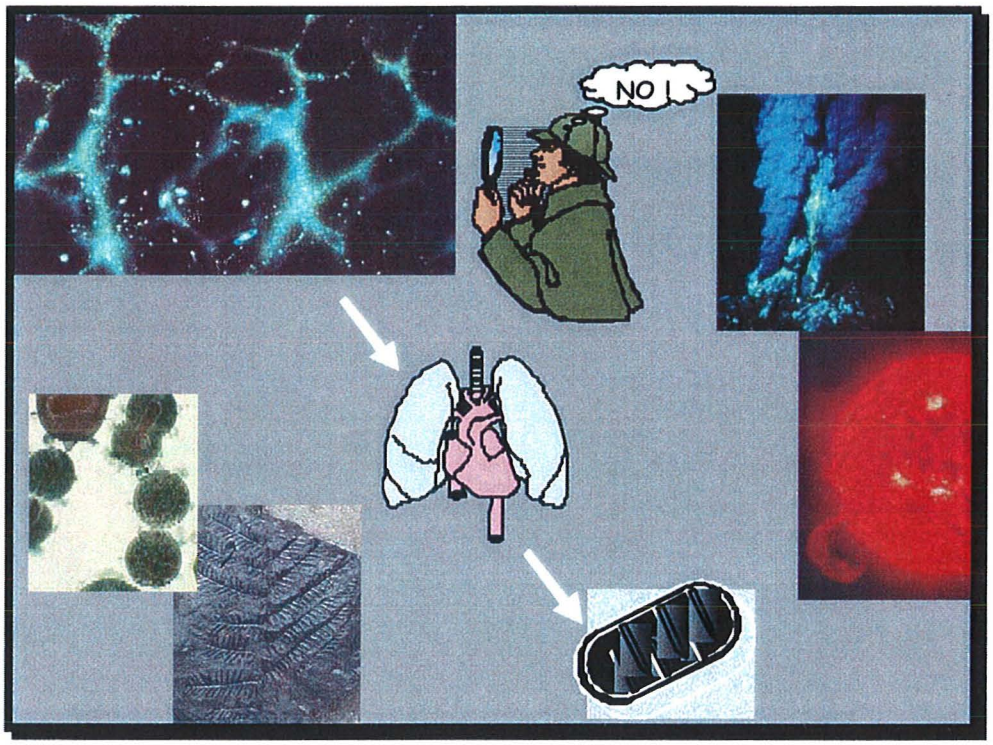
Nitric Oxide and Oxygen Transport

Evolution, Physiology and Clinical Implications



Connie C.W. Hsia, M.D.

Internal Medicine Grand Rounds

University of Texas Southwestern Medical Center at Dallas

May 15, 2003

This is to acknowledge that Connie Hsia, M.D. has not disclosed any financial interest or other relationships with commercial concerns related directly or indirectly to this program. Dr. Hsia will be discussing off-label uses in her presentation.

Connie C.W. Hsia, M.D.

Professor of Internal Medicine

Pulmonary and Critical Care Medicine

Dr. Hsia's academic interests are in the area of pulmonary exercise physiology and pathophysiology, regulation of gas exchange, mechanisms of compensatory lung growth as well as structure-function relationships during lung growth.

Introduction

Biological processes regulated by nitric oxide (NO) are ubiquitous, inter-connected and highly conserved across all life forms. Its ubiquity reflects the ancient origin of this simple gas dating back to the beginning of planetary formation. Understanding the evolutionary origin and progression of NO signaling helps us understand its diverse present day physiological functions and maximizes the potential for harnessing its actions in the prevention and/or treatment of disease. This review covers:

a) The "discovery" of NO as a signaling molecule
b) The origin and evolution of NO-related biological functions
c) NO in cellular respiration and O_2 transport
d) Clinical applications of basic knowledge

a) Discovery of NO as a signaling molecule

NO is a free radical with one unpaired electron (known as paramagnetic). It is highly unstable and reacts readily with other molecules that also possess an unpaired electron, including superoxide anion O_2^- and transitional metals (Fe, Cu, Mg, etc). NO was recognized as an atmospheric pollutant long before its ubiquitous biological importance was recognized. For example, the orange smog that hangs over early morning traffic after the sun rises in large cities is caused by NO produced by car exhaust reacting with ozone (O_3) to produce nitrogen dioxide (NO_2); the latter forms nitrate (NO_3^-) when dissolved in water:

$$\text{Car exhaust} \rightarrow \underset{\text{colorless}}{2\,NO + 2\,O_3} \rightarrow \underset{\text{orange}}{2\,NO_2 + 2\,O_2} \xrightarrow{H_2O} H^+ + NO_3^-$$

Another example is silo filler's disease [1]. NO is generated during anaerobic bacterial fermentation of silage and oxidized to NO_2, which causes a heavy orange gas to fill the bottom of the silo. Concentration of NO_2 may rise as high as 4,000 parts per million. Occupational exposure to this gas can lead to methemoglobinemia, acute pulmonary edema and death. A diffuse nodular interstitial infiltrate develops on chest x-ray. Following the acute episode there may be a recrudescence of signs and symptoms 2-3 weeks later. If the worker recovers the lung may be left permanently damaged by fibrosis and closure of small bronchioles.

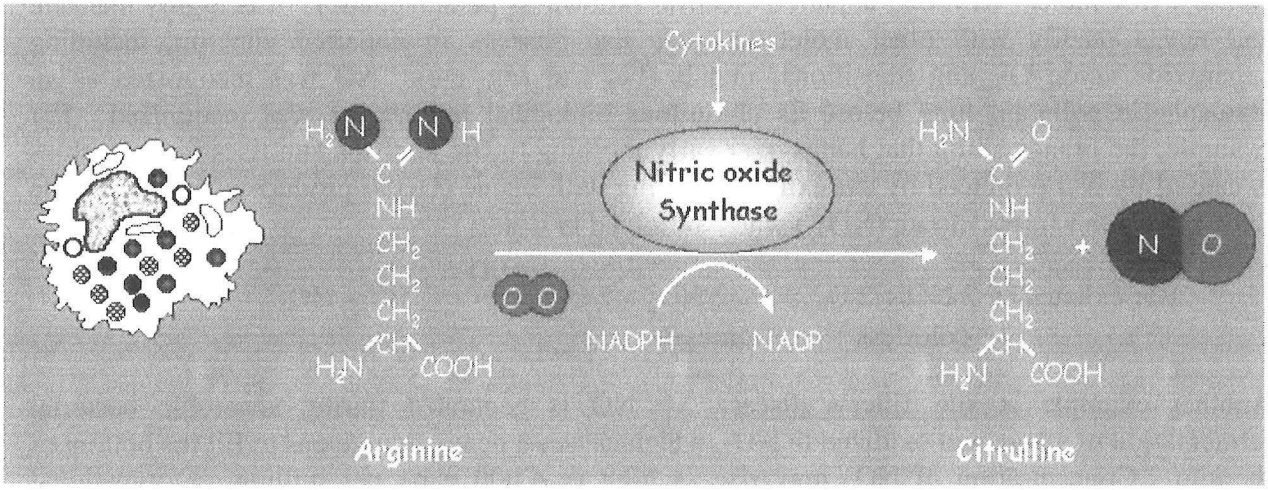
NO can be inactivated by conversion to non-reactive anions such as nitrite (NO_2^-) and nitrate (NO_3^-).

$$NO + H_2O \rightarrow 2\,H^+ + NO_2^-$$

These reactions have been utilized to preserve meat for thousands of years. Binding of NO to the heme of myoglobin produces the characteristic red color of cured meat. Binding of NO to bacterial heme proteins blocks cellular respiration of pathogens such as *Clostridium botulinum* and is responsible for the antimicrobial effect of curing.

The British chemist Sir Humphrey Davy first described the physiologic effects of inhaling a bolus of NO. He felt a burning sensation in the mouth and throat associated with severe chest pain. Having nearly died from this experience, he vowed never again to attempt so rash an experiment [2].

In 1956, Magee and Barnes [3] reported that nitrite in preserved meat when cooked at a high temperature generates the carcinogen nitrosamine. The sale of bacon plummeted following publication of their paper. In 1981, Green et al. [4] noted that people who maintained a low nitrate diet still excreted substantial amounts of urinary nitrate, which increased further in one subject who developed infectious diarrhea. This observation suggested that endogenous nitrate formation was increased by inflammation. Injecting bacterial endotoxin to rats reproduced the increase [5]. Later Marletta et al [6, 7] discovered that endotoxin-induced nitrate excretion requires the presence of macrophages and the amino acid arginine, which was converted to nitrates and citrulline. From this it was deduced that NO was produced within macrophages by a specific enzyme (later identified as the inducible isoform of NO synthase, iNOS) induced by cytokines to mediate the inflammatory response [8].



A second line of investigation in the late 1970's involved the sympathetic cholinergic vasodilatation response. In intact perfused vascular smooth muscle, sympathetic stimulation causes release of acetylcholine (Ach), which diffuses to smooth muscle causing relaxation and arterial dilation. However, in isolated arterial smooth muscle removed from the animal and stripped of adventitia and endothelium and studied in tissue baths, Ach caused arterial smooth muscle constriction. This paradox was unexplained. In 1976, a technician working for Robert F. Furchgott (Downstate Medical Ctr., Brooklyn) forgot to remove the endothelium during isolation of the rabbit aorta smooth muscle, and exogenous Ach caused a potent relaxation. From this serendipitous observation Furchgott deduced that Ach causes the endothelial cells to release a small molecule (termed endothelium-derived relaxation factor, EDRF) that diffuses to the adjacent muscle to cause myosin relaxation [9].

Also around the same time, Ferid Murad (UT Houston) independently found that nitroglycerin and related vasodilators elicits vascular smooth muscle relaxation only after being converted to NO [10], and that NO binds to the heme component of guanylyl cyclase, thereby activating the enzyme and stimulating the formation of cGMP, a known 2nd messenger that induces relaxation

as well as inhibits platelet aggregation. The action of NO can be blocked by the rapid reaction with superoxide anion and hemoglobin.

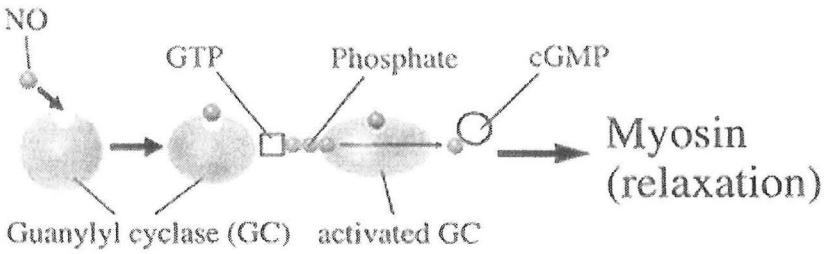


Figure is taken from http://www.nobel.se/medicine/laureates/1998/press.html.

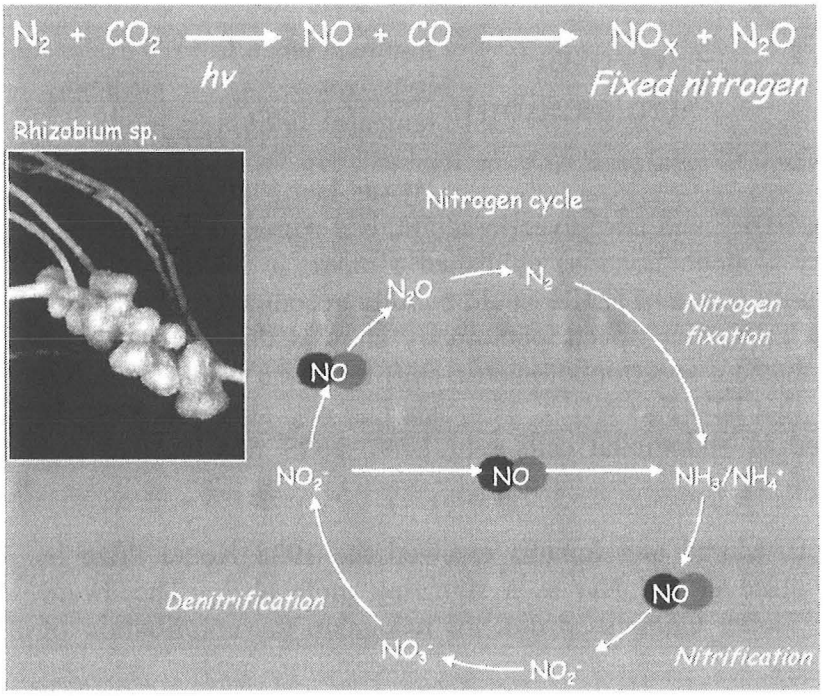
By the late 1980's, these three lines of investigation: inflammation, EDRF and nitroglycerin action, had converged on NO. In 1987, Salvador Moncada (University College London) published a report in Nature with the tentative conclusion that the vasodilatory action of EDRF could be fully accounted for by NO [11]. Six months later, Louis Ignarro (UCLA) using spectrophotometry showed that EDRF, when added to a hemoglobin solution, produced a spectrophotometric shift identical to that produced by NO, thereby proving definitively that EDRF is NO [12]. Moncada had also shown that NO is derived from L-arginine, synthesized in endothelial cells. In 1990, NOS was isolated and purified by Bredt and Snyder [13].

For their pioneering work, Furchgott, Murad and Ignarro received the 1998 Nobel Prize in Medicine and Physiology for the "discovery of NO as a signaling molecule". The award provoked some controversy, as the Nobel Committee did not recognize the contribution of Moncada [14].

b) Origin and evolution of NO-related biological functions

Biologic Function of NO	*(NOT an exhaustive list!)*	
Signal transduction	Circadian rhythm	
	Feeding	
	Motility	
	Sensory perception	
	Memory	
	Cell growth and development	
	Apoptosis	
	Smooth muscle relaxation	blood pressure homeostasis
		airway dilatation
		GI peristalsis
Host defense	Macrophage anti-microbial activity	
Reproduction	Erectile function	
	Egg fertilization	
Platelet aggregation		
Respiration	Ventilatory drive	
	Ventilation-perfusion matching	
	Oxygen delivery	
	Mitochondrial electron transport	

Why is NO signaling so pervasive?



The answer lies in the ancient origin of this molecule. When the earth was formed 4.5 billion years ago (BYA), its atmosphere was rich in nitrogen (N_2), hydrogen (H_2), methane (CH_4), carbon dioxide (CO_2) and water vapor. As the earth cooled the water vapor condensed to form oceans, leaving predominantly N_2 and CO_2 in the atmosphere. High energy radiation (*hv*) from coronae discharge of the sun combined with lightning in the atmosphere caused N_2 to react with CO_2, forming NO and CO, which then further react to form the various nitrogen oxides (NO_X) as well as nitrous oxide (N_2O), forms known as "fixed nitrogen" that are essential for organic life [15, 16]. Hence NO was present in earth's atmosphere long before the appearance of O_2 and was an important intermediate compound in the fixation of nitrogen.

Relative Distribution of Some Chemical Elements in the Earth's Crust and in the Human Body

Element	Earth's Crust (% by wt)*	Human Body (% by wt)**	Fold Enrichment
Oxygen	46.4	63	1.35
Silicon	28.2		
Aluminum	8.32		
Iron	5.63		
Hydrogen	0.14	9	64
Carbon	0.02	19	950
Nitrogen	0.002	5	2500

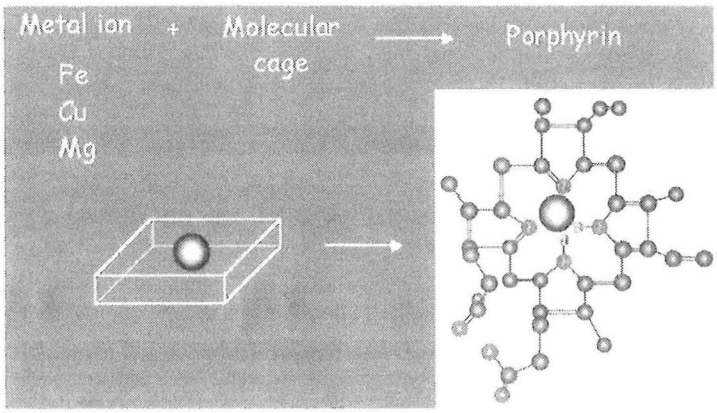
* *The Encyclopedia of Chemical elements*, CA Hampel Editor, Rheinhold Book Corp

** *The New York Public Library Science Desk Reference*, P Barnes-Svarney Editor

There is a huge enrichment of nitrogen in the body relative to that in the earth's crust. Enrichment is generated by the nitrogen cycle. Nitrifying bacteria and plants incorporate these nitrates from soil into amino acids and proteins for entry into metabolic pool of both plants and animals (nitrification). Ultimately the cycle is completed by return of nitrogen to the soil and atmosphere (denitrification) [17]. Nitrogen oxides including NO are essential components of this cycle.

In this "primordial soup" of the primitive earth, a global scale version of the present day deep-sea hydrothermal vents, the first prokaryocytes appeared about 3.8 BYA, such as methanococcus and cyanobacteria. These bacteria utilized H_2 to produce methane, hydrogen cyanide, hydrogen

sulfide and other equally attractive compounds. Other bacteria (such as rhizobium species that persist today) evolved nitrogenases to biologically fix atmospheric N_2 and NO, and dioxygenases to break down NO in order to protect themselves from the toxic nitrogenous compounds. These bacteria also ingested metal ions such as iron (Fe), magnesium (Mg), and copper (Cu) that are highly effective in mopping up free radicals. These metals came to catalyze some of the metabolic reactions. With time, some bacteria evolved a molecular cage to trap the metal ion, known as the porphyrin ring. Porphyrin is a planar group of 4 connected rings, each containing a nitrogen atom facing the metal ion in the center.



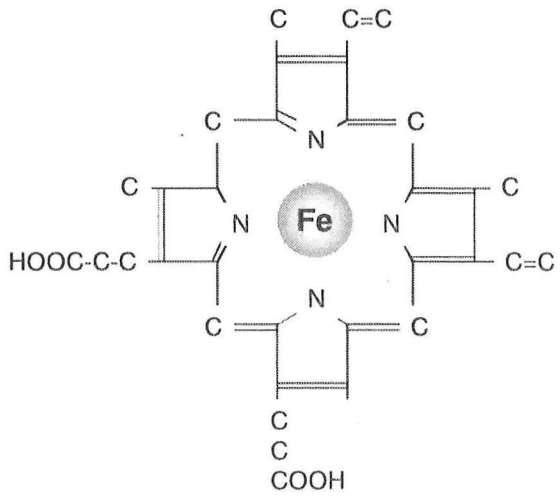
These rings became associated with protein complexes, which determine the specific function of the ring. By changing the 3D conformation of the protein complexes the metal binding sites of porphyrin become more or less exposed, thereby modifying the reaction and function of the ring.

The heme pigments or hemoproteins consist of a porphyrin ring containing an iron center. The earliest hemoproteins (also called flavohemoglobins) scavenged excess NO to prevent toxicity to the cells. Many of these flavohemoglobins are still present in bacteria and plant species today [18].

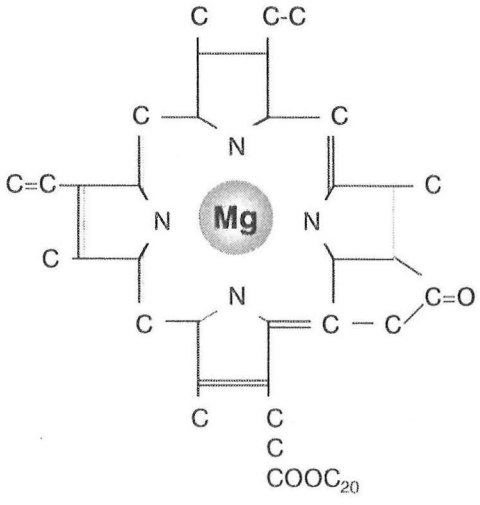
Other porphyrins evolved into cytochromes, which participated in the electron transport chain or functioned as enzymes to catalyze metabolic reactions. Cytochromes can have Fe, Mg or Cu at the center. The earliest cytochromes utilized NO as the terminal electron acceptor in the electron transport chain to generate ATP [18]. However, excess NO can also bind to and oxidize the metal ion in the cytochromes, thereby poisoning electron transport and causing cell death. To prevent NO toxicity, some cytochromes functioned as scavengers of NO to eliminate excess NO from the cells.

Some porphyrins with an Mg

Heme



Chlorophyll



center evolved into chlorophyll, which initiated photosynthesis. The earliest photosynthetic bacteria appeared about 3.5 BYA, known as the blue-green algae (cyanobacteria). Initially these bacteria utilized hydrogen sulfide during photosynthesis:

$$2\,H_2S + CO_2 \xrightarrow{photons} H_2O + 2S + CH_2O$$

Sulfur Carbohydrate

Hydrogen sulfide is oxidized to yield elemental sulfur. Released electrons are energized by photons during photosynthesis to reduce CO_2 and water and synthesize carbohydrates. The resulting elemental sulfur deposits are still being mined today.

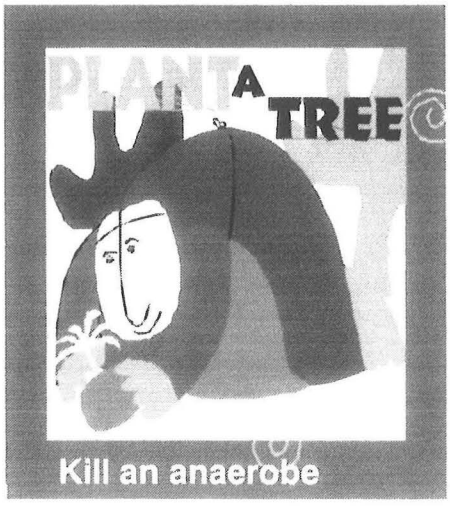
By about 2 BYA, nearly all of the biochemical mechanisms that we know today had already evolved in ancient bacteria. Then, a different process evolved as some cyanobacteria began to oxidize water instead of hydrogen sulfide to yield O_2 molecules instead of sulfur. Released electrons are energized by photons to high enough levels during photosynthesis that they can reduce CO_2 and water to synthesize carbohydrates for supporting growth and metabolism:

$$H_2O + CO_2 \xrightarrow{photons} O_2 + CH_2O$$

Because they need sunlight, fossils of ancient cyanobacteria colonies can be found in shallow waters and are known as stromalite formations. At first the O_2 released by cyanobacteria was mopped up by reaction with metals, causing rusting of earth's crust over millions of years and creating the layered red rock formations we see today. From these so called "banded iron formations" geologists are able to deduce the atmospheric O_2 concentrations at different periods in earth's history. It took approximately 1.5 billion years for the atmospheric O_2 concentration to stabilize at the present level of 21%. However, it remains a mystery as to why atmospheric O_2 concentration did not continue to rise beyond 21%.

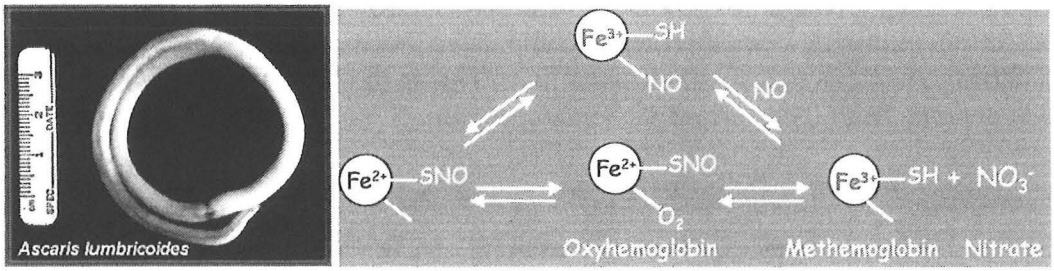
Oxygen Holocaust

Oxygen was a major global environmental pollutant in the ancient anaerobic environment. Because of its reactivity oxygen was a poison for many existing organisms at that time, causing the first and the greatest mass extinction of life forms on earth beginning around 2.5 BYA, known as the Oxygen Holocaust [19]. Some species sequestered in anaerobic environments like ocean floors or the bottom of lakes survived. Other species survived by developing adaptive mechanisms to detoxify oxygen. New physiologic roles opened up for metalloproteins in general and hemoproteins in particular. Oxygen-binding hemoproteins probably functioned initially to sop up O_2 in porphyrin cages, thus protecting cells from its toxic effects.



Functional Evolution of Hemoglobins

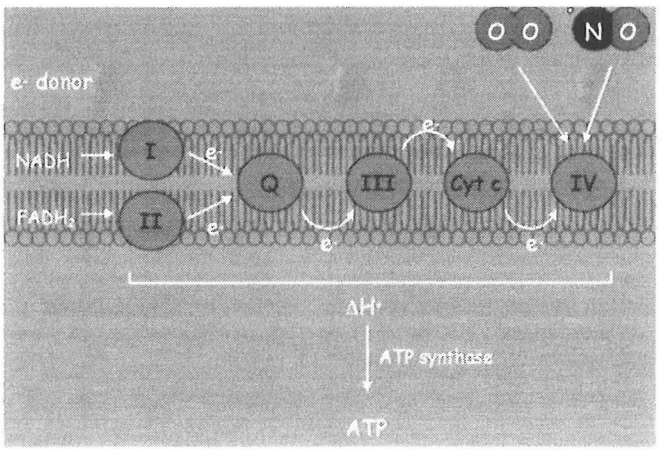
Ascaris Hemoglobin



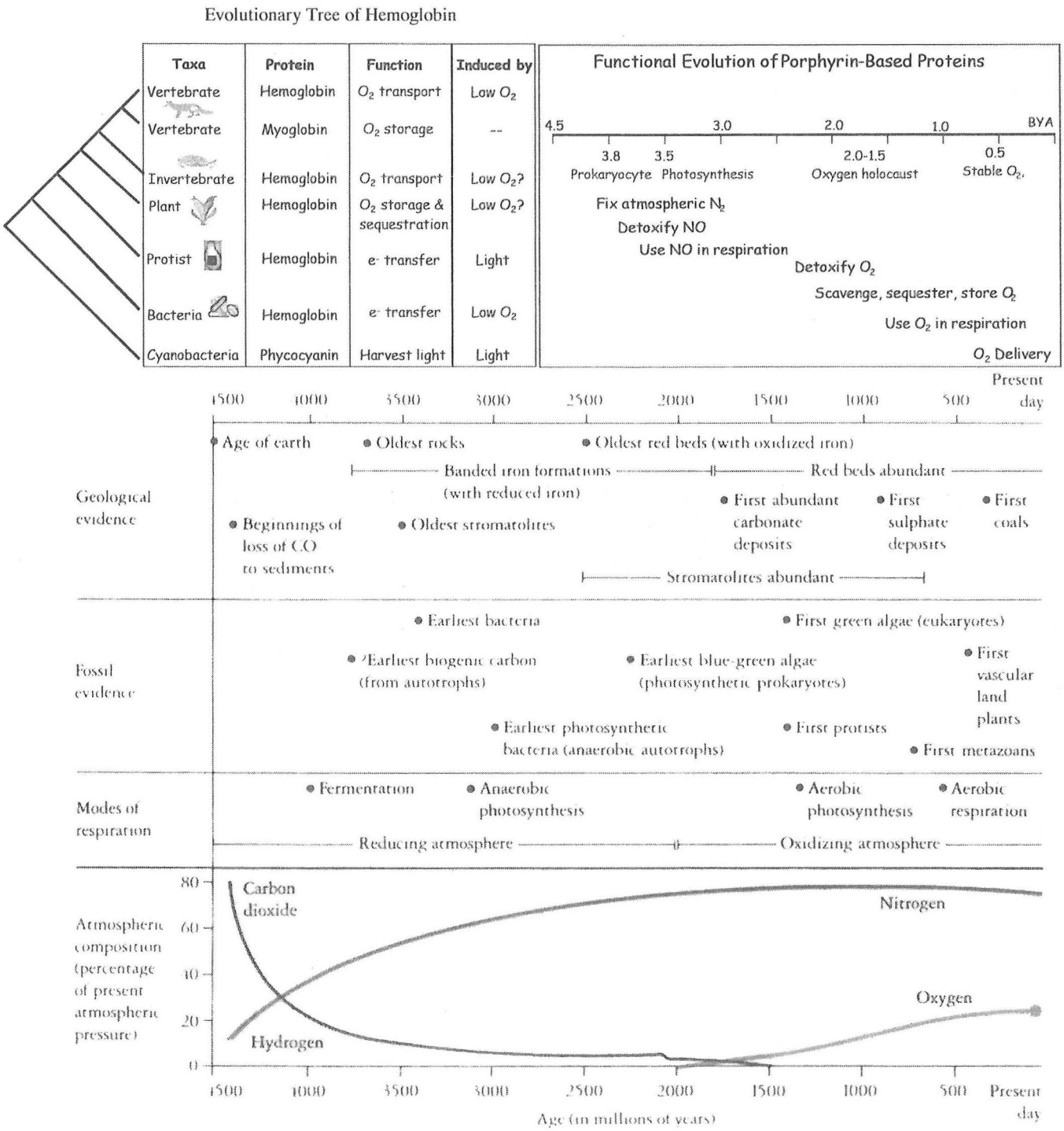
It has only recently been recognized that there are remnants of these hypothesized, ancient scavenging hemoglobins still in existence. At least one of these suggests an early role for NO in O_2 scavenging and detoxification. The parasitic anaerobic intestinal nematode *Ascaris lumbricoides* infects nearly 1 billion persons worldwide. Its hemoglobin in the perienteric fluid binds the heme iron 25,000 times more tightly than human hemoglobin (P_{50} =0.0001 torr compared to human hemoglobin P_{50}=26.5 torr). This means that O_2 once loaded onto the heme can never get off. The reaction of Ascaris hemoglobin with O_2 is driven by NO. Recent data from Minning et al [20] demonstrate that Ascaris hemoglobin consumes both NO and O_2 in an NADPH-dependent manner, simultaneously oxidizing the iron of oxyhemoglobin (Fe^{2+}) to form methemoglobin (Fe^{3+}), while removing O_2 from the heme binding site and converting it to nitrate (NO_3^-). These reactions keep the perienteric fluid anaerobic. Thus, ascaris hemoglobin is essentially a NO-dependent deoxygenase that uses endogenously produced NO as a substrate to detoxify O_2. A sulfide-fixing clam, *Lucina pectinata* [21] and even cyanobacteria [22] also have been shown to possess hemoglobins with very high O_2 affinities suggestive of a scavenging function although the role of NO has not been directly examined in these organisms.

Rise of Aerobic Respiration

In spite of its toxicity to anaerobic organisms, O_2 has an energetic advantage. Chemical potential available from O_2 is about 18 times that available from anaerobic metabolism; hence, some cells began to utilize O_2 as the terminal electron acceptor in oxidative phosphorylation (aerobic respiration). As O_2 became a useful metabolic agent but still in short supply in the atmosphere, the same scavenging hemoproteins originally used to eliminate O_2 from the cell might have been modified to make O_2 more available for cellular energy production. One consequence was that the primitive cytochrome/hemoglobin apparatus was modified to scavenge, sequester and deliver available O_2, giving rise to a diverse variety of heme-based respiratory pigments (hemoglobin, erythrocruorin, hemocyanin, chlorocruorin, hemerythrin, etc). The need to increase O_2 availability also gave rise to the induction of the synthesis of heme proteins by hypoxia, a ubiquitous response found in all hemoglobins in plants and animals [23, 24].



During the transition from anaerobic to aerobic cellular respiration, some organisms were capable of using either O_2 or NO as the terminal electron acceptor in the electron transport chain. Later these primitive hematoproteins could have been recruited for O_2-based metabolism in transferring electrons to sites of O_2 reduction. This is the function of contemporary cytochromes, which evolved from these primitive hematoproteins.



History of atmospheric composition and biological evolution. Taken from PW Atkins [25].

Evolution of NO synthase (NOS) [26]

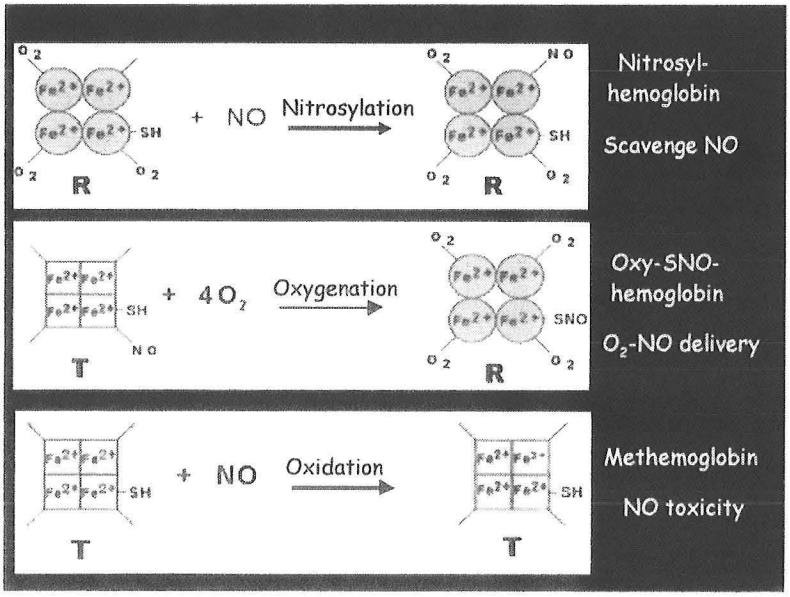
In vertebrates and higher mammals there are 3 isoforms of NOS representing 3 different genes: The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutively expressed at constant low levels and are Ca^{++} dependent. eNOS is stimulated by endothelial cell surface receptors or physical forces (shear stress). The inducible isoform (iNOS,) is expressed in macrophages, vascular smooth muscle, endothelial cell, by cytokines and microbial lipopolysaccharide, and is Ca^{++} independent. In invertebrates (mollusks, insects) only one NOS isoform is found in any given species, while no NOS gene is found in many prokaryote, yeasts, plants or nematode including C. elegans [27, 28]. Thus, generation of NO by NOS evolved later and only in higher life forms.

c) NO in cellular respiration and O_2 transport

Once earth's atmosphere reached normoxia (21% O_2) about 500 million years ago (MYA), complex highly compartmentalized aerobic organisms flourished. These organisms require regulated differential O_2 delivery to different tissue compartments in accordance with regional metabolic demand. The interactions between NO and O_2 binding sites on hemoglobin provided an ingenious and effective means of accomplishing this goal.

It has become apparent that almost all of the important biological functions of NO are a consequence of the reactivity of NO with metallic ions held in the centers of porphyrin rings of hematoproteins like hemoglobin, cytochromes such as those in the electron transport chains of mitochondria and enzymes like guanylyl cyclase in smooth muscle. NO bound to hemoglobin in the same heme pockets that O_2 and CO_2 bind to and this reaction has been utilized to study the binding properties of hemoglobin. Table below taken from Meyer et al. [29].

Molecule	Molecular Weight	Affinity, Keq x 10^{-4} M^{-1}	Reaction Velocity, k_{on} x 10^{-5} $M^{-1}s^{-1}$
O_2	32	5.6	47
CO	28	2,000	0.9
NO	30	4,500,000	255



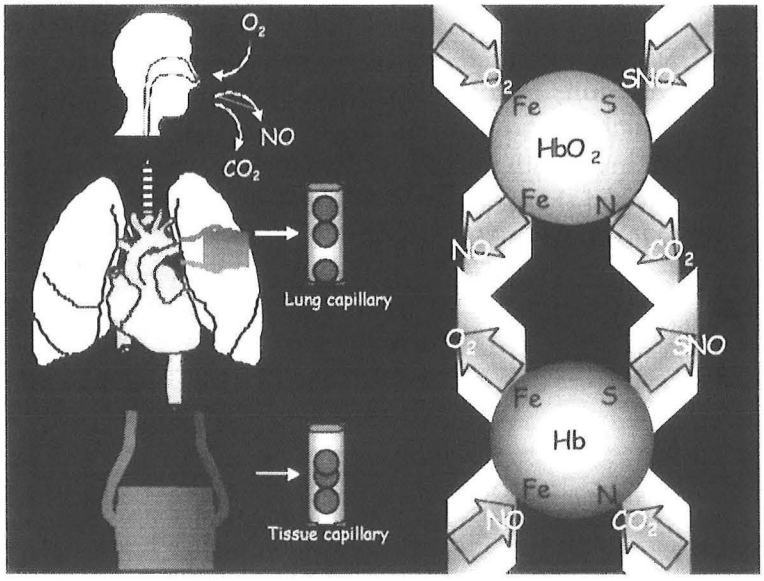
NO has the highest affinity and reaction velocity with hemoglobin of the three molecules shown above. The final product is different for the reaction of NO with oxy-hemoglobin (heme shown as circles) and reduced (deoxy-) hemoglobin (heme shown as squares). The three main types of NO-hemoglobin reaction are illustrated above [30]:

a) *Nitrosylation* NO can bind to the same heme binding site as O_2 in a competitive reaction to produce nitrosyl-hemoglobin. This reaction accounts for the scavenging of NO by hemoglobin

b) *Allosteric interaction of with O_2* NO can also bind to a sulf-hydryl group on the β93 cysteine residue of the globin chain to produce S-nitroso (SNO-) hemoglobin. This reaction is allosterically regulated by heme binding to O_2, and facilitates peripheral delivery of O_2 and NO (see below).

c) *Oxidation* NO in excess can oxidize the ferrous ion in heme to ferric ion, forming methemoglobin, which is incapable of binding O_2. This is one mechanism of NO cytotoxicity.

Allosteric Interaction Between NO and O_2 [31]



In the alveolar capillaries, O2 diffuses across the alveolar membrane, capillary plasma and red cell membrane to bind to the heme iron of deoxy-hemoglobin. Binding of one O_2 molecule facilitates the binding of more O_2 molecules to the remaining heme binding sites. This facilitative property, known as "cooperativity", is responsible for the sigmoidal shape of the oxyhemoglobin dissociation curve. The O_2-heme binding also alters the CO_2 dissociation curve in such a way as to promote the unloading of CO_2 from hemoglobin. The CO_2 released from hemoglobin diffuses into the alveolar air and is eliminated by ventilation. Since normally O_2 is far more abundant than NO in the alveoli, uptake of O_2 tends to displace NO from the heme binding site.

When 3 of the 4 heme binding sites on each hemoglobin are occupied by O_2, the quaternary structure of the molecule undergoes a conformational change, from the tense (T) state of deoxy-hemoglobin to the relaxed (R) state of oxy-hemoglobin. As a result of the conformational change, the globin subunits unfold to expose the sulf-hydryl (-SH) group of the β93 cysteine residue on the globin chain, which can now bind to NO to form S-nitroso-oxy-hemoglobin (or SNO-hemoglobin). This reaction allows hemoglobin to carry NO in a stable and "safe" chemical bond without the risk of being oxidized to nitrate with the formation of methemoglobin.

In the peripheral circulation, CO_2 produced by cell metabolism diffuses into capillary red cells and binds to hemoglobin. The reaction facilitates the dissociation of O_2 from heme binding sites. As O_2 is unloaded, the conformation of hemoglobin reverts back to the tense (T) state, causing NO to also dissociate from the β93–SH group. The released NO travels via one of several routes:

a) It can be carried by plasma thiols into the capillary endothelium to effect local vasodilation.

b) It can be transferred onto one of the empty heme iron binding sites of deoxy-hemoglobin to be carried back to the lung.

c) It can act directly on blood components such as platelets to inhibit platelet aggregation.

Through these interconnected reactions among NO, O_2 and CO_2, hemoglobin can no longer be considered as just a passive O_2 carrier. Rather, hemoglobin actively regulates regional O_2 delivery and microvascular tone in accordance with local O_2 demand. Thus, a hypoxic organ that produces a large amount of lactate and CO_2 will induce greater local unloading of O_2 and NO from the microcirculation. The released NO will in turn cause greater local dilatation of microvessels allowing greater regional perfusion and greater delivery of O_2 and NO.

Interaction of NO with red cell membrane

Recently, another NO-related regulatory function of the red cell membrane has been described by Jonathan Stamler's group at Duke University. When physiologic amounts of NO are added to deoxygenated red cells in situ, both S-nitroso (SNO) hemoglobin and nitrosyl-hemoglobin are formed. Upon fractionation of the red cell, most of the nitrosyl-hemoglobin is recovered from the cytosolic fraction, while SNO is found mainly in the membrane fraction. The finding suggests that red cells are capable of compartmentalizing NO within the membrane.

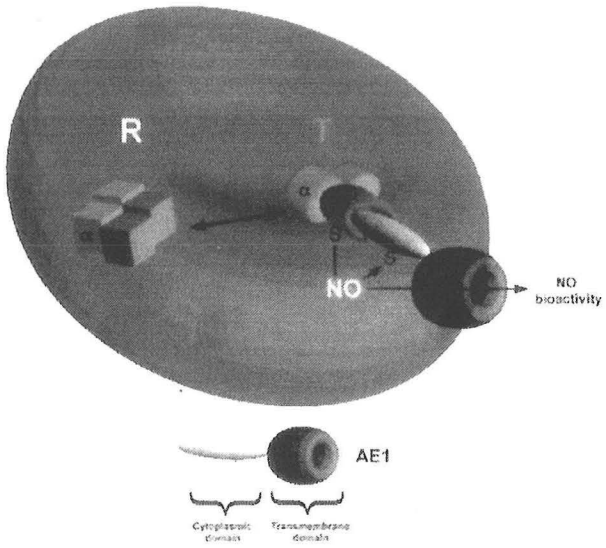


Figure is taken from Pawloski and Stamler [30].

It is postulated that some SNO-hemoglobin molecules can bind to the chloride-bicarbonate anion-exchanger 1 (AE1) protein that resides within the red cell membrane. In this reaction the N-terminus of the cytoplasmic domain of AE1 is inserted into the pocket between the beta subunits normally used for 2,3-DPG binding. This binding stabilizes SNO-hemoglobin into the T state (deoxy-) conformation, causing the release of NO from Cysβ93 and its transfer to the –SH binding site on the cytoplasmic domain of AE1. From this juxta-membrane site NO could be released to plasma thiol carriers in a controlled fashion as the red cells traverse through the microcirculation. The significance of this trafficking step is not yet fully understood. However, it is clear that the bioavailability of NO is carefully regulated by its

carrier along each step of oxygen transport.

Role of NO in Hypoxia-induced hyperventilation

Ventilation increases linearly with decreasing oxyhemoglobin saturation (about 0.6 l/min/% saturation). Deoxygenation of hemoglobin is associated with increased SNO formation and biological activity. Lipton et al [32] shows that the central brainstem respiratory center nucleus tractus solitarius (NTS) is rich in NOS and SNOs. NOS expression in NTS is increased after chronic hypoxia; endogenous SNOs act stereoselectively on the NTS to produce the same type of ventilatory response as seen in hypoxia. In addition, the hypoxic ventilatory responses can be attenuated by NOS inhibition and in endothelial NOS knockout mice. All these results suggest that SNO may be a signaling molecule between endothelial cells and respiratory neurons.

However, NO may have different effects on central vs. peripheral chemoreceptors. While SNO appears to stimulate central chemoreceptors, NO is inhibitory to the carotid body by modifying the Ca^{++} channel activity of glomus cells in a manner similar to the activity of carbon monoxide on these cells [33]. Acute hypoxia reduces NOS activity in carotid body extracts, and may contribute to the augmentation of sensory discharge from glomus cells.

Mechanisms of NO Toxicity

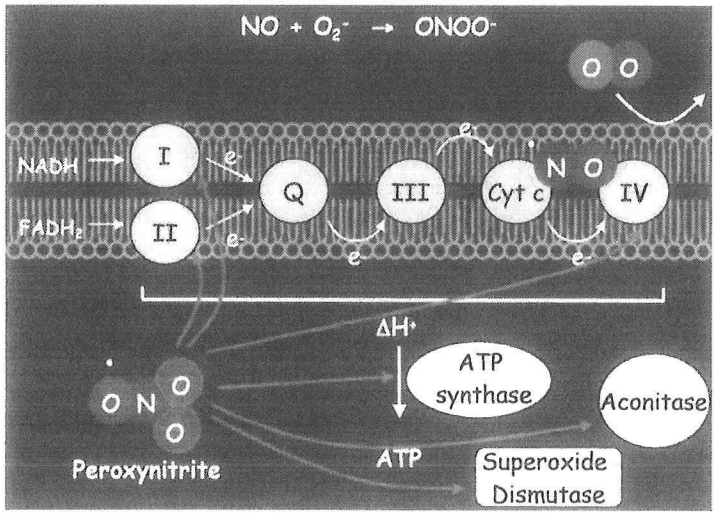
1) NO can oxidize the heme iron of hemoglobin to produce methemoglobin. This effect increases in direct proportion to the cumulative dose of NO exposure.

2) NO rapidly reacts with O_2 to form NO_2, which is a potent pulmonary irritant.

3) NO can directly cause mutagenesis via DNA strand breakage and base alterations [34].

4) NO can specifically bind to complex IV of the electron transport chain to inhibit ATP production.

5) NO reacts with superoxide anion to form peroxynitrite ($ONOO^-$), which can oxidize all the cytochrome enzymes of the electron transport chain in addition to inactivating key enzymes such as ATP synthase, aconitase and superoxide dismutase, causing arrest of cellular respiration [35]. This mechanism is the basis of the potent anti-microbial activity of iNOS produced by macrophages, but overproduction as in severe sepsis may produce tissue damage resulting in the multi-organ failure (see below).



d) Clinical Application

Diseases in which a deficiency of NO bioactivity play a role:
- Systemic hypertension
- Pulmonary hypertension
- Circulatory problems and impotence as in diabetes mellitus
- Bacterial infection

Diseases in which excess NO bioactivity play a role:
- Inflammation, e.g., asthma
- Septic shock and multi-organ failure.

Inhaled NO

Inhaled NO preferentially reaches well-ventilated areas of the lung to selectively induce local vasodilation; hence it improves oxygenation by matching ventilation to perfusion. By positron emission tomography and radiolabeled NO, it has been shown that most of the inhaled NO is taken up by lung and airway tissue; only a minor fraction reaches the plasma as nitrate [36]. Some portion of the inhaled NO reacts with airway proteins including glutathione to form s-nitrosoglutathione (GSNO), which sequesters NO bioactivity and delays its clearance from the lung.

Inhaled NO is effective and safe when given to full term infants with primary pulmonary hypertension and ventilator-dependent infants with chronic lung disease [37]. It is also a useful agent to test pulmonary vascular reactivity during diagnostic cardiac catheterization. In adult respiratory distress syndromes (ARDS), inhaled NO reduces pulmonary vascular resistance and improves oxygenation without altering systemic blood pressure. However, despite the significant short-term hemodynamic benefit, inhaled NO has not been shown to improve mortality [38].

Adding pulsed inhaled NO to chronic supplemental oxygen therapy in patients with COPD and pulmonary arterial hypertension may confer greater improvement of oxygenation and reduction of pulmonary vascular resistance than oxygen therapy alone [39]. Long term administration over 3 months resulted in significant hemodynamic improvement with little side effect [40].

Inhaled NO therapy is also a useful adjunct during the immediate post-transplant period to reduce right ventricular afterload and improve intrapulmonary shunting [41]. In laboratory animals transplant rejection is associated with the formation of nitrogen oxides; whether this is related to the inflammatory activities of rejection is not known.

Of note is the fact that car exhaust measured 30 ft. from inner city highways during morning rush hour contains sufficient NO (about 550 parts per billion) to induce a six-fold increase in guanylyl cyclase activation *in vitro* [42], supporting a significant physiologically effect of NO inhalation.

Potential adverse effects of inhaled NO therapy include impaired renal function, methemoglobinemia, left heart failure, intracerebral hemorrhage, accumulation of nitrite within

airways, pro-apoptotic and pro-inflammatory tendencies, and a rebound increase in pulmonary arterial pressure upon discontinuing the treatment [41, 43]. Concerns over its short duration of action and potential toxicity have prompted the search for alternative donors of NO bioactivity that possess less reactivity, longer duration of action and greater ease of administration.

Aerosolized S-nitrosoglutathione (GSNO)

GSNO is an endogenous source of SNO present in high concentrations in the lung and brain. Physiologic concentrations of GSNO relaxes airway smooth muscle, improves airway ciliary motility, inhibits airway epithelial amiloride-sensitve sodium transport while activating Ca^{++} dependent epithelial chloride transport, promotes neutrophil apoptosis and has antimicrobial activity. Endogenous GSNO level in the airway tends to be low in several lung diseases including cystic fibrosis, asthma and hypoxemic respiratory failure. A short term double-blind placebo-controlled study in patients with cystic fibrosis [44] showed that a single inhalation of GSNO (0.05 ml/kg) modestly increased oxygen saturation for 30 minutes, associated with an increased expired NO concentration and no change in blood pressure, heart rate or spirometry results. The report of early benefit needs to be confirmed for longer durations.

It is possible that repletion of SNO will ultimately be more efficacious than repletion of NO itself, since the thiol binding of SNO protects NO from scavenging by heme and minimizes potentially toxic reactions with O_2 radicals, thereby prolonging the biological action of NO and promoting the transport of NO bioavailability beyond the airways and alveoli to distant sites. For example, increasing the SNO reservoir may increase peripheral oxygen delivery and stimulate ventilatory response to hypoxia in central respiratory chemoreceptors.

Inhaled O-nitrosoethanol (ENO)

ENO is synthesized as a liquid but easily partitions into the gas phase because of its low boiling point (17ºC). It is stable in pure oxygen and does not generate nitrogen oxides. It reacts with glutathiones in tissue to form s-nitrosoglutathione (GSNO) and ethanol; this reaction preserves NO bioactivity while mitigating endogenous NO toxicity. Therefore ENO potentially acts as a reservoir of NO. In neonatal pigs inhalation of ENO markedly increases the concentration of indigenous SNO within the airway lining fluid. In pigs with acute hypoxic pulmonary vasoconstriction, ENO inhalation acutely improves oxygenation and pulmonary hemodynamics with no rebound deterioration upon stopping the treatment. Blood ethanol level was undetectable [45]. ENO administered over 4 h to neonates who required assisted ventilation with an oxygenation index of >25 produced sustained improvements in arterial oxygenation and systemic hemodynamics with no rebound effect [46]. Increases in methemoglobinemia were modest and toxic NO(x) was not detected.

Blood-based administration of NO

A different approach is to supplement SNO bioactivity of stored blood products or blood substitutes. *Ex vivo* treatment of stored red cells with NO or SNO could improve systemic O_2 delivery following blood transfusion, and potentially lessen the risks associate with transfusion

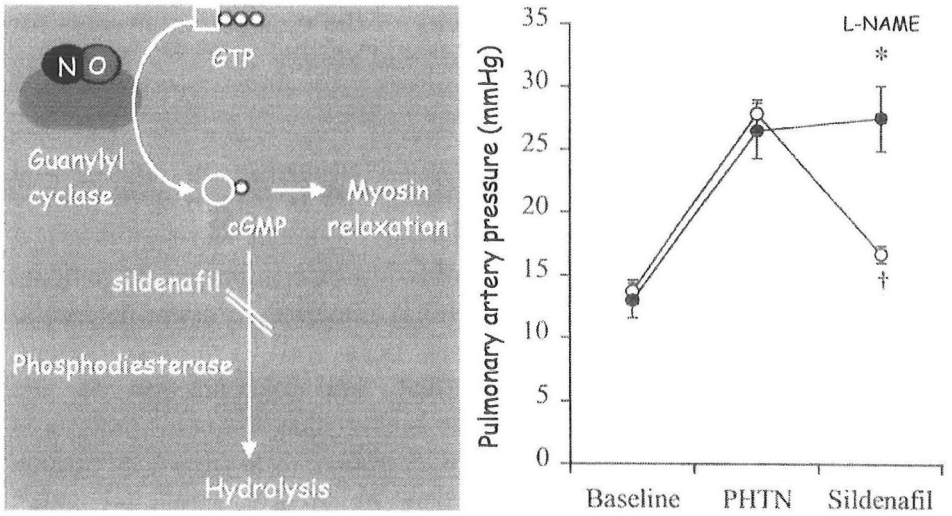
particularly in critically ill patients. SNO supplementation may also inhibit platelet aggregation, which may or may not be desirable.

The clinical use of recombinant hemoglobin solution (blood substitute) has been hampered by the side effect of severe vasoconstriction and hypertension, which is at least partly due to a disturbed SNO-O_2 equilibrium. Modifying the SNO- carrying and releasing properties of these compounds may reduce these side effects and facilitate the development of these compounds.

Manipulation to increase endogenous NO production

Sildenafil

NO binds to the metal ion in guanylyl cyclase to catalyze the conversion of GTP to cGMP, which mediates myosin relaxation in smooth muscle. Phosphodiesterase type 5 (PDE5) hydrolyzes cGMP. Sildenafil, a selective PDE5 inhibitor approved for use in erectile dysfunction, prevents the degradation of cGMP and enhances endogenous NO bioactivity. In lambs with thromboxane-induced acute pulmonary hypertension (PHTN), cumulative doses of sildenafil induce significant reductions in pulmonary vascular resistance with minimal effect on systemic blood pressure. The vasodilatory effect is blocked by L-NAME, an inhibitor of endogenous NO production [47].



In patients with primary or secondary pulmonary hypertension, a single oral dose of sildenafil (75 mg) is as effective as inhaled NO (80 ppm) in reducing pulmonary vascular resistance. Sildenafil decreases pulmonary capillary wedge pressure where inhaled NO increased it. Sildenafil plus inhaled NO increases the cardiac index whereas inhaled NO alone does not increase cardiac index [48]. In a randomized controlled open label trial in patients with pulmonary fibrosis and secondary PHTN [49], inhaled NO (10-20 ppm) and sildenafil (50 mg) both selectively reduced pulmonary vascular resistance, maintained ventilation-perfusion matching and raised arterial O_2 tension. In contrast, intravenous prostacyclin increased ventilation-perfusion mismatch. Sildenafil is obviously more convenient to administer than inhaled NO, but long term efficacy is not known.

There is also preliminary evidence that sildenafil inhibits hypoxia-induced pulmonary vasoconstriction [50]. Clinical studies are currently underway to test its efficacy in preventing and/or treating high altitude-induced pulmonary edema.

Manipulations to ameliorate excessive NO bioactivity

Asthma

NO is normally produced by constitutive NO synthase (cNOS) of vascular endothelium in alveolar walls where gas exchange occurs and walls of conducting airways. Spillover of this continuous synthesis of NO into the blood from lung tissues is rapidly scavenged by hemoglobin to maintain localized NO action. Spillover into alveolar and bronchial air is continuously excreted by ventilation. So we are in fact continuously polluting the atmosphere with metabolically produced NO from the lung just as does car exhaust but at a much lower rate. The average NO concentration in the expired air of a normal person is about 10 parts per billion (ppb) above that of ambient air. The average concentration exiting from the Lincoln tunnel at rush hour might be as high as 500 ppb. With a rapid NO gas analyzer it is possible to measure continuous NO excretion throughout a breath and using special maneuvers to measure how much NO is coming from alveoli and how much from conducting airways. It is possible to determine the diffusing capacity of the alveolar region of the lung for NO (DL_{NO}) just as diffusing capacity is measured for carbon monoxide (DL_{NO}) and oxygen (DL_{O2}) [51]; normally DL_{No} is ~120 ml/min/mmHg. It is also possible to measure diffusing capacity of the conducting airways for NO (DU_{NO}), a measure of NO exchange between airway lumen and airway walls [52]. Normally DU_{NO} is very low, about 0.5 ml/min/mmHg.

The purpose of NO synthesis in the lung appears to be the maintenance of a low airways and pulmonary vascular resistance. Yet an elevated level of exhaled NO is a marker of airway inflammation in asthma [53]. A significant correlation exists between exhaled NO and skin test scores in asthmatic patients who have not received steroids, allowing discrimination of patients with and without airway hyper-responsiveness [54]. Exhaled NO is elevated in acute asthmatic exacerbation and decreases following steroid treatment, and has been used to monitor therapy with inhaled corticosteroids in asthma. It seems paradoxical that concentration of this bronchodilator in expired air of asthmatics should be 10 times higher than normal. Asthma is recognized as an inflammatory disease and upregulation of iNOS occurs in response to various inflammatory mediators, not only in macrophages but also in airway epithelial cells. Upregulation of both cNOS and iNOS appears to be involved in elevating the exhaled NO [55]. NO excretion during a mild asthma exacerbation is poorly correlated with measures of airways function. Treatment with steroids, which only affects iNOS, significantly reduces exhaled NO. NO excretion remains high in most treated subjects but related to a compensatory upregulation of cNOS, which now became significantly correlated with measures of airways function, i.e., the higher the NO excretion the better the airways function.

Silkoff et al [55] hypothesize that *"the relation between cNOS-derived NO and severity of asthma may be analogous to that between insulin production and the severity of non-insulin dependent diabetes mellitus which is thought to result from decreased sensitivity to insulin. Here insulin production is elevated, (being) highest in patients (whose fasting blood sugar and glucose tolerance are closest to normal). In early disease, glucose levels are maintained nearly normal*

by overproduction of insulin. As the disease progresses... the ability to maintain high insulin output diminishes, resulting in progressive hyperglycemia. By analogy, the high level of cNOS-derived NO in asthma may result from a decreased sensitivity of airway smooth muscle to the relaxing effects of NO. Progression of asthma may...relate, in part, to a decreasing ability to maintain high cNOS activity." The feedback control mechanisms of NO synthesis in the lung are still poorly understood but constitute an area of active research.

Exhaled NO is elevated in bronchiectasis and in chronic obstructive pulmonary disease (COPD) during an exacerbation, but is typically not increased in stable COPD [56]. Exhaled and nasal NO are low in cystic fibrosis and expression of iNOS in airway epithelium is reduced. However, iNOS expression is abundant in the subepithelial tissues and NO metabolites are elevated in the breath condensate and sputum. These results suggest increased scavenging of endogenous NO by superoxide radicals, resulting in rapid conversion to peroxynitrite, so that only a small fraction of NO produced in the lung tissue reaches the airway lumen. Glucocorticoid therapy suppress NO production in cystic fibrosis airways by reducing iNOS expression and by inhibiting neutrophils recruitment [57].

Septic shock

Since NO and O_2 bind to the same mitochondrial cytochrome oxidase but NO has a much higher binding affinity than O_2, NO functionally regulates mitochondrial ATP production by controlling the entry of O_2 into the electron transport chain. A small increase in mitochondrial NO concentration can greatly inhibit the rate of oxidative phosphorylation. In sepsis, cytokines such as TNF and IL-1 induce neutrophils and macrophages to produce massive amounts of reactive oxygen species as well as reactive nitrogen species including NO and peroxynitrite that directly bind to multiple components of the mitochondrial electron transport chain, effectively terminating oxidative phosphorylation and precipitating cell necrosis [35]. There may also be depletion of cellular stores of electron donors such as nicotinamide adenine dinucleotide (NAD/NADH) as a result of activation of the nuclear enzyme, poly-(ADP-ribose) polymerase-1 [58]. Excess NO bioactivity also mediates widespread vasodilation causing systemic hypotension that is resistant to intravenous fluids and inotropic agents. Experimentally iNOS expression in elevated in septic shock and the hypotension is reversible by inhibition of iNOS activity. The multi-organ failure of sepsis is now recognized as a manifestation of generalized mitochondrial dysfunction, sometimes termed cytopathic hypoxia [59]. A full treatment of this concept is beyond the scope of the present discussion but the concept has radically altered treatment strategies in septic shock, aimed at manipulating mitochondrial enzyme function.

Summary (If you don't wish to read anything else in this syllabus):

1. The biology of NO is more ancient than even that of oxygen. NO, as an intermediate in each step of the nitrogen cycle, was a key player in the evolution of life on planet Earth.

2. The diverse physiological functions of NO are the result of its chemical reactivity as a free radical with an unpaired electron, its avid binding to superoxide and metal ions, and its position as a gatekeeper of the entry of O_2 into the electron transport chain.

3. NO has a high affinity for the iron binding sites in heme proteins (hemoglobin, myoglobin, guanylyl cyclase, cytochrome P450, cytochrome oxidase, etc), all of which share a common ancestral origin. NO binding can activate or inactivate protein function.

4. NO is a small molecule that diffuses rapidly to its site of action but the distance over which it acts is highly restricted due to rapid scavenging by local hemoglobin or myoglobin, which avidly bind NO and in the presence of O_2 convert it to nitrite or nitrate.

5. The ancient biochemical mechanisms that utilized NO as a mediator of anaerobic energy production were later adapted to utilize O_2 in aerobic respiration. This evolutionary link explains the dynamic interactions between the transport of NO and O_2.

6. Endogenous NO synthesis is widespread via three different synthase genes, a constitutive form in vascular endothelium, a neuronal form in the central nervous system and peripheral nerves, and an inducible form in leukocytes and macrophages.

7. The respiratory chemoreceptors respond to NO and O_2 in a reciprocal manner to increase ventilatory drive during hypoxia.

8. Allosteric interactions among NO, SNO, O_2, CO_2, H^+ and hemoglobin allow fine regulation of O_2 uptake, CO_2 elimination and ventilation-perfusion matching in the lung. Similar reciprocal interactions regulate CO_2 uptake, O_2 off-loading and peripheral regional vasodilation in accordance with regional metabolic demand.

9. NO binds to the iron-containing heme of guanylyl cyclase, induces a molecular conformational change at this site, which enzymatically converts GTP to cyclic GMP in smooth muscle, causing myosin relaxation. This reaction mediates vasodilatation as well as bronchodilatation.

10. NO binding to the metal ions of cytochrome P450 in liver and kidney modulates drug metabolism.

11. NO at high concentrations binds to the heme of mitochondrial cytochromes, blocking electron transport and shutting off oxidative metabolism. Macrophages kill invading bacteria by this mechanism. On the other hand, excessive NO generated by macrophages and neutrophils during sepsis may shut down mitochondrial respiration and induce multi-organ failure in the host by the same mechanism.

12. Understanding the origin and nature of NO-O_2 interactions has provided new insight into the pathophysiology of diseases and led to new therapeutic approaches aimed at either replenishing NO bioactivity or eliminating excess NO bioactivity.

References

1. Morrissey, W. L., Gould, I. A., Carrington, C. B., and Gaensler, E. A. (1975) Silo-Filler's disease. Respiration, **32**: 81-92.

2. Sprigge, J. S. (2002) Sir Humphry Davy; his researches in respiratory physiology and his debt to Antoine Lavoisier. Anaesthesia, **57**: 357-364.

3. Magee, P. N. and Barnes, J. M. (1967) Carcinogenic nitroso compounds. Adv Cancer Res, **10**: 163-246.

4. Green, L. C., Ruiz De Luzuriaga, K., Wagner, D. A., Rand, W., Istfan, N., Young, V. R., and Tannenbaum, S. R. (1981) Nitrate biosynthesis in man. Proc Natl Acad Sci U S A, **78**: 7764-7768.

5. Wagner, D. A., Young, V. R., and Tannenbaum, S. R. (1983) Mammalian nitrate biosynthesis: incorporation of 15NH3 into nitrate is enhanced by endotoxin treatment. Proc Natl Acad Sci U S A, **80**: 4518-4521.

6. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., and Wishnok, J. S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry, **27**: 8706-8711.

7. Iyengar, R., Stuehr, D. J., and Marletta, M. A. (1987) Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. Proc Natl Acad Sci U S A, **84**: 6369-6373.

8. Lancaster, J. R., Jr. (1992) Nitric oxide in cells. Am. Scientist, **80**: 248-259.

9. Furchgott, R. F. (1999) Endothelium-derived relaxing factor: discovery, early studies, and identification as nitric oxide. Biosci Rep, **19**: 235-251.

10. Katsuki, S., Arnold, W., Mittal, C., and Murad, F. (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res, **3**: 23-35.

11. Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature, **327**: 524-526.

12. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A, **84**: 9265-9269.

13. Bredt, D. S. and Snyder, S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci U S A, **87**: 682-685.

14. Howlett, R. (1998) Nobel award stirs up debate on nitric oxide breakthrough. Nature, **395**: 625-626.

15. Nna Mvondo, D., Navarro-Gonzalez, R., Mckay, C. P., Coll, P., and Raulin, F. (2001) Production of nitrogen oxides by lightning and coronae discharges in simulated early Earth, Venus and Mars environments. Adv Space Res, **27**: 217-223.

16. Kasting, J. F. and Siefert, J. L. (2001) Biogeochemistry. The nitrogen fix. Nature, **412**: 26-27.

17. Reutov, V. P. (1999) Biochemical predetermination of the NO synthase and nitrite reductase components of the nitric oxide cycle. Biochemistry (Moscow), **64**: 528-542.

18. Durner, J., Gow, A. J., Stamler, J. S., and Glazebrook, J. (1999) Ancient origins of nitric oxide signaling in biological systems. Proc Natl Acad Sci U S A, **96**: 14206-14207.

19. De Duve, C., (1996) The birth of complex cells. www.biosci.uga.edu/almanac/archive/fall_96/bio_340/docs/deduve.html.

20. Minning, D. M., Gow, A. J., Bonaventura, J., Braun, R., Dewhirst, M., Goldberg, D. E., and Stamler, J. S. (1999) Ascaris haemoglobin is a nitric oxide-activated 'deoxygenase'. Nature, **401**: 497-502.

21. Peterson, E. S., Huang, S., Wang, J., Miller, L. M., Vidugiris, G., Kloek, A. P., Goldberg, D. E., Chance, M. R., Wittenberg, J. B., and Friedman, J. M. (1997) A comparison of functional and structural consequences of the tyrosine B10 and glutamine E7 motifs in two invertebrate hemoglobins (Ascaris suum and Lucina pectinata). Biochemistry, **36**: 13110-13121.

22. Thorsteinsson, M. V., Bevan, D. R., Potts, M., Dou, Y., Eich, R. F., Hargrove, M. S., Gibson, Q. H., and Olson, J. S. (1999) A cyanobacterial hemoglobin with unusual ligand binding kinetics and stability properties. Biochemistry, **38**: 2117-2126.

23. Dordas, C., Rivoal, J., and Hill, R. D. (2003) Plant haemoglobins, nitric oxide and hypoxic stress. Ann Bot (Lond), **91**: 173-178.

24. Hardison, R. C. (1996) A brief history of hemoglobins: plant, animal, protist, and bacteria. Proc Natl Acad Sci U S A, **93**: 5675-5679.

25. Atkins, P. W. (1987) Molecules. Scientific American Library. New York.

26. Griffith, O. W. and Stuehr, D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol, **57**: 707-736.

27. Moroz, L. L. (2001) Gaseous transmission across time and species. Am. Zool., **41**: 304-320.

28. Torreilles, J. (2001) Nitric oxide: one of the more conserved and widespread signaling molecules. Front Biosci, **6**: D1161-1172.

29. Meyer, M., Schuster, K. D., Schulz, H., Mohr, M., and Piiper, J. (1990) Pulmonary diffusing capacities for nitric oxide and carbon monoxide determined by rebreathing in dogs. J. Appl. Physiol., **68**: 2344-2357.

30. Pawloski, J. R. and Stamler, J. S. (2002) Nitric oxide in RBCs. Transfusion, **42**: 1603-1609.

31. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature, **380**: 221-226.

32. Lipton, A. J., Johnson, M. A., Macdonald, T., Lieberman, M. W., Gozal, D., and Gaston, B. (2001) S-nitrosothiols signal the ventilatory response to hypoxia. Nature, **413**: 171-174.

33. Prabhakar, N. R. (1999) NO and CO as second messengers in oxygen sensing in the carotid body. Respir. Physiol., **115**: 161-168.

34. Murata, J., Tada, M., Iggo, R. D., Sawamura, Y., Shinohe, Y., and Abe, H. (1997) Nitric oxide as a carcinogen: analysis by yeast functional assay of inactivating p53 mutations induced by nitric oxide. Mutat Res, **379**: 211-218.

35. Stuart-Smith, K. (2002) Demystified. Nitric oxide. Mol Pathol, **55**: 360-366.

36. Mccarthy, T. J., Dence, C. S., Holmberg, S. W., Markham, J., Schuster, D. P., and Welch, M. J. (1996) Inhaled [13N]nitric oxide: a positron emission tomography (PET) study. Nucl Med Biol, **23**: 773-777.

37. Lonnqvist, P. A., Jonsson, B., Winberg, P., and Frostell, C. G. (1995) Inhaled nitric oxide in infants with developing or established chronic lung disease. Acta Paediatr, **84**: 1188-1192.

38. Klinger, J. R. (2002) Inhaled nitric oxide in ARDS. Crit Care Clin, **18**: 45-68, vi.

39. Ashutosh, K., Phadke, K., Jackson, J. F., and Steele, D. (2000) Use of nitric oxide inhalation in chronic obstructive pulmonary disease. Thorax, **55**: 109-113.

40. Vonbank, K., Ziesche, R., Higenbottam, T. W., Stiebellehner, L., Petkov, V., Schenk, P., Germann, P., and Block, L. H. (2003) Controlled prospective randomised trial on the effects on pulmonary haemodynamics of the ambulatory long term use of nitric oxide and oxygen in patients with severe COPD. Thorax, **58**: 289-293.

41. Lindberg, L., Sjoberg, T., Ingemansson, R., and Steenb, S. (1996) Inhalation of nitric oxide after lung transplantation. Ann Thorac Surg, **61**: 956-962.

42. Friebe, A., Malkewitz, J., Schultz, G., and Koesling, D. (1996) Positive effects of pollution. Nature, **382**: 120.

43. Koh, E., Niimura, J., Nakamura, T., Yamakage, H., and Takahashi, H. (1998) Long-term inhalation of nitric oxide for a patient with primary pulmonary hypertension. Jpn Circ J, **62**: 940-942.

44. Snyder, A. H., Mcpherson, M. E., Hunt, J. F., Johnson, M., Stamler, J. S., and Gaston, B. (2002) Acute effects of aerosolized S-nitrosoglutathione in cystic fibrosis. Am J Respir Crit Care Med, **165**: 922-926.

45. Moya, M. P., Gow, A. J., Mcmahon, T. J., Toone, E. J., Cheifetz, I. M., Goldberg, R. N., and Stamler, J. S. (2001) S-nitrosothiol repletion by an inhaled gas regulates pulmonary function. Proc Natl Acad Sci U S A, **98**: 5792-5797.

46. Moya, M. P., Gow, A. J., Califf, R. M., Goldberg, R. N., and Stamler, J. S. (2002) Inhaled ethyl nitrite gas for persistent pulmonary hypertension of the newborn. Lancet, **360**: 141-143.

47. Weimann, J., Ullrich, R., Hromi, J., Fujino, Y., Clark, M. W., Bloch, K. D., and Zapol, W. M. (2000) Sildenafil is a pulmonary vasodilator in awake lambs with acute pulmonary hypertension. Anesthesiology, **92**: 1702-1712.

48. Michelakis, E., Tymchak, W., Lien, D., Webster, L., Hashimoto, K., and Archer, S. (2002) Oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension: comparison with inhaled nitric oxide. Circulation, **105**: 2398-2403.

49. Ghofrani, H. A., Wiedemann, R., Rose, F., Schermuly, R. T., Olschewski, H., Weissmann, N., Gunther, A., Walmrath, D., Seeger, W., and Grimminger, F. (2002) Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial. Lancet, **360**: 895-900.

50. Zhao, L., Mason, N. A., Morrell, N. W., Kojonazarov, B., Sadykov, A., Maripov, A., Mirrakhimov, M. M., Aldashev, A., and Wilkins, M. R. (2001) Sildenafil inhibits hypoxia-induced pulmonary hypertension. Circulation, **104**: 424-428.

51. Tamhane, R. M., Johnson, R. L., Jr., and Hsia, C. C. (2001) Pulmonary membrane diffusing capacity and capillary blood volume measured during exercise from nitric oxide uptake. Chest, **120**: 1850-1856.

52. Shin, H. W. and George, S. C. (2002) Impact of axial diffusion on nitric oxide exchange in the lungs. J Appl Physiol, **93**: 2070-2080.

53. Bates, C. A. and Silkoff, P. E. (2003) Exhaled nitric oxide in asthma: from bench to bedside. J Allergy Clin Immunol, **111**: 256-262.

54. Simpson, A., Custovic, A., Pipis, S., Adisesh, A., Faragher, B., and Woodcock, A. (1999) Exhaled nitric oxide, sensitization, and exposure to allergens in patients with asthma who are not taking inhaled steroids. Am J Respir Crit Care Med, **160**: 45-49.

55. Silkoff, P. E., Sylvester, J. T., Zamel, N., and Permutt, S. (2000) Airway nitric oxide diffusion in asthma: Role in pulmonary function and bronchial responsiveness. Am J Respir Crit Care Med, **161**: 1218-1228.

56. Kharitonov, S. A. and Barnes, P. J. (2000) Clinical aspects of exhaled nitric oxide. Eur Respir J, **16**: 781-792.

57. Linnane, S. J., Thin, A. G., Keatings, V. M., Moynihan, J. B., Mcloughlin, P., and Fitzgerald, M. X. (2001) Glucocorticoid treatment reduces exhaled nitric oxide in cystic fibrosis patients. Eur Respir J, **17**: 1267-1270.

58. Fink, M. P. (2002) Bench-to-bedside review: Cytopathic hypoxia. Crit Care, **6**: 491-499.

59. Fink, M. P. (2000) Cytopathic hypoxia. A concept to explain organ dysfunction in sepsis. Minerva Anestesiol, **66**: 337-342.