



# THE VITAL ROLE OF NITRIC OXIDE

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Nitric oxide (NO) was among the first gases to be discovered. It was discovered by Joseph Priestley in 1772—two years after his discovery of oxygen (O<sub>2</sub>). For more than two centuries this colorless and odorless gas was considered highly toxic. Several chemists have died of what we now call toxic-shock syndrome, after purposely or accidentally inhaling large amounts of it. No one anticipated that small quantities of this lethal agent would have a crucial function in humans and probably all other animals. During the last ten years, this belief was dramatically changed when several discoveries were made, revealing a significant biological role for NO. First, in 1980, Furchgott & Zawadzki reported that endothelial cells release a substance which is responsible for the relaxation of vascular smooth muscle (they called this substance endothelium-derived relaxing factor-EDRF). Then in 1987 Palmer, Ferrige & Moncada suggested that EDRF is NO which is produced by the oxidation of L-arginine. In 1992 Malinski finally proved this suggestion by direct *in situ* measurement of NO in a single endothelial cell. Since then about 27,000 scientific papers have been published dealing with different aspects of NO in biological systems.

NO in aqueous systems is moderately reactive; it reacts with oxygen to produce nitrite and subsequently nitrate. The average half-life of NO in tissue is about 3–6 seconds and in

blood 1–2 seconds. This short life makes *in situ* studies of NO in living systems extremely challenging.

Before 1987, NO biosynthesis was thought to be restricted to bacteria engaged in nitrification or denitrification reactions. However, recently we learned that NO can be released by many different cells in mammalian systems. NO can play different physiological roles depending on the place of its release. It can be a neurotransmitter when (for seconds) a small puff of it is generated by the neurons of the central and peripheral nervous system. It can regulate blood pressure and inhibit blood coagulation when (for minutes) a larger burst of it is generated in the endothelium, a monolayer of cells lining the cardiovascular system. Also, NO can act as a cytostatic agent when (for hours) a continuous blast of it is biosynthesized by the immune system; its presence may halt the proliferation of cancer and pathogens.

Nitric oxide synthase (NOS) is the enzyme that converts the co-substrates L-arginine and  $O_2$  into the co-products NO and L-citrulline. Biosynthesized within the cell, NO may react with a select few types of molecules inside the cell, or outside (after free diffusion through the cell membrane). The most rapid scavenger of NO is superoxide. The peroxyxynitrite ( $OONO^-$ ) formed quickly rearranges to nitrate. Reaction with  $O_2$  is much slower and leads to production of nitrite and nitrate. NO also may react with a few metal ions (iron, copper or manganese) which are usually bound to proteins. The selective reactivity of NO with such proteins, and its reaction with  $O_2^-$  and  $O_2$  dominate the chemistry of NO in the biological systems.

To date NOS has been found in many cell types in various parts of the body (Table 1). The monolayer of cells lining the cardiovascular system (the endothelium) produce the largest amount of NO. The endothelium should be considered one of the largest specialized organs in the body; the total weight of endothelial cells in the human body is about 1.5 kg and is comparable with the weight of a liver. In the brain NO is produced by both neuronal and endothelial NOS.

**Table I. Nitric oxide synthases**

Constitutive NOS	Inducible NOS
vascular endothelium	vascular endothelium
brain	vascular smooth muscle
platelets	macrophages
adrenal gland	kupffer cells
peripheral nerve	hepatocytes
mast cells	endocardium
masangial cells	mesangial cells
myocardium	lymphocytes
	chondrocytes
	fibroblasts
	neutrophils
	megakaryocytes

NOS is also found in myocytes and skeletal muscles. NO can also act as a cytostatic agent in the immune system. NO can be produced (after induction) by almost every cell in the human body, where it plays a primary role in host defense. In this role, NO may defend the body against invading bacteria, viruses and even cancer.

## ***Nitric Oxide as a Regulator of Cardiovascular System***

Vascular endothelial cells contain calcium-dependent constitutive NOS. In order to maintain normal blood pressure, NOS synthesizes NO in bursts lasting a few minutes. Synthesis of NO is stimulated by chemical agonists like bradykinin, acetylcholine, ATP and several other agents which can stimulate the flux of calcium. However, physical agonists like shear stress, flow, electrical current, acupuncture, light and electromag-

netic fields can also stimulate NO release in cardiovascular system. After NOS is turned on by a calcium flux and it biosynthesizes NO for about a minute, it is turned off by phosphorylation.

NO synthesized by endothelial cells diffuses out in all directions. About 70–90% of NO released by endothelium is washed away by the blood where it is used to prevent platelet aggregation and the subsequent formation of blood clots. The remaining amount of NO diffuses to the wall of arteries and veins (smooth muscle) and triggers a cascade of events leading to smooth muscle relaxation. Relaxation of the surrounding smooth muscle allows the blood vessel to dilate (increase of vessel diameter), resulting in lowered blood pressure. The cardiovascular system maintains a constant level of NO at a given blood flow. When blood flow increases, the endothelium releases more NO to maintain its constant concentration in the blood stream. When this normal level is not produced, because production is blocked by pathological states such as deposition of cholesterol on the wall of the arteries (atherosclerosis), the vascular muscles do not relax to the appropriate degree and vasoconstriction ensues. Vasoconstriction increases blood pressure, and decreases flow, and is responsible for hypertension.

Nitroglycerin, discovered by Alfred Nobel, has been used as a drug for cardiac treatment for more than 100 years after the accidental discovery that his nitroglycerin factory workers had low blood pressure. Nobel was one of the first patients purposely treated with nitroglycerin for his heart problem. After the discovery of the biological role of NO, it is now possible to explain the beneficial role of nitroglycerin in the treatment of heart disease. Nitroglycerin undergoes metabolic degradation in biological tissue, and one of the degradation products is NO, which increases muscle relaxation and can improve blood flow even in atherosclerotic arteries.

Platelets in the blood can also release NO. NO released by platelets prevents blood coagulation, formation of

thrombi and subsequent blockage of arteries. The pathology of this process leads to coronary thrombosis and is a major cause of stroke. The heart itself releases a significant amount of NO, on a beat-to-beat basis, during the systolic (compression), as well as the diastolic (decompression) period. Under normal conditions NO is produced in the beating heart by endothelial cells which are always located in close proximity 10–20  $\mu\text{m}$  from cardiac muscle cells (myocytes). Without the mechanical transduction of NO production, cardiac-output would change abruptly as preload changed abruptly. This very desirable gradual response to abrupt changes in cardiac preload, mediated by NO, has often been called “heart memory.”

It has been noted for over 100 years that a heart disconnected from the cardiovascular system and central nervous system can beat spontaneously for several seconds. The vital functions of the heart are preserved as the concentration of NO is maintained above 300 nmol/L level. This finding has serious implication for heart preservation strategies for transplantation. Current cold storage preservation solutions permit short time storage of a heart (about 4 hours). This is a severe limitation, because surgery itself for heart transplants requires 2–3 hours. Recently, we have developed new solutions for heart storage which preserve the NOS. These new solutions extend the time for heart storage up to 12 hours.

Under conditions when the heart has to provide extensive work (pumping blood under heavy stress, exercise, etc.) NO is produced by myocytes in addition to endothelial cells. Myocyte NO production is not stimulated by mechanical forces of the heart, but by adrenaline. The release of adrenaline is triggered by the nervous system. The heart is more sensitive to a low supply of NO than to a low supply of oxygen. Cutting-off of NO production/supply in the heart will terminate a human life within 10–15 seconds, much faster than cutting-off of oxygen supply (5–7 min).

## *Nitric Oxide in Nervous System*

NO generated in certain neurons in the peripheral nervous system serves as a neurotransmitter to help control the cardiovascular, respiratory and digestive systems. Neurons throughout the body transfer information by means of signaling molecules. Neuronal activity, and therefore all the functions attributed to the central and peripheral nervous system are based on the synthesis and release of neurotransmitters. In the brain, this action can lead to an increase or decrease of the electrical signals produced. Electrical signals are due to the flow of positive or negative ions into and out of neurons which change the electrical potential on the neuron membrane. The net effect of this process is either excitation or inhibition of neurons.

In the central nervous system, NO acts as a neurotransmitter in the cerebellum. Its action mediates the interaction between endothelial cells and smooth-muscle cells in a blood vessel. NO released from the postsynaptic neurons stimulates neighboring neurons. NO has also been implicated in another part of the brain—the hippocampus. The hippocampus is involved in learning formation of memory. NO appears to be essential for establishing long-term potentiation memory, but not short-term potentiation memory. In long term potentiation, the strength of synaptic contact increases as a consequence of the frequent use of memory. It has also been suggested that NO can act as retrograde messenger in this process. The inhibition of NO release in the hippocampus prevents long-term potentiation and decreases learning capabilities.

The vital role of NO in the brain can change diametrically under pathological (disease) conditions. During a stroke, when the supply of blood to the brain is limited, a massive release of NO is observed 4–6 min after the onset of the stroke; NO concentration in the brain can reach as high as 2–4  $\mu\text{mol/L}$  about 100 times higher than physiologic NO concentration. This highly nonphysiological NO concentration initiates a cascade of events leading to quasi-reversible or irreversible brain damage.

## ***Nitric Oxide as a Part of the Immune System***

The role of NO in the immune system is much different from its role in the cardiovascular or neuronal system. In humans, most every type of cell in the body can express NOS.

The signal to translate the DNA sequence for the NO-producing enzyme comes from certain cytokines, which are produced by the infected cells. Any kind of infection (including bacteria, viruses, or cancer) will lead to the production of cytokines. Cytokines carry the message of the infectious state to the surrounding cells of humans, which will start to produce NOS enzyme. Immediately after translation is complete and the prosthetic groups are in place, the enzyme will continuously produce large amounts of NO for an extended period of time (several hours). The total NO production will be therefore much higher than that produced by endothelial cells (few minutes) or neurons (few seconds). The enzyme produces a sufficient concentration of NO to locally inhibit DNA synthesis; hence, the profound cytostatic effect of NO on the proliferation of rapidly dividing tumor cells or pathogens. In addition, DNA synthesis is a fundamental step in normal cell proliferation. Even normally high NO concentrations from constitutive NOS can inhibit the ribonucleotide reductase, thus halting the proliferation of smooth muscles around major arteries and cardiac myocytes. NO is not toxic even at higher biological concentration. Therefore, it is unlikely that NO can actually kill tumor cells, it will just limit their proliferation.

### ***Pathology of Nitric Oxide Release***

NO is one of the 10 smallest molecules found in biological systems. There is now little question that NO is essential to the everyday activities of many cells and tissues in the body. Therefore, any pathology of NO production in the body can lead to

many diseases (Table II). Because of this, NO is also called a vital-poison, the right amount of NO production is essential for life, but too much or too little can be deadly poisonous. In most life-threatening diseases like hypertension, atherosclerosis, and diabetes, the net concentration of NO is lower than in a healthy system. This does not necessarily mean that the expression of the enzyme is lower. In some of these diseases, enzyme expression is even higher (hypertension).

Why? Endothelial L-arginine concentration is deficient in hypertensive mammals usually due to the formation atherosclerotic plaques on the membrane. Huk & Malinski et al. (1997), have demonstrated in L-arginine-starved *in vivo* environments that the NO-producing enzyme can also donate an electron to its other substrate oxygen ( $O_2$ ) to form superoxide ( $O_2^-$ ). When NO and  $O_2^-$  are produced simultaneously, in close proximity, they chemically react very quickly.

The product of this reaction is called peroxynitrite ( $OONO^-$ ). In the presence of certain reactive centers,  $HOONO$  may undergo homolytic cleavage to a hydroxyl free radical ( $\bullet OH$ ) and nitrogen dioxide free radical ( $\bullet NO_2$ ), or heterolytic cleavage to a nitronium cation ( $NO_2^+$ ) and hydroxide anion ( $OH^-$ ). Three of these cleavage products ( $\bullet OH$ ,  $\bullet NO_2$  radicals and  $NO_2^+$ ) are among the most reactive and most damaging species in biological systems and may be major contributors to the severe damage of the heart and brain. The low concentration of NO produced in the heart in atherosclerotic cardiovascular system is a prime cause of heart attack. Also, a continuously produced high concentration of NO in the heart under emotional stress can lead to heart attack. Heart failure is a major cause of death (in USA 185 deaths per 100,000 population per year).

Another serious disease associated with NO is septic shock, which threatens the life of 50–70 million people per year worldwide. The affliction is usually initially caused by a bacterial toxin, which enters the blood circulation through incidental infections, wounds, and surgical procedures. Up to 60% of the people with septic shock do not survive this condi-



**Table II. Pathology of Nitric Oxide Release**

<b>Nitric Oxide Concentration</b>	
too low	too high
hypertension	septic shock
atheriosclerosis	hypotension
diabetes	excessive bleeding
ischemia (stroke, heart Alzheimer's disease)	meningitis
Parkinson's disease	rheumatoid arthritis
fibrosis	
cancer	

tion. Septic shock is due to the extensive release of NO. The immune system, attempting to fight infection, releases so much NO that the system goes out of control. Too high a concentration of NO dramatically decreases blood pressure, which is followed by failure of vital organ system, especially the liver, kidney and heart.

During a heart attack, brain stroke, and any kind of condition which limits the supply of blood and O<sub>2</sub> (ischemia) to an organ, a massive release of NO can be observed. Therefore, if blood flow can not be restored within several minutes, the NOS starts to produce superoxide in addition to NO. The simultaneous, proximal release of both NO and O<sub>2</sub> may lead to serious, sometime irreversible damage especially in the brain or heart.

Knowing the activity and targets of NO in the body, it is possible to develop new drugs which can enhance or inhibit the chemistry of NO production. New drugs, based on inhibition of NO production have been already developed as well as drugs to increase NO concentration (nitric oxide donors) in the body when needed. NO donors which are more effective and controllable will replace nitroglycerin. Also, the proper NO concentration in the body can be maintained by scaveng-

ing  $O_2^-$  or inhibiting the major sources of  $O_2^-$ . Many natural vitamins and other compounds can be used as effective scavengers of  $O_2^-$  production. Even though there are many sources of  $O_2^-$  in humans, a proper diet with foods containing a low amount of fat will help to prevent deposition of cholesterol on the endothelial membrane and subsequent damage by NOS, a major source of  $O_2^-$ .

The variety of vital roles played by NO is a direct consequence of its unique chemical properties in the biological cells. NO is small and chargeless, therefore, it can freely diffuse through the cell membrane as well as through cytoplasm just like  $O_2$  and  $N_2$ . NO has modest chemical reactivity (making it a somewhat selective reducing or oxidizing agent), allowing its survival at least for several seconds in the biological matrix. NO has an unpaired electron and binds to quickly to  $O_2^-$  and more slowly to several metals in the biological matrix including iron in hemoglobin. This reaction leads to the eventual disposal of NO in the body. The story of the role of NO is fascinating and far from being complete. It is also an excellent example of utilization of chemistry in solving important medical problems.

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