Species by Mitochondria

Experimental evidence accumulated between the late 1960s and late 1970s suggests that a small percentage of the O₂ used by mitochondria is not completely reduced to water but is converted to superoxide anion O₂^{•-} because of the escape of electrons at complexes I and III of the electron transport chain. Theoretical considerations and experimental evidence indicate that the redox state of the mitochondrial respiratory chain may be a major determinant in the control of this process (reviewed by Turrens [53]). Studies using carbon monoxide have also suggested that the reduction of the electron transport chain as a consequence of cytochrome C oxidase inhibition may enhance O₂^{•-} formation. [54]. Studies in isolated mitochondria have indicated that treatment with NO generates O₂^{•-} in a similar manner [55]. It has been suggested that NO acts as a rheostat that sets the concentration of O₂ at which an early reduction of the electron transport chain will occur without inhibition of respiration. When RAW 246.7 cells and HUVECs are incubated at 3% O₂ (giving 30μM O₂ in the culture medium), the early reduction of the electron transport chain correlates with an NO-dependent increase in O₂^{•-} levels [52]. These findings suggest that NO plays a dual role in mitochondrial bioenergetics, on one hand affecting O₂ consumption and, on the other, favoring the generation of O₂^{•-} by decreasing electron flux through the cytochrome C oxidase. Regardless of the precise mechanism, in the presence of superoxide dismutase (SOD) the NO-induced O₂^{•-} could lead to the formation of hydrogen peroxide (H₂O₂) and thus initiate downstream signaling events. In this sense O₂^{•-} generated by the action of NO on the electron transport chain may represent a second messenger by which mitochondria may

modulate signal transduction cascades and gene transcription. A similar second messenger role has been ascribed to $O_2^{\cdot-}$ formed by the action of other cellular oxidases, particularly by NADPH oxidases (reviewed by Griendling et al [56]). However, the relative contribution to cellular signaling of these sources of $O_2^{\cdot-}$ vis a vis that generated in the electron transport chain has yet to be assessed.

3.5 Modulation of Mitochondrial Membrane Potential $\Delta\Psi_m$.

Studies in intact lymphoid cells [33] and astrocytes [34] showed that inhibition of respiration by NO results in a temporary small increase in $\Delta\Psi_m$. This phenomenon depends on the capacity of some cell types to maintain ATP levels by glycolysis when respiration is compromised. Generation of a $\Delta\Psi_m$ under these conditions requires entry of the glycolytically-generated ATP to the mitochondrial matrix via the adenine nucleotide translocator and its subsequent hydrolysis by the F_0F_1 ATPase which, now acting in reverse, extrudes protons from the mitochondrial matrix. An increase in $\Delta\Psi_m$ has previously been detected in association with the initiation of apoptosis [57]. The possibility that NO may also be involved in this phenomenon is underscored by findings showing that several proapoptotic factors stimulate NO production [58, 59]. Furthermore, the possibility that a high $\Delta\Psi_m$ promotes the formation of $O_2^{\cdot-}$ by complex III [60], suggests that this force may also contribute to the NO-stimulated increase in $O_2^{\cdot-}$ release observed at decreasing O_2 . Conversely, there are many reports indicating that NO causes mitochondrial membrane depolarization in association with the induction of apoptosis. Although some of these seemingly contradictory observations may be attributed to the methodology used to detect changes in $\Delta\Psi_m$, there may be cases in which these opposing actions of NO may result from differences in the metabolic or redox environment of the target cell.

3.6 Activation of AMP-Kinase

Insufficient energy output results in bioenergetic crisis. This phenomenon may stem from a variety of biological situations, including increased energy demand, restriction of nutrient or oxygen supply (ischemia and hypoxia), and mitochondrial dysfunction. Bioenergetic crisis causes an increase in intracellular AMP levels, and this in turn leads to the activation of the AMP-activated protein kinase (AMPK), an enzyme which plays a central role in the control of intracellular energy metabolism [61]. AMP binding to the enzyme promotes its phosphorylation by the tumor suppressor LKB1, resulting in full activation. Once activated, the enzyme turns off biosynthetic pathways and at the same time turns on catabolic pathways, thus conserving ATP levels.

3.7. Nitric oxide and LLLT.

It has been proposed that LLLT might work by photodissociating NO from the cytochrome c oxidase, thereby reversing the signaling consequences of excessive NO binding [62]. Light can indeed reverse the inhibition caused by NO binding to cytochrome oxidase, both in isolated mitochondria and in whole cells [63]. Light can also protect cells against NO-induced cell death. These experiments used light in the visible spectrum, with wavelengths from 600 to 630 nm. NIR also seems to have effects on cytochrome oxidase in conditions where NO is unlikely to be present.

Light mediated vasodilation was first described in 1968 by R F Furchgott, in his nitric oxide research that led to his receipt of a Nobel Prize thirty years later in 1998 [64]. Later studies conducted by other researchers confirmed and extended Furchgott's early work and demonstrate the ability of light to influence the localized production or release of NO and stimulate vasodilation through the effect NO on cGMP. This finding suggests that properly designed illumination devices may be effective, noninvasive therapeutic agents for patients who would benefit from increased localized NO availability. However the wavelengths that are most effective on this light mediated release of NO are different from those used in LLT being in the UVA and blue range [65].

Some wavelengths of light are absorbed by hemoglobin and that illumination can release the NO from hemoglobin (specifically from the nitrosothiols in the beta chain of the hemoglobin molecule) in red blood cells (RBCs) [66-68]. Since RBCs are continuously delivered to the area of treatment, there is a natural supply of NO that can be released from each new RBC that passes under the light source and is exposed to the appropriate wavelength of photo energy. Since the half life of the NO released under the area of illumination is only 2 to 3 seconds, NO release is very local, preventing the effect of increased NO from being manifested in other portions of the body. Vasodilation from NO is based its effect on the enzyme guanylate cyclase (GC), which forms cGMP to phosphorylate myosin and

relax smooth muscle cells in the vascular system. Once available levels of GC are saturated with NO, or once maximum levels of cGMP are achieved, further vasodilation through illumination will not occur until these biologic compounds return to their pre-illumination status. Again the wavelengths that have been shown to mediate this effect tend to be in the UVA and blue ranges not the red and NIR wavelength ranges that are mainly used for LLLT [69].

Tiina Karu provided experimental evidence [62] that NO was involved in the mechanism of the cellular response to LLLT in the red region of the spectrum. A suspension of HeLa cells was irradiated with 600-860 nm, or with a diode laser 820 nm and the number of cells attached to a glass matrix was counted after 30 minute incubation. The NO donors sodium nitroprusside (SNP), glyceryl trinitrate (GTN), or sodium nitrite (NaNO_2) were added to the cellular suspension before or after irradiation. Treating the cellular suspension with SNP before irradiation significantly modifies the action spectrum for the enhancement of the cell attachment property and eliminates the light-induced increase in the number of cells attached to the glass matrix, supposedly by way of binding NO to cytochrome c oxidase. Other in vivo studies on use of 780-nm for stimulating bone healing in rats [70], the use of 804-nm laser to decrease damage inflicted in rat hearts after creation of heart attacks [71], have shown significant increases of nitric oxide in illuminated tissues after LLLT. On the other hand studies have been reported on the use of red and NIR LLLT to treat mice with arthritis caused by intra-articular injection of zymosan [72], and studies with 660-nm laser for strokes created in rats [73] have both shown reduction of NO in the tissues. These authors explained this observation by proposing that LLLT inhibited iNOS.

4. CONSEQUENCES FOR LLLT

Many published papers describe increased blood flow during and after LLLT treatments both in animal models and in patients. One key question that has not been answered as yet is: does this increased blood flow arise from light mediated release of NO? If so what is the source? Is it NO that is photodissociated from hemoglobin in circulating erythrocytes, or NO that is photodissociated from other labile NO stores in the blood vessel wall, or is it derived from dissociation of NO that has bound to cytochrome c oxidase in the mitochondria of cells in the illuminated area? It appears that the optimum wavelengths are different for these three processes. Blue light at 441-nm appears to be best for dissociating NO from hemoglobin, UVA light at 366-nm appears to be best for dissociating NO from blood vessel walls, and red or NIR light appears to be best for dissociating NO from cytochrome c oxidase.

One observation about the effects of LLLT as it is normally used that needs explanation is the selectivity for injured or diseased tissues. Illumination of normal tissue in general has little effect. For instance illumination of normal skin or mucosa does not induce hyperplasia, and illumination of uninjured nerves does not generally induce anesthesia. This selectivity could be partially explained by the action of light on mitochondria of cells that are injured, predisposed to apoptosis or hypoxic. In these damaged cells it is possible that the ratio of NO to O_2 bound to cytochrome c oxidase is biased away from O_2 and towards NO. If this was the case the mitochondrial respiration could be reduced dramatically for only small changes in the ration, and dissociation of only small amounts of NO away from the active sites in the cytochrome c oxidase enzyme would result in large and relatively sustained increases in respiration and consequent rises in ATP, metabolism and cellular activity.

There are reports that LLLT can induce angiogenesis or growth of new blood vessels that are necessary in wound healing and especially in repair of ulcers and other non-healing wounds for which LLLT is frequently carried out. AS mentioned in Section 3.3 the binding of nitric oxide to cytochrome c oxidase can inhibit the stabilization of hypoxia-inducible factor (HIF) 1- α that would otherwise occur in cells with low oxygen concentrations. Stabilization of HIF1- α is one of the main mechanism for cells to initiate the formation of new blood vessels as a response to tissue hypoxia. Vascular endothelial growth factor is an important gene whose transcription is regulated by HIF1- α .

It has been known for some time that LLLT is particularly effective at reducing swelling or edema in tissues and in improving lymphatic drainage. This can also be explained by the effect of nitric oxide in activating the lymphatic drainage and in relaxing the lymphatic smooth muscle cells. Lymphatic endothelial cells (LECs) specifically express the $\alpha 1\beta 1$ isoform of soluble guanylate cyclase (sGC) [77], and NO induced LEC proliferation, migration, and cGMP production in LECs are specifically dependent on sGC $\alpha 1\beta 1$. Moreover, the specific sGC inhibitor NS-2028

completely prevents ultraviolet B-irradiation-induced lymphatic vessel enlargement, edema formation, and skin inflammation *in vivo*. These findings identify a crucial role of the NO/sGC α 1 β 1/cGMP pathway in modulating lymphatic vessel function. Mechanical activity of lymph vessels with or without the endothelium were investigated with macrophage conditioned medium [78]. Rat peritoneal macrophages stimulated with LPS suppressed significantly the basal tone of the lymphatic bioassay rings precontracted by U46619. The induced vasodilation of the lymph nodes was significantly reduced by preincubation of the macrophages with N omega-nitro-L-arginine methyl ester indomethacin, or dexamethasone. Simultaneous preincubation of L-NAME and indomethacin caused a synergistic reduction of the M phi-induced vasodilation of the lymphatic bioassay rings. These findings suggest that macrophages activated by bacterial LPS produce a marked relaxation of lymphatic smooth muscles through the co-release of nitric oxide and vasodilative prostaglandins.

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