Tamás Rőszer

The Biology of Subcellular Nitric Oxide

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If one part suffers, every part suffers with it; if one part is honored, every part rejoices with it.

1 Corinthians 12:26

Foreword

It is with great pleasure that I write this Foreword for the book by Dr. Tamás Rőszer in which every aspect of the intracellular biology of nitric oxide is comprehensively reviewed.

The biological activity of nitric oxide was originally recognised when it was discovered to be the mediator of vascular endothelium-dependent relaxation. As its actions in a variety of other biological systems were unravelled, nitric oxide became known as a mediator of cell-to-cell communication. In the last fifteen years, however, its role as an orchestrator of communication between intracellular organelles has become apparent, opening up an increasingly exciting area of research.

This book provides an elegant overview of current knowledge of the biology of subcellular nitric oxide, not only in mammalian cells but also in plants and fungi. I have no doubt that it will become a reference point, not only for teaching but also for the development of future research.

The Wolfson Institute for Biomedical Research,
University College London

Prof. Sir Salvador Moncada,
FMedSci, FRS

Preface

The latest progress in the field shows that NO is generated within distinct cell compartments, including specific plasma membrane regions, mitochondria, chloroplasts, peroxisomes, the Golgi-complex and intracellular membrane systems. NO synthesis plays specific roles in these compartments and, in turn, cell organelles also control intracellular NO levels. NO is an important biological signal, but a highly reactive molecule as well; thus its biological effects depend on its concentration and the chemical microenvironment of NO synthesis. A key determining factor of cellular NO effects is the subcellular compartmentalization of NO synthesizing enzymes.

To understand the role of cell compartments in NO biology, we may make an everyday analogy: the energy of fire, which can be used for heating in a fireplace or for lighting with a candle. The same factor (the energy of the fire) is required in different quantities in a fireplace and in a candle, to serve different needs. Organelles determine the effects of NO in a similar way, since they produce and tolerate different levels of NO in spatially separated locations in the cell. Organelles effectively control and maintain NO levels within a physiological range and orchestrate temporal and spatial patterns of NO synthesis. Disturbances of this organelle-specific NO homeostasis evoke cellular degeneration.

The rapid development and complexity of subcellular NO biology made it timely to produce a book dedicated to the better understanding of NO in organelle biology and the molecular mechanisms by which cell compartments give home to NO-signaling microdomains and ensure balanced NO production.

I would like to thank the Senior Editor of Springer Life Sciences, Dr. Meran Owen. I am also grateful for the help Tanja van Gaans provided in this project. Valuable image contributions provided by Dr. Madhu Dikshit (Central Drug Research Institute, CSIR, Lucknow), Dr. Mateusz Kolanczyk (Max Planck Institute for Molecular Genetics, Berlin), Dr. Jason E. Lee and Dr. Pravin B. Sehgal (New York Medical College, Valhalla), Dr. Justin Percival (University of Washington, Seattle) and Dr. Iván Schmelczer (Debrecen University, Hungary) are acknowledged. I also wish to thank Dr. Gáspár Bánfalvi (Debrecen University, Hungary) for his support in carrying out my NO-research; the many colleagues at Debrecen University and research groups

of the Hungarian Academy of Sciences, with whom I have worked for years; and Dr. Mercedes Ricote (Spanish National Cardiovascular Research Center, Madrid) for support in my current scientific work. Livia I. Lelkes provided valuable editorial assistance; her careful and timely work is highly appreciated.

Madrid, Spain
15 August 2011

Dr. Tamás Rőszer

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Abbreviations

ATP:	Adenosine triphosphate
BH_4:	Tetrahydrobiopterin
cAMP:	Cyclic adenosine monophosphate
CAT:	Catalase
CcO:	Cytochrome-c oxidase
cGMP:	Cyclic guanosine monophosphate
DAF-2:	*4,5*-diaminofluorescein diacetate (NO-indicator)
FAD:	Flavin adenine dinucleotide
FMN:	Flavin mononucleotide
GSH:	Reduced glutathione
H_2O_2:	Hydrogen peroxide
L-NAME:	N_ω-nitro-L-arginine methyl ester
L-NMMA:	N_ω-momomethyl-L-arginine
L-NNA:	N_ω-nitro-L-arginine
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
NiR:	Nitrite reductase
NO_2^-:	Nitrite
NO_3^-:	Nitrate
NR:	Nitrate reductase
O_2:	Oxygen
O_2^-:	Superoxide
$OH^\bullet$:	Hydroxyl radical
OH^-:	Hydroxide ion
$ONOO^-$:	Peroxynitrite
PKG:	Protein kinase G (cGMP-dependent protein kinase)
SEM:	Scanning electron microscopy
SOD:	Superoxide dismutase
TEM:	Transmission electron microscopy

Part I
General Concepts

Chapter 1
Introduction

1.1 Synthesis of NO in Biological Systems

Nitric oxide (NO) is a toxic free radical gas and an important biomolecule. It is involved in signal transmission between cells, pathogen killing, cellular energy expenditure, cytoprotection and cell death (Ignarro 2002; Bian and Murad 2003; Fang 2004; Calabrese et al. 2009; Murad and Barber 2009; Taylor and Moncada 2010; Luo and Zhu 2011).

Although it has been known since the 1960's that NO is an intermediate product of bacterial denitrification, and that NO emission was measured from plants in the early 1970's, these first studies could not attribute a specific biological role to NO (Barbaree and Payne 1967; Payne et al. 1971; Klepper 1979). Interestingly, organic nitrate esters, which release NO, were used in the treatment of angina pectoris due to their vasodilator effects long before NO's role in circulation was recognized (Ignarro 1989b; Marsh and Marsh 2000). In the late 1980's, three independent research lines converged in the same direction and established that NO is produced within cells and that NO plays specific biological roles in mammals and the human body (Fig. 1.1). These studies have established the major functions of NO in the circulation, the nervous system and the immune response (Griffith et al. 1984; Moncada et al. 1989; Moncada and Palmer 1991; Furchgott 1993). In the cardiovascular system, NO is emitted from the endothelial cells and evokes relaxation of the vascular smooth muscle cells, thereby increasing arterial blood flow (Moncada et al. 1989). In the nervous system, NO is a neurotransmitter and is required for intercellular signal transmission (Marletta et al. 1990). Overproduction of NO evokes cell death and neuron loss (Moncada et al. 1989). Phagocytosing immune cells also produce NO and use it as a weapon against cellular pathogens (Rosen et al. 1995). These findings led to the birth of NO biology. In 1992, NO was proclaimed the "Molecule of the Year" by the leading scientific journal *Science*, hallmarking a starting point of a new era in biomedicine, which began the search for other gas transmitters and biological functions of free radicals (Koshland 1992). In 1998, the Nobel Prize in Physiology or Medicine was granted to three pioneering researchers of the newborn NO biology (Bradbury 1998; Xu and Liu 1998). NO-research has extended to organisms

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Fig. 1.1 The biological attributes of NO. The classical NO-image depicts a vagabond molecule that can freely cross cell borders and cause cell death, transmit messages between cells (e.g. between neurons or endothelia and vascular smooth muscle cells) and can protect the body from pathogens as a weapon of cellular immunity. Artwork by Péter Dráviczky

other than mammals, and NO-mediated regulatory networks have been identified in various invertebrates, plants and more recently in prokaryotes (Martinez 1995; Shapiro 2005; Amaroli et al. 2006; Crane et al. 2010; Moreau et al. 2010; Andreakis et al. 2011).

Today, various faces of NO are known: a poisonous free radical that evokes chemical injury of cell proteins, lipids and DNA, thereby induces apoptosis, and leads to necrosis or eliminates pathogenic cells (Rivero 2006; Rameau et al. 2007; Calabrese et al. 2009). On the contrary, NO is an important mediator involved in synaptic plasticity, neuronal cell path finding, sensory organ physiology, pain modulation, motor functions, pulmonary-, renal and cardiovascular biology (Seddon et al. 2008; Baylis 2009; Milsom et al. 2010; Tjong et al. 2011). Among many other functions, this molecule is required for the establishment of symbiotic relationships between prokaryote and eukaryote cells, development of antibiotic tolerance in bacteria, cellular accommodation to hypoxia in various organisms or successful fusion of gamete cells (Lewis et al. 1996; Gusarov et al. 2009; Del Giudice et al. 2011; Gupta and Igamberdiev 2011). Of biomedical importance, the overproduction of NO occurs in certain inflammatory reactions, autoimmune conditions, cell degeneration and ischemia-reperfusion injury (Uesugi et al. 2000; Hirai et al. 2001; Balercia et al. 2004; Milsom et al. 2010; Nagy et al. 2010). Mitigation of NO synthesis is of interest in the medical intervention of several pathologies (Chabrier et al. 1999; Bian and Murad 2003; Atochin and Huang 2010; Nagy et al. 2010; Joubert and Malan 2011; Takizawa et al. 2011). The lack of NO synthesis leads to various disorders including compromised pathogen defense, endothelial dysfunction, atherosclerosis, cardiac events, inherited motor disorders and muscle dystrophies (Salzman 1995; Donnelly et al. 1997; Deckel 2001; Dudley et al. 2006; Tidball and Wehling-Henricks 2007; Loot et al. 2009; Atochin and Huang 2010; Michel and Vanhoutte 2010; Percival et al. 2010).

1.2 Mechanisms of NO Production

NO can be released from various nitrogen oxides, such as NO_2^- or nitrous acid under acidotic conditions (Fig. 1.2a). This non-enzymatic NO emission is reliable only in a limited number of acidotic compartments, such as the apoplasm of the plant cells and the stomach of mammals, where the release of NO from nitrogen oxides displays certain biological effects (Duncan et al. 1995; Shapiro 2005) (Chaps. 2 and 3).

Apart from this abiotic NO release, NO can be generated by enzymatic processes (Fig. 1.2b, 1.2c). Dissimilatory nitrite reductase (a key enzyme of the denitrification process) and in some cell types nitrate reductase are capable of reducing NO_2^- to NO (Shapiro 2005; Starkenburg et al. 2008; Kim et al. 2010) (Chaps. 3–5). Under hypoxic conditions the NO_2^-/NO reduction can also be catalyzed by the mitochondrial electron transport chain and deoxygenated hemoglobins (Valdez et al. 2004; Shiva et al. 2007; Gupta and Igamberdiev 2011; Tiso et al. 2011) (Chaps. 4, 5 and 10). Collectively, these mechanisms consist of the so-called reductive way of NO generation, which occurs mainly under O_2 limitation in prokaryotes, plants, fungi and in animal cells (Payne et al. 1971; Li et al. 1997; Kozlov et al. 1999; Jasid et al. 2006; Kim et al. 2010; Tiso et al. 2011).

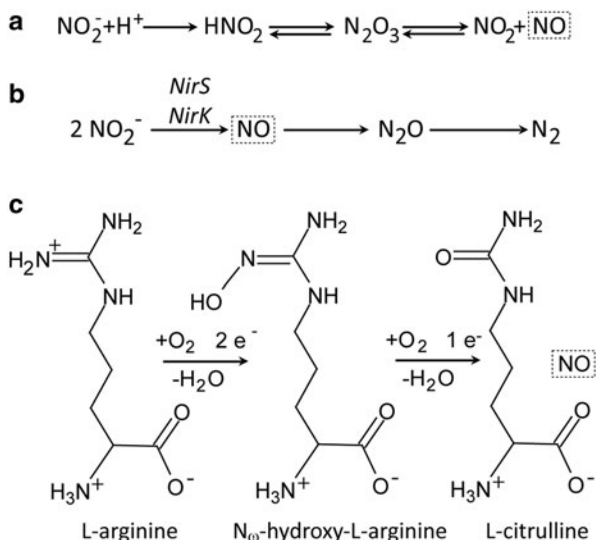


Fig. 1.2 Forms of NO generation in biological systems. Low pH (e.g. in the mammalian stomach or plant apoplast) favors non-enzymatic NO release from nitrous acid (HNO_2), the protonated form of NO_2^- (**a**). In reductive NO generation NO_2^- is reduced to NO by various reductases (**b**); for example by dissimilatory nitrite reductases (NirS, NirK) in the bacterial denitrification chain. The oxidative NO generation is catalyzed by NOS and the NO-giving substrate is the amino acid L-arginine (**c**)

In mammals, the biologically important NO generating enzymes are the NO-synthase (NOS, EC 1.14.13.39) proteins (Andrew and Mayer 1999). The first studies in the field have identified three NOS isoforms, the endothelial (eNOS or NOS3), the neuronal (nNOS or NOS1) and the inducible (iNOS, NOS2) isoforms; all of them are encoded by distinct genes (Xu and Liu 1998). Both eNOS and nNOS are expressed constitutively in various cell types. Although their transcription can be upregulated under certain conditions (Huber-Abel et al. 2011), their activity is triggered by increased intracellular Ca^{2+} levels (Andrew and Mayer 1999). In contrast, the activity of iNOS is not dependent on the Ca^{2+} supply and the induction of its transcription (e.g. by inflammatory stimuli) is the key determinant of the NO synthesis in iNOS-containing cells (Ganster et al. 2001). Today, several NOS molecules are known from various species representing the entire phylogenic tree: bacteria, unicellular eukaryotes, myxomycota, fungi, plants, metazoans and several invertebrate species express NOS enzymes (Malvin et al. 2003; Crane et al. 2010; Gonzalez-Domenech and Munoz-Chapuli 2010; Andreakis et al. 2011). Some invertebrate-type NOSs are expressed constitutively but pathogen inducible NOS is also known (Rodriguez-Ramos et al. 2010). Vertebrate-type NOSs have evolved from a common invertebrate-type ancestral NOS and the eNOS is considered the evolutionarily most recently evolved NOS (Gonzalez-Domenech and Munoz-Chapuli 2010). In vertebrates, several splice variants and post-translational modifications of the three NOS isoforms are also known, many of them display specific subcellular distribution (Lu et al. 2010; Percival et al. 2010).

Members of the NOS enzyme family share similarities in their domain structure and catalytic properties (Andreakis et al. 2011). The active NOS is a homodimer.

Each monomer is built up from a heme-containing oxygenase, and a flavoprotein reductase domain (Andrew and Mayer 1999). The active NOSs oxidize the guanidino group of L-arginine to form L-citrulline and elaborate NO (Moncada et al. 1989). Although L-arginine/L-citrulline conversion can occur in other biochemical pathways, the conversion of the guanidino nitrogen to NO is a distinctive property of the NOS molecules (Sudhamsu and Crane 2009). The catalysis requires O_2, NADPH, FAD, FMN and BH_4; and also Ca^{2+} or Ca^{2+}/calmodulin in the case of many NOS molecules. The presence of O_2, substrate-, and cofactor supply are the main prerequisites of an ongoing NOS activity. In the case of Ca^{2+}-dependent NOS enzymes, the binding of Ca^{2+}/calmodulin triggers NO synthesis (Fleming 2010; Luo and Zhu 2011). Moreover phosphorylation and association with several adaptor proteins ensure the balanced NO production (Chap. 6).

1.3 Cellular Targets of NO: How Far from NO Synthesis?

1.3.1 The Many Targets of NO

The major cellular target of NO is the heme-containing lyase enzyme, the soluble or type 2 guanylyl cyclase (EC 4.6.1.2) (Arnold et al. 1977; Katsuki et al. 1977). This enzyme catalyzes the conversion of guanosine triphosphate (GTP) to 3′–5′ cyclic guanosine monophosphate (cGMP), an important intracellular second messenger molecule (Schaap 2005) (Fig. 1.3). Increased cGMP synthesis regulates cGMP-dependent protein kinase (PKG), phosphodiesterases and ion channels, thus modulating the phosphorylation state of several proteins and affecting cellular ion homeostasis (Ke et al. 2001; Gertsberg et al. 2004). Other heme-containing proteins can also be targets of NO: e.g. oxyhemoglobin, cytochromes, catalase; or iron-sulphur enzymes, such as aconitase and NADH-dehydrogenase (Kremser et al. 1995; Poderoso et al. 1996; Cooper 1999). The NO/oxyhemoglobin interaction is an important mechanism to eliminate excess NO by oxidizing it to NO_2^- (Gow et al. 1999).

Another important reaction of NO is the S-nitrosylation of proteins (Fig. 1.3). In this reaction NO forms a nitrosyl group with the thiol group of cysteine residues of proteins (Foster et al. 2003). S-nitrosylation represents a dynamic post-translational modification of proteins which transduces NO-signals with various biological effects: for example hemoglobin S-nitrosylation yields a long-distance acting NO-carrier molecule, which can release NO in hypoxic capillaries (Gow 2005). S-nitrosylation of ADP-ribosyl cyclase leads to reduced synthesis of the second messenger cyclic-ADP-ribose, an important modulator of intracellular Ca^{2+} transients (White et al. 2002). Ion channels, cell junctions, apoptotic proteins can also be subjects of S-nitrosylation, determining their cellular effects (Sun et al. 2001; Lee et al. 2010; Donoso et al. 2011; Straub et al. 2011). S-nitrosylation of nuclear proteins has also been described, which mediates epigenetic changes and controls gene expression

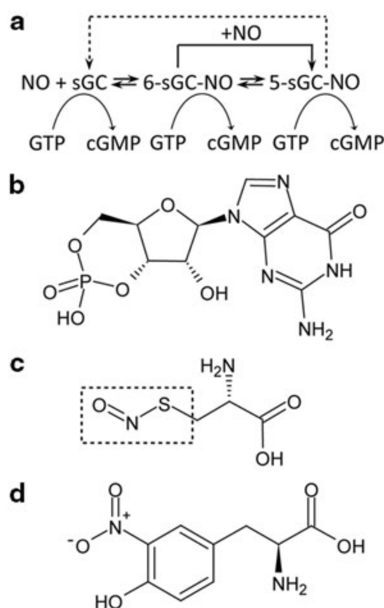


Fig. 1.3 Molecules of the NO-mediated signal transduction. Soluble guanylyl cyclase (*sGC*) is an important target of NO (**a**). The initial binding of NO to the heme group of the sGC molecule initiates GTP-cGMP conversion. A six-coordinate sGC-nitrosyl intermediate is formed which is further converted by NO-dependent and independent mechanisms to a penta-coordinate active complex (Tsoukias 2008). The sGC activation increases the intracellular level of the second messenger cGMP (**b**). NO and NO-derivatives also evoke S-nitrosylation of cysteine residues (**c**) by forming S-nitrosyl groups (in *dotted frame*), or cause tyrosine nitration (3-nitrotyrosine, **d**)

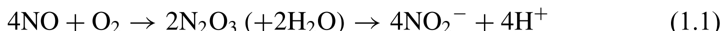
(Nott and Riccio 2009). Additionally, NO can modulate gene expression through various transcription factors (Bar-Shai and Reznick 2006; Chiranand et al. 2008; Biedasek et al. 2011). Tyrosine nitration is also an effect of NO-derivatives, such as peroxynitrite ($ONOO^-$). Nitration of tyrosine residues may impair protein function, by reducing enzyme activities or diminishing signal transduction (Tórtora et al. 2007). Moreover, $ONOO^-$ can evoke necrotic cell death (Virag et al. 2002).

1.3.2 Limited Diffusion of NO Expands the Frames of NO Biology

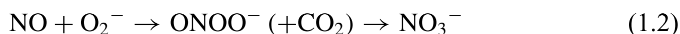
The many targets of NO can reside in the cytoplast, can be associated with the plasma membrane, and can be located in the mitochondria or the chloroplasts. Since NO acts through several mechanisms by affecting distinct subcellular units, one can raise the question how a diffusible molecule can reach these targets without evoking a chaotic signal transmission? The answer can rely in the spatial separation of distinct NO synthesizing compartments within the cell.

Both reductive and oxidative NO synthesis occurs in specific subcellular compartments. Near NO synthesizing enzymes, the downstream targets such as guanylyl cyclase or proteins for S-nitrosylation are enriched (Iwakiri et al. 2006; Fleming 2010; Straub et al. 2011). The accumulation of NO within cell organelles without a free diffusion to the cytoplasm has also been documented in several studies (Lopez-Figueroa et al. 2000; Jasid et al. 2006). These phenomena support the idea that the cells contain several independent NO-signaling microdomains and the locally produced NO acts locally, without diffusing toward distant cellular locations.

However, the canonical NO-image depicts a highly diffusible and rapidly spreading molecule, which crosses cell borders and reaches target molecules far from the source of NO generation (Wood and Garthwaite 1994; Lancaster 1997). NO is a lipid soluble molecule and can escape from the cells; however the half-life of NO highly determines its diffusion distance. The simplest model for estimating NO half-life takes into account only the non-catalyzed degradation of NO, the so-called autoxidation process (1.1, 1.2), which leads to NO decomposition to NO_2^-, NO_3^- and $ONOO^-$.



$$4NO + O_2 \rightarrow 2N_2O_3\ (+2H_2O) \rightarrow 4NO_2^- + 4H^+ \tag{1.1}$$



$$NO + O_2^- \rightarrow ONOO^-\ (+CO_2) \rightarrow NO_3^- \tag{1.2}$$

In this model, the concentration of O_2 is the key limiting factor of the half-life of NO. In a cell-free solution for example ~ 830 s is the estimated half-life of 1 μM NO in the presence of 200 μM O_2 (Shapiro 2005). In the cytoplasm and cell organelles however, the O_2 concentration is much lower: ranging from 1 to 50 μM, and giving an extreme estimated half-life of NO such as > 15 h in the mitochondria (Shapiro 2005). Other estimates predict 440–830 s half-life of NO in mammals (Hakim et al. 1996) and 670 s in plant cells (Shapiro 2005). The measured half-life of NO is still ~ 200 s in a cell-free medium under conventional cell culture conditions (Chin and Deen 2010). However, the measured half-life of NO ranges from 0.2 ms to 2–5 s in most biological systems (Griffith et al. 1984; Ignarro 1989a, b; Thomas et al. 2001; Balbatun et al. 2003). In tissues, NO is eliminated not only by autoxidation but also by other enzymatic mechanisms, such as conversion to N_2O by NO-reductases, oxidation to NO_2^- by cytochrome-c oxidase and oxyhemoglobin or generation of reactive nitrogen species by reacting with hydrogen peroxide and O_2^- (Joshi et al. 2002; Kim-Shapiro et al. 2006; Tsoukias 2008).

By knowing the half-life (t) of NO, we can predict its radius of action (Δx) using the Einstein-Smoluchowski Eq. (1.3), where *D* is a diffusion constant of NO (3,400 $\mu m^2\ s^{-1}$ in water and 2,000–3,300 $\mu m^2\ s^{-1}$ in various biological media).

$$\Delta x = \sqrt{2 \times D \times t} \tag{1.3}$$

Using various half-life values, this calculation gives an average diffusion radius for NO ranging from some micrometers (reliable in tissues) to millimeters (e.g. in cell