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).

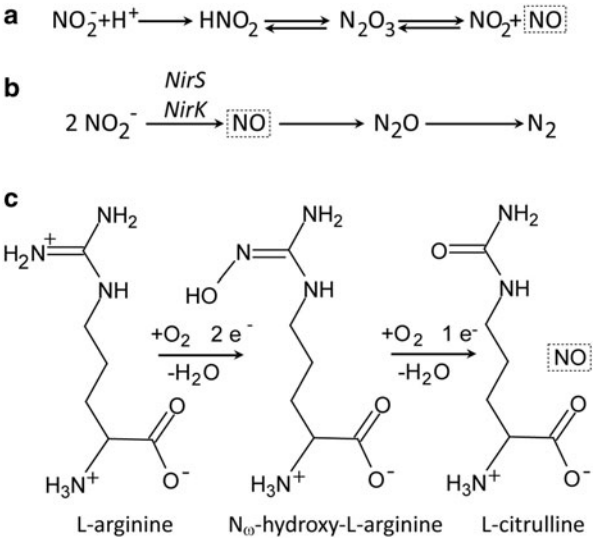

Fig. 1.2 Forms of NO generation in biological systems. Low pH (e.g. in the mammalian stomach or plant apoplast) favors non-enzymatic NO release from nitrous acid (HNO_2), the protonated form of NO_2^- (**a**). In reductive NO generation NO_2^- is reduced to NO by various reductases (**b**); for example 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

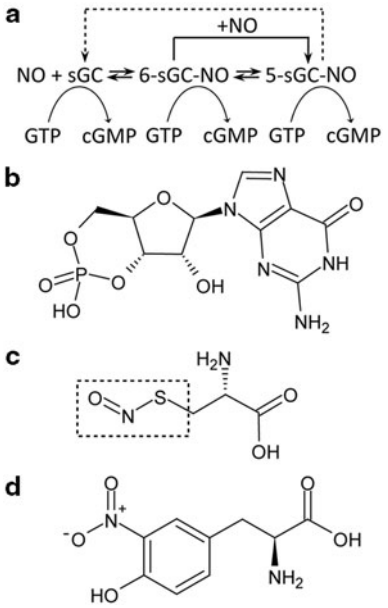

Fig. 1.3 Molecules of the NO-mediated signal transduction. Soluble guanylyl cyclase (*sGC*) is an important target of NO (**a**). The initial binding of NO to the heme group of the sGC molecule initiates GTP-cGMP conversion. A six-coordinate sGC-nitrosyl intermediate is formed which is further converted 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

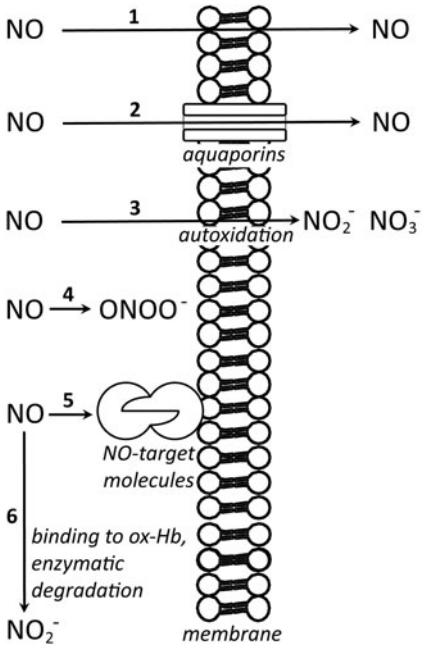

Fig. 1.4 Diffusion and degradation of NO: determinants of the radius of action. NO can diffuse through the membranes and act long distances from its source (*1*). Water channels (aquaporins) help NO to cross membranes (*2*) (Hachez and Chaumont 2010). However, autoxidation in the membrane (*3*) and in the cytoplasm (*4*) limits the diffusion of NO. NO can also bind to various targets (*5*) or degrade (*6*) without leaving the membrane-bound cellular unit. *ox-Hb* oxyhemoglobin

cultures) (Lancaster 1997). Subcellular location of NO synthesis thus determines the diffusion distance of NO: at the cell surface, e.g. in neurons and endothelial cells NO may release to the extracellular space and act as an intercellular mediator, with higher radius (10–30 μm) (Vaughn et al. 1998) than within e.g. a chloroplast (less than 7 μm) (Jasid et al. 2006).

Collectively, in membrane-bordered cell compartments NO is eliminated by various site-specific mechanisms, thereby the escape of NO is limited (Fig. 1.4). Of note, the autoxidation process is more effective in biological membranes than in water-based solvents, leading to the degradation of NO while it penetrates the cell membranes (Lancaster 2000). Together, these effects lead to the concentration of NO within the membrane-bound cell compartments.

NO is no longer the vagabond molecule it was previously considered: upstream activators of NO synthesis, targets of NO and the mechanisms ensuring NO elimination are grouped in subcellular compartments (Fig. 1.5). This book is an introduction to this novel aspect of NO biology and provides an overview on the evolution, biology and clinical relevance of the subcellular NO-signaling microdomains.

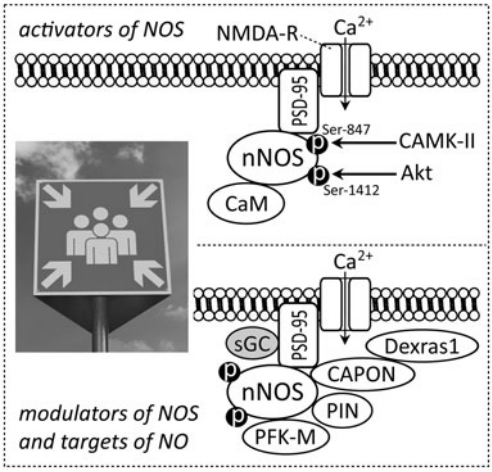

Fig. 1.5 NO-signaling microdomains: cellular assembly points for modulators and targets of NO synthesis. Upstream activators and downstream target molecules of NO synthesis are arranged in signaling microdomains within subcellular compartments. The example shows the assembly of nNOS and its associated proteins in the postsynaptic neuronal cell membrane. Activators of NO synthesis: NMDA-receptor (NMDA-R), Ca^{2+}/calmodulin (CaM), protein kinases (CAMK-II, Akt). Soluble guanylyl cyclase (*sGC*) is the major target of NO. Various NOS-associated proteins are modulators and possible targets of NO synthesis (CAPON, PIN, Dexras-1, PFK-M). The core molecule of this signaling complex is PSD-95. For further details see Chap. 6

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Part II
Nitric Oxide Synthesis in Prokaryote Cells

Chapter 2
Nitric Oxide is a Bioproduct in Prokaryotes

2.1 Prokaryotes are NO Producer Organisms

It was known since the 1960's that various denitrifying bacteria generate NO as an intermediate product of their dissimilatory NO_3^- metabolism (Barbaree and Payne 1967; Payne et al. 1971; Balderston et al. 1976). Today several prokaryote species are known as NO producers; many of them synthesize NO by reduction of NO_2^-, while others contain NOS-like enzymes and show oxidative NO generation from L-arginine or Nω-hydroxy-L-arginine (Zumft 1993; Sudhamsu and Crane 2009; Crane et al. 2010). Various ecological niches house these NO producer prokaryotes: marine environments (Baumann et al. 1983; Romanenko et al. 2005; Weon et al. 2006; Marinho et al. 2009; Santos et al. 2010), poorly ventilated or flooded soils (Zumft 1997), contaminated and eutrophized waters (Shapleigh 2006; Kampschreur et al. 2007, 2008), fermented meat or milk products (Morita et al. 1997; Gündoğdu et al. 2006; Gotterup et al. 2007) and the surface of mucosal barriers (Salzman 1995; Cuzzolin et al. 1997) are all colonized by NO synthesizing bacteria.

Better understanding of prokaryote NO production has agricultural, biotechnological and medical impact. Importantly, mitochondria and chloroplasts preserved some key features of prokaryote NO synthesis, giving an evolutionary vista to the NO synthesizing prokaryote world. Although NO was recognized as a bacterial bioproduct much before the discovery of the physiological impact of NO in mammals, the importance of bacterial NO synthesis gained attention only in very recent years (Sudhamsu and Crane 2009).

2.2 Bacteria Synthesize NO and Contribute to Chemical NO Release from Nitrogen Oxides

Bacteria may produce NO endogenously, but their ability to metabolize nitrogen oxides also contributes to abiotic NO release in their surroundings. Of biotechnological impact, the fermentation process of milk products or cured meat involves both of these two distinct mechanisms: NO can be synthesized by the bacteria, or the

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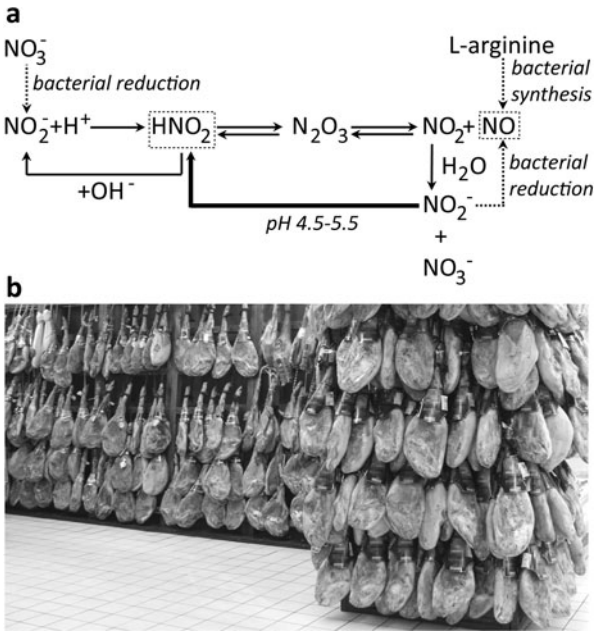

Fig. 2.1 Chemical and bacterial NO liberation in the fermentation process. *Top*: Meat fermentation starts with the addition of NO_3^- and NO_2^- containing salts, which at the prevailing pH of the meat are chemically transformed to nitrous acid (HNO_2) and NO. Bacterial reduction of NO_2^- or oxidation of L-arginine also contributes to the NO production. In fermented milk products, the bacterial NO synthesis is more prominent than the chemical NO release. In meat, the generated HNO_2 has an antimicrobial effect, while NO forms a complex with myoglobin and develops the characteristic red color of cured meat. *Bottom*: Iberian ham is an example of cured meat

bacterial NO_3^- metabolism can facilitate chemical NO release from nitrogen oxides (Fig. 2.1).

Chemical NO release occurs during the meat curing process, when NO_3^- and NO_2^- containing salts are added to the meat (Møller et al. 2003). This treatment leads to the replacement of a predominantly Gram-negative flora of aerobic saprophytes by Gram-positive bacteria of the lactic acid group, which convert NO_3^- to NO_2^- (Gündoğdu et al. 2006). In the slightly acidic environment of the meat (pH 5.5–6.5) NO_2^- forms nitrous acid (HNO_2) and NO (Shank et al. 1962). As a result NO establishes a pentacoordinate complex with the hem-group of meat myoglobin, producing nitrosylmyoglobin, the stable red pigment of cured meat (Møller et al. 2003). The presence of nitrogen oxides (especially HNO_2) in the meat also exerts a bacteriostatic effect, which determines the quality of the bacterial flora and is required for the proper curing and preservation of the meat (Hu et al. 2007). In this process, the bacterial activity only provides a substrate (NO_2^-) supply and maintains the appropriate conditions (e.g. pH and O_2 saturation) for chemical NO elaboration (Fig. 2.1).

However, various isolates of lactic acid bacteria from fermented milk and meat products also produce NO enzymatically and thereby are capable of converting myoglobin to nitrosylmyoglobin (Møller et al. 2003; Gündoğdu et al. 2006). The most important NO synthesizing bacteria in fermented products are *Pediococcus acidilactici* S2, *Pediococcus acidilactici* S3, *Lactobacillus plantarum* T119, and *Lactobacillus fermentum* (Møller et al. 2003; Gündoğdu et al. 2006; Hu et al. 2007).

Similarly, various isolates of cured meat-associated staphylococci are also NO producers (Gotterup et al. 2007). These bacteria elaborate NO as a byproduct of their dissimilatory NO_3^- metabolism, by reducing NO_2^-. Interestingly, *Lactobacillus fermentum* produces NO in the absence of NO_2^- or NO_3^-, using L-arginine as a substrate (Khouw and McCurdy 1969). In this way, NO also occurs as a bacterial product in the fermentation process (Fig. 2.1).

This combined chemical and bacterial NO generation also has physiological relevance, as it has been pointed out in the antimicrobial defense mechanism of the mammalian stomach. Physiologically the salivary glands secrete NO_3^- (derived from the dietary NO_3^- of green vegetables), which is concentrated in the saliva. Although NO_2^- is not secreted by the saliva, it is measurable in the oral cavity (Fritsch et al. 1985). This NO_2^- is a product of bacterial reduction of dietary NO_3^-. This NO_3^-/NO_2^- reduction is missing from germ free rats, and reduced by the administration of wide-spectrum antibiotics in humans (Dougall et al. 1995), confirming that microbial activity is responsible for NO_2^- production (Duncan et al. 1995). The posterior dorsal surface of the tongue (dorsum linguae) harbours large numbers of facultative anaerobic bacteria (*Staphylococcus spp., Streptococcus spp. Rothia spp., Veillonella spp.*) which reduce NO_3^- to NO_2^- under hypoxic conditions (Li et al. 1997; Doel et al. 2005). Various denitrifier bacteria of the oral cavity further reduce NO_2^- to NO and N_2O (Mitsui and Kondo 1998; Mitsui and Kondo 1999; Bayindir et al. 2005). These nitrogen oxides display antimicrobial activity; however, the more potent microbicidal effect evolves when the NO_2^- enriched saliva reaches the stomach (Benjamin et al. 1994; Duncan et al. 1995; McKnight et al. 1997). In the acidic environment of the stomach NO_2^- is being protonated and forms various nitrogen oxides (dominantly NO), which act as antimicrobial agents and facilitate the elimination of pathogens ingested with the food (Benjamin et al. 1994; McKnight et al. 1997) (Fig. 2.2). For example *Candida albicans* and *Escherichia coli* are much more susceptible to the combined effect of nitrogen oxides and gastric acid than the acidic destruction alone (Benjamin et al. 1994). High NO_2^- intake can lead to NO-evoked injury of the stomach epithelia at the esophageal-gastric junction, underscoring the role of salivary NO_2^- levels in the control of intragastric chemical NO release (Asanuma et al. 2005) (Fig. 2.2).

2.3 NO-Generating Microbes: Health, Biotechnological and Ecological Impact

Denitrifying bacteria, which generate NO as a byproduct are present in various mucosal barriers (e.g. in the airways and the upper alimentary tract) (Salzman 1995) and in the dental plaques (Bayindir et al. 2005). Under pathological conditions NO releasing bacteria are abundant in infected areas of mucosal layers (Genc et al. 2006). Importantly, NO production increases the antibiotic resistance and stress adaptation of certain pathogenic bacteria (Gusarov et al. 2009). However, we are still far from definitely understanding the physiological and pathological roles of

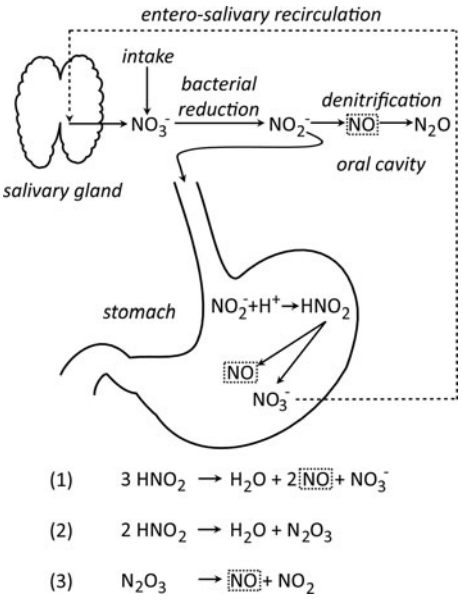

Fig. 2.2 Combined bacterial and chemical NO generation in the upper digestive system. NO_3^- is either secreted by the saliva or derived directly from dietary uptake. Bacterial reduction of NO_3^- to NO_2^- or NO and N_2O takes place in the oral cavity. Within the acidic environment of the stomach NO_2^- forms nitrogen oxides—including NO—by reactions (*1*–*3*), providing an antimicrobial defense mechanism. The acidic pH of the stomach also favors NO_3^- generation, which is absorbed and then concentrated again in the saliva (Fritsch et al. 1985). This entero-salivary pathway (Duncan et al. 1995) recirculates approximately 25% of the dietary NO_3^-

microbial NO synthesis. It is already known that NO synthesis by the gingival eNOS and iNOS has a protective role in the oral mucosa, since NO is an important agent against *Porphyromonas gingivalis*, the main periodontal pathogen (Gyurko et al. 2003; Skaleric et al. 2006). Consequently, altered NO homeostasis in the oral mucosa is accompanied with periodontal disease (Ohashi et al. 1999; de Sa Siqueira et al. 2010; Parwani et al. 2011) and inhibition of eNOS (Sun et al. 2010). Although the iNOS expression is increased in the *P. gingivalis* infected mucosa (Sun et al. 2010), the salivary NO_2^- level is lower in patients with periodontitis than in healthy subjects (Aurer et al. 2001). This may reflect the increased NO_2^- consumption of bacterial communities of the oral cavity, although the impact of the microbial NO emission in this pathology is still elusive.

Recently, the relevance of bacterial NO emission in industrial biotechnology has also been defined, showing that it impacts the biological degradation of waste materials (Kampschreur et al. 2008) and the production of renewable fuels (Ahmed and Lewis 2007). In wastewater treatment systems, ammonia-oxidizing (nitrosifying), NO_2^- oxidizing (nitrifying) and denitrifying bacteria produce NO and NO_2^- availability and O_2 limitation generally favors their NO emission (Stüven and Bock 2001; Kampschreur et al. 2007, 2008, 2009) (Fig. 2.3). The negative effects on some biodegradation processes from bacterial generation of NO have been shown (Tas and Pavlostathis 2008; Okutman Tas and Pavlostathis 2010). Microbial conversion of biomass-generated synthesis gas to ethanol, a candidate renewable fuel, is also affected by NO production of microbes (Ahmed and Lewis 2007).

Soil bacteria are also NO producers: in acidic forest soil (pH 4.0) denitrifying bacteria are responsible for NO release (Krämer and Conrad 1991). In the slightly alkaline agricultural soil (pH 7.8) reduced ventilation (low O_2 availability) and soil

Fig. 2.3 Examples of ecological niches occupied by NO-synthesizing bacteria. Various NO producer prokaryotes are present in wastewater treatment systems, contaminated and eutrophized waters, cultivated agricultural soils, sandstones, poorly ventilated or flooded soils. NO may be generated in the major conversions of the biogeochemical nitrogen cycle: denitrification, nitrification and ammonium oxidation. Some N_2-fixing bacteria and cyanobacteria under hypoxia also reduce NO_2^- to NO and NOS-containing bacteria may generate NO by L-arginine oxidation. Local NO concentrations in bacterial communities suggests that NO is an important bioactive compound in natural environments. The production and consumption of NO is shared by separate regions of stratified microbial communities. (Schreiber et al. 2008)

fertilization (excess NO_3^- and NO_2^-) favors NO generation (Krämer and Conrad 1991) by both denitrifier and NO_2^- reducing nitrifier bacteria (Remde and Conrad 1991; Martinez-Espinosa et al. 2011) (Fig. 2.3). Bacterial NO production is also involved in the establishment of symbiotic interaction between rhizobiont bacteria and plant roots (Del Giudice et al. 2011). Soil bacteria with the ability to reduce NO_2^-

to NO have been detected even at 6000 m heights of the Himalayas (Henry et al. 2006). Anaerobic microniches within the generally aerobic uppermost soil layer (0.05–0.1 m) are the sites of this reductive NO synthesis (Remde et al. 1993). Bacterial NO_2^-/NO reduction therefore affects NO_2^- content and thereby determines the quality of the upper soil layer (Martinez-Espinosa et al. 2011). Moreover, bacterial NO emission has more general environmental impact since NO is an air pollutant gas (Jaegle et al. 2004; Martinez-Espinosa et al. 2011). Its release from the soil shows that seasonal periodicity and environmental factors (e.g. rain) affect the rate of NO emissions (Jaegle et al. 2004). Of global importance, rainforest soils exhibit high NO emission rates. For example, the total NO_x emission of the soils in tropical Africa is ~3 TgN (10^{12} g biomass)/year, although the majority of NO_x is scavenged and consumed within this ecosystem (Jaegle et al. 2004).

Interestingly, NO producing bacteria may grow in the soil around buildings and may also colonize corroding surfaces of buildings or sandstones of historical monuments in air polluted regions (Bock 1987; Meincke et al. 1989) (Fig. 2.3). These bacteria metabolize nitrogen compounds derived from the contaminated air and the generated NO is released to the atmosphere (~0.4–4 ng/h/g) (Baumgärtner et al. 1990) or forms nitric acid and leads to the corrosion of the mineral content of the sandstones (Meincke et al. 1989). Although the growth of these bacteria is rather slow, their endolithic NO production may have impact on the deterioration of our technical environment (Sand and Bock 1991) and at a greater time scale on the preservation of archeological objects (McNamara et al. 2006).

2.4 Mechanisms of Reductive NO Synthesis in Prokaryotes

2.4.1 Denitrifying Bacteria Reduce NO_2^- to NO: NO Synthesis in Anaerobiosis

Denitrification is a form of dissimilatory NO_3^- metabolism (Fig. 2.4), in which NO_3^- is being sequentially reduced to NO_2^-, NO, nitrous oxide (N_2O) and dinitrogen (N_2) by four nitrogen oxide reductases (NO_3^-, NO_2^-, NO, and N_2O reductases, respectively) (Zumft 1997). Among prokaryotes, the denitrifying organisms are mostly facultative anaerobic, chemolithotrophic, phototrophic, diazotrophic or organotrophic bacteria and archaea[1] (Zumft 1997). Denitrification allows anaerobic nitrate-respiration and may also be a mechanism to dispose of excess reducing equivalents and modulate intracellular redox state (Shapleigh 2006).

The ability of NO production by a prokaryote was first shown in the marine denitrifying bacterium *Pseudomonas perfectomarinus* (Barbaree and Payne 1967) [its taxonomic name has been revised and corrected to *Pseudomonas perfectomarina*

[1] Mitochondria of fungi also contain denitrifying enzymes.

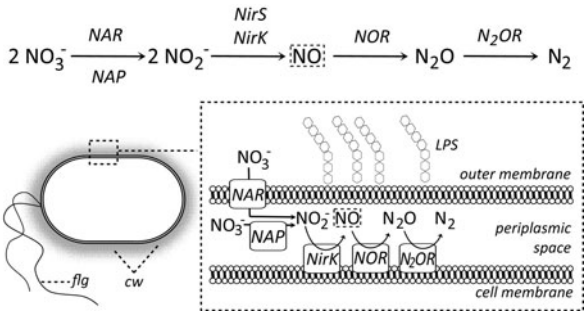

Fig. 2.4 Generation of NO by the denitrification system. Denitrification is a form of dissimilatory NO_3^- metabolism, in which NO_3^- is being sequentially reduced to NO_2^-, NO, N_2O and N_2 by four nitrogen oxide reductases. NAR: NO_3^--reductase, NAP: periplasmic NO_3^--reductase, NirK, NirS: dissimilatory NO_2^--reductases, NOR: NO-reductase, N_2OR: N_2O-reductase. In Gram-negative bacteria, these enzymes are associated with the cell membrane, the outer membrane and the periplasmic space. *flg* flagellum, *cw* cell wall, *LPS* lipopolysaccharide

(Baumann et al. 1983), and more recently, it is considered a member of the *Pseudomonas stutzeri* subgroup (Lalucat et al. 2006)]. This facultative anaerobe species is a Gram-negative, rod-shaped bacterium, first isolated from marine sediments (Zobell and Upham 1944). It belongs to the class of γ-proteobacteria within the phylum of proteobacteria (Lalucat et al. 2006). Under low oxygen tension and in the presence of NO_3^- or NO_2^- it is able to respire anaerobically by means of denitrification (Liu et al. 1983), and thereby produces NO. Several closely related *Pseudomonas* species are known from marine sediment [e.g. *P. stanieri*, *P. nautica*, *P. doudorofii* (Baumann et al. 1983)] although these species are non-denitrifiers (Baumann et al. 1983) thus they possibly lack the ability to generate NO. Recently, several novel denitrifying *Pseudomonas* species, (*P. balearica, P. migulae, P. brenneri, P. pohangensis, P. pachastrellae* and a close relative of *P. segetis*), have been described from aerated seawater, the seashore and in association with marine sponges (Romanenko et al. 2005; Weon et al. 2006; Gao et al. 2010), suggesting that NO-producing bacteria may be more widespread in the marine environment than it was previously expected.

In *Pseudomonas perfectomarina* (*stutzeri*), the NO synthesis is detectable in cells grown on NO_3^- or NO_2^- media under anoxic conditions, and NO liberation is associated with the NO_2^- reducing cell fraction (Payne et al. 1971). The responsible NO-generating enzyme is a membrane-bound NO_2^- reductase (NiR), which catalyzes the one-electron reduction of NO_2^- to NO (Balderston et al. 1976; Liu et al. 1983). The mechanism of NO generation by denitrification has been studied in various *Pseudomonas* species (Matsubara and Zumft 1982), the α-proteobacteria *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, the β-proteobacteria *Achromobacter cycloclastes* and *Ralstonia eutropha* and some representatives of archaea (Risgaard-Petersen et al. 2006). In these species, NO occurs as an intermediate product of denitrification (Shapleigh 2008) and the reduction of NO_2^- to NO is catalyzed by hem-containing cytochrome-cd_1 NiR (NirS or type 1 NiR) or copper-containing

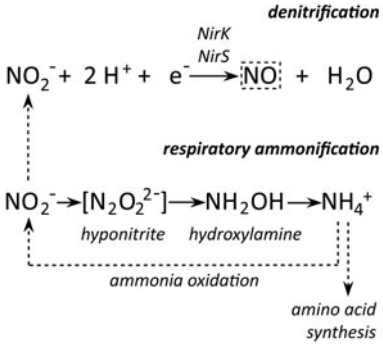

Fig. 2.5 Two distinct ways of NO_2^- reduction: denitrification and respiratory ammonification. Reductive NO synthesis occurs in the denitrification process, catalyzed by dissimilatory NO_2^- reductases (e.g. NirK or NirS). Similar NO_2^-/NO reduction may also occur in eukaryote cells (e.g. by a NirK-like mitochondrial enzyme in fungi). In respiratory ammonification, NO_2^- may undergo a six-electron reduction to NH_4^+, catalyzed by cytochrome c NiR (NrfA). In certain bacteria, ammonia oxidation may generate NO_2- which can be reduced by NirK to NO

NiR (NirK or type 2 NiR) (Liu et al. 1983; Coyne et al. 1990; Hallin and Lindgren 1999; Moreno-Vivian et al. 1999; Cabello et al. 2004). These NiRs are expressed in cells grown anaerobically under denitrifying conditions (in the presence of nitrogen oxides) and activity of NirK is inhibited by O_2 (Liu et al. 1983).

This form of reductive NO synthesis occurs in denitrifiers of hypoxic niches, such as marine sediments, flooded or waterlogged soils and the aerobic/anaerobic interface of eutrophized waters. In non-denitrifier bacteria, NO_2^- may undergo a six-electron reduction to ammonia catalyzed by cytochrome-c NiR (NrfA) (Simon 2002). This process is the so-called respiratory ammonification (Fig. 2.5), which takes place in the cytoplasm and is required for anabolic purposes (Simon 2002). Reduction of NO_2^- in respiratory ammonification fails to produce NO.

Subcellular Localization and Biological Effects of Reductive NO Synthesis Immunogold labeling with colloidal-gold probes has shown that bacterial NiRs are associated with the periplasmic space or with the cell membrane (Fig. 2.4) (Coyne et al. 1990). Accordingly, NO_3^- reductases (the membrane-bound NO_3^- reductase NAR and the periplasmic NO_3^- reductases NAP), moreover, NO-reductase (NOR) and nitrous oxide reductase (N_2OR)[2], are all enriched in the cell membrane of denitrifying bacteria (Balderston et al. 1976; Matsubara and Zumft 1982; Zumft and Frunzke 1982; Korner and Mayer 1992; Hendriks et al. 1998; Pohlmann et al. 2000; Richardson et al. 2001; Gonzalez et al. 2006; Hino et al. 2010; Simpson et al. 2010). The cell membrane with the periplasmic space thus represents the major subcellular pool of NO synthesis.

[2] The literature uses the "Nos" abbreviation to indicate nitrous oxide reductase. However we have reserved the "NOS" acronym for NO-synthase in this book. To avoid the confusion of these two distinct enzymes, we have applied the "N_2OR" abbreviation for nitrous oxide reductase.

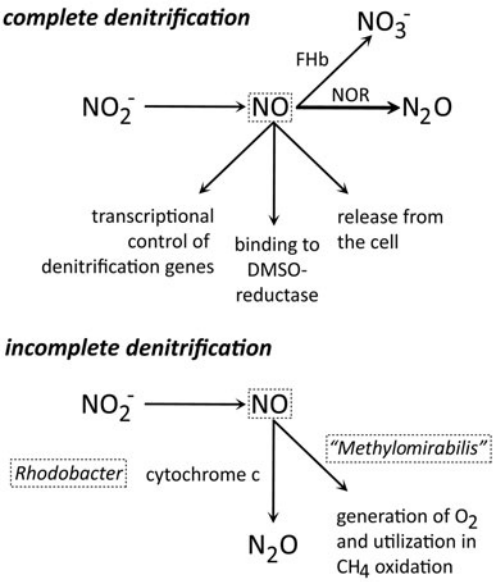

Fig. 2.6 Possible fates of NO generated by denitrification *Top*: In bacteria with a complete denitrification enzyme chain, the majority of NO is converted to N_2O (by NORs). NO may also increase the transcription of genes encoding denitrification enzymes, may bind to membrane proteins (e.g. DMSO-reductase) or may release from the cell. Bacterial flavohemoglobins (*FHb*) scavenge excess NO and convert it to NO_3^-. *Bottom*: In bacteria lacking NORs, NO may be reduced by cytochrome c or may be consumed for O_2 generation

Since NO occurs transiently as a byproduct of NO_3^- dissimilation, one can debate that NO would gain functional impact in denitrifier bacteria. Some studies however, suggest that the locally produced NO affects distinct functions of the cell membrane and the periplasmic space. For instance, in the denitrifier 2.4.3. strain of *Rhodobacter sphaeroides*, one possible target of NO is dimethyl sulfoxide (DMSO) reductase (Fig. 2.6). This enzyme is located in the periplasmic space (McEwan et al. 1991), near the site of reductive NO synthesis. DMSO reductase is a molybdenum (VI) containing enzyme, and functions as a terminal reductase in anaerobic respiration by using DMSO as a terminal electron acceptor. In *Rhodobacter*, the DMSO respiratory pathway has a role in redox homeostasis and it also enables the cells to utilize a variety of carbon sources during anaerobic growth (Kappler et al. 2002). It has been shown that NO carries out a one electron reduction of molybdenum in DMSO reductase and stabilizes molybdenum (V) without inhibiting enzyme activity (Bastian et al. 1995). It is possible that the molybdenum (V)-NO complex is a transition state analog of the enzyme-substrate complex, however the functional impact of NO on DMSO reductase still remains elusive (Bastian et al. 1995).

Another candidate function of reductive NO synthesis has been proposed in a recently discovered anaerobic methane-oxidizing bacterium, "Candidatus *Methylomirabilis oxyfera*" (Ettwig et al. 2010; Wu et al. 2011). This prokaryote reduces NO_2^- to NO by its denitrifying system, however NO is not converted further to N_2O, instead a yet unidentified enzyme metabolizes NO to produce O_2 and N_2 (Ettwig et al. 2010) (Fig. 2.6). The O_2 produced in this unique "bypassed" denitrification system is utilized in methane (CH_4) oxidation (Ettwig et al. 2010; Wu et al. 2011). Enzymes of the methane oxidation pathway are associated with intracellular membranes and the periplasm (Brantner et al. 2002), thus the periplasmic NO pool may be consumed

locally. In this organism, anaerobic NO synthesis thereby provides an endogenous O_2 supply for CH_4 oxidation (Wu et al. 2011). The finding that an anaerobic bacterium is capable of producing O_2 from NO, using NO_2^- as a substrate opens the possibility that O_2 may be generated by other means in addition to the canonical O_2-yielding pathways, i.e. photosynthesis, chlorate respiration and the conversion of reactive oxygen species (Ettwig et al. 2010).

Denitrifying enzymes and a set of proteins required for the assembly of the denitrification system are expressed in response to hypoxic shift and in the presence of nitrogen oxides (NO_3^-, NO_2^-, NO, N_2O) (Sabaty et al. 1999). It has been shown that among these nitrogen oxides, NO is a key signal, which acts at the level of gene expression of the denitrification system (Fig. 2.6). In denitrifying bacteria NO controls the transcription of genes encoding NiRs and NORs (e.g. *nirSJF* and *norZ*, *qnorB*, *cnorB*, respectively). These NO-target genes are associated with various NO-responsive transcriptional regulators in proteobacteria. The NO-activated signal pathway in the genera *Pseudomonas*, *Paracoccus* and *Rhodobacter* involves regulators of the FNR (fumarate and nitrate reductase regulatory protein) family of transcription factors (Zumft 1993). In *Ralstonia eutropha* the sigma-54 dependent transcriptional regulator NorR controls NOR transcription in a NO-dependent manner (Pohlmann et al. 2000; Cramm et al. 2006).

How Denitrifiers Avoid and Benefit from NO Cytotoxicity The amount of NO produced by denitrification may reach the micromolar range, which can evoke nitrosative stress. However, denitrifier bacteria convert NO instantly to N_2O by NORs. This reduction step combines two NO molecules to N_2O and H_2O. The two reducing equivalents necessary for the two NO/N_2O conversions are delivered by Fe^{2+}/Fe^{3+} transitions of the hem and non-hem iron of the NORs. In prokaryotes with incomplete denitrification systems, the NOR-like activity of cytochrome-c is responsible for reducing excess NO to N_2O (Cross et al. 2001) (Fig. 2.6). Increased NO_2^- and NO availability and anaerobic conditions increase the expression of NOR, which is the most effective NO-converting enzyme under anaerobiosis. Under aerobic or microaerophilic conditions, the bacterial flavohemoglobin (FHb) is responsible for NO elimination. FHb is a NO-dioxygenase, which converts NO and O_2 into NO_3^- with concomitant oxidation of Fe^{2+} to Fe^{3+} heme within the FHb molecule (Gardner et al. 1998; Hausladen et al. 1998; Forrester et al. 2011). Bacteria expressing NORs and FHb are thereby able to avoid NO-toxicity (Hausladen et al. 1998) (Fig. 2.6).

Denitrifiers also emit some amount of NO to their surroundings (Fig. 2.6), which may limit the growth of nondenitrifying bacteria in heterogenous bacterial communities (Choi et al. 2006). Bacterial growth is inhibited by NO, although the NO sensitivity shows species variation (Shank et al. 1962). Derivatives of NO (such as HNO_2) and NO itself may also kill bacteria (Shank et al. 1962; Cuzzolin et al. 1997). It is possible, that NO liberation of denitrifying prokaryotes provides an adaptive advantage by limiting the spread of competitor bacteria within the same niche. Various marine *Pseudomonas* species form biofilms and are associated with sponge species. In this bacterial-sponge symbiotic relationship, the *Pseudomonas* cells generate antimicrobial substances and provide an “immunological” defense mechanism

for the poriferan cell colony (Marinho et al. 2009; Santos et al. 2010). Denitrifier bacteria and their NO_2^-/NO converting activity have been found in the alimentary tract of earthworms (Matthies et al. 1999), where it contributes to the defense against microbes.

2.4.2 An Apparent Paradox: Nitrogen Fixation and NO Synthesis by Denitrification may be Present in the Same Bacterium

Interestingly, reduction of NO_2^- to NO may also occur in N_2-fixing bacteria. Fixation of N_2 and denitrification are antagonistic processes; however reductive NO synthesis by means of denitrification can be present in rhizobiont N_2 fixing bacteria. For instance, *Azospirillum brasilense* contains a plasmid-encoded NirK (Petrova et al. 2010) and elaborates NO by the reduction of NO_2^- (Molina-Favero et al. 2008). A set of genes encoding denitrification enzymes has also been found in the bacterial plasmid DNA, although the main function of the denitrification system may solely be the production of NO in this bacterium (Petrova et al. 2010). It is possible that denitrification genes (including the NO producing NirK) spread with horizontal transfer among rhizospheric N_2-fixing bacteria (Petrova et al. 2010). Since root development involves NO as a signal molecule in vascular plants (Chap. 3), the bacterial NO emission increases root branching and helps establish plant-bacteria rhizobial symbiosis in various legumen species (Molina-Favero et al. 2008; Del Giudice et al. 2011). The NO_2^- supply for reductive NO synthesis is maintained by the activity of both plant and bacterial NO_3^--reductase (NR) in the legumen root nodule (Horchani et al. 2011). Interestingly, NR is also capable of reducing NO_2^- to NO (with ~1% effectivity) if the NO_3^- supply is limited and NO_2^- is available in excess (Shapiro 2005). In N_2-fixing bacteria, the NO production has an important adaptive benefit since bacterial NO emission facilitates root development of the host plant and stabilizes rhizobial plant-bacteria symbiosis by forming root nodules (Del Giudice et al. 2011). Possibly, this is the only one example of NO-mediated intercellular communication in the bacterial world.

2.4.3 Anaerobic Ammonia Oxidation ("anammox") Also Generates NO

Ammonia can also be oxidized anaerobically, by using NO_2^- as a terminal electron acceptor (van der Star et al. 2008; Martinez-Espinosa et al. 2011). Only the members of the Planctomycetales order are able to catalyze this unique process (van der Star et al. 2008), in which they combine two nitrogen compounds (NH_4^+ and NO_2^-) to generate N_2. This is the so-called anammox process, which occurs in anoxic aquatic environments (Lam et al. 2009), including wastewater treatment systems (Kampschreur et al. 2009).

The presence of a gene encoding NirS (EMBL accession number CAJ74898) has been shown in the anammox bacterium "Candidatus *Kuenenia stuttgartiensis*" (Strous et al. 2006; Kartal et al. 2007). It means that similar to the denitrifiers and aerobe ammonia oxidizers, anammox bacteria reduce NO_2^- to NO by NiRS (Fig. 2.5). To date however, the role of NO production in anammox bacteria is unexplored. It is known that the anammox bacterium "Candidatus *Brocadia anammoxidans*" tolerates high NO doses which otherwise inhibit nitrogen metabolism in other NO-forming denitrifiers or aerobic ammonia oxidizers (Schmidt et al. 2002; Kartal et al. 2010). This suggests that anammox bacteria have a rather effective NO-detoxifying mechanism. Supporting this possibility, genes encoding NOR (reduces NO to the less toxic N_2O) and bacterial hemoglobin (sequesters NO) have been identified in "Candidatus *Kuenenia stuttgartiensis*" (Strous et al. 2006). A recent study also shows that NO has metabolic impact on these bacteria, since the excess NO is used for ammonia oxidation and N_2 production in a still hypothetical pathway (Kartal et al. 2010).

2.4.4 Aerobe Bacteria are Also Capable of Reducing NO_2^- to NO

Reductive NO Synthesis in Ammonia Oxidizing and Nitrite Oxidizing Bacteria Until recently, the non-denitrifying bacteria were considered unable to catalyze NO_2^-/NO reduction. Unexpectedly, some ammonia oxidizing (nitrosyfier) and NO_2^- oxidizing (nitrifier) bacteria show reductive NO synthesis, using NO_2^- as a substrate (Remde and Conrad 1990).

Representatives of these aerobic ammonia oxidizer bacteria are *Nitrosomonas europaea*, *Nitrosovibrio spp.* and *Nitrosospira spp*. They colonize soil, various solid surfaces (e.g. walls of buildings) and aquatic environments and they are important players in mineralization (Meiklejohn 1950; Meincke et al. 1989; Remde and Conrad 1990). *Nitrosomonas europaea* is also used in bioremediation since it is capable of metabolizing various organic waste materials and—at least partially—this bacterium is responsible for NO emission of wastewater treatment systems (Stüven and Bock 2001). These nitrosifying bacteria produce NO_2^- by oxidation of ammonia, and then reduce NO_2^- to NO by NiRs. A NiR protein has been purified from *Nitrosomonas europaea* (Ritchie and Nicholas 1974) and a NirK coding gene has also been identified within its genome (Beaumont et al. 2005). It is likely that NirK, the "classic" denitrifier enzyme is responsible for NO_2^-/NO conversion (Remde and Conrad 1990). Importantly, increasing the NO_2^- supply enhances the expression of NirK through a unique NO_2^- sensitive transcription factor (Beaumont et al. 2004). The ability of these bacteria to reduce NO_2^- to NO ensures the removal of toxic NO_2^- (product of their nitrification process) and increases their NO_2^- tolerance (Cantera and Stein 2007b). However, mutant *N. europaea* cells lacking NirK are still capable of NO_2^-/NO reduction (Beaumont et al. 2002), suggesting that as yet undefined alternative mechanisms may also be responsible for NO synthesis.

Aerobe nitrifier bacteria oxidize NO_2^- to NO_3^- and thereby contribute to the transformation of soil NO_2^- to NO_3^-, the most important nitrogen source for vascular

plants. The α-proteobacter *Nitrobacter winogradskyi* is a representative of these NO_2^- oxidizer prokaryotes. Recently, a putative NirK-encoding gene (Nwin_2648) has been identified within its genome (Starkenburg et al. 2008). Low O_2 availability (0–4% O_2) and the presence of NO_2^- increase its transcription and the putative NirK can reduce NO_2^- to NO (Starkenburg et al. 2008). Similar NirK-encoding sequences are known from other *Nitrosomonas* and *Nitrosospira* isolates (Cantera and Stein 2007a). However, to date the possible function of the NO synthesis in these bacteria is unknown.

Recently ammonia-oxidizing archaea have been found in terrestrial and marine environments. The genome of two ammonia-oxidizer archaeon (*Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*) also contains NirK homologs, suggesting that reductive NO synthesis can be widespread in aerobic microbes (Bartossek et al. 2010).

2.4.5 *Reductive NO Synthesis Without NiRs: Cyanobacterial NO Production*

Cyanobacterial hemoglobin (cyanoglobin) also reduces NO_2^- to NO under anoxia (Thorsteinsson et al. 1999; Sturms et al. 2011), thereby cyanobacteria are capable of producing NO in the lack of NiRs (Busch et al. 2002). Both NO_2^- or HNO_2, which are in equilibrium under acidic conditions react with Fe^{2+} in the hem group of deoxyhemoglobin, which leads to the release of NO, as shown by eq. 2.1 (Sturms et al. 2011).

$$\mathrm{Hb[Fe^{2+}] + NO_2^- + H^+ \rightarrow Hb[Fe^{3+}] + NO + OH^-} \tag{2.1}$$

Since cyanoglobins are peripheral membrane proteins and they are components of the membrane-associated terminal cytochrome oxidase (Hill et al. 1996), the reductive NO generation is possibly restricted to the cell membrane.

Since NO_2^- is toxic for most microorganisms (Martinez-Espinosa et al. 2011), the primary function of cyanobacterial NO_2^- reduction is the efficient elimination of NO_2^- when this nitrogen oxide is abundant in the environment and the oxygen supply is limited (e.g. in eutrophized waters) (Sturms et al. 2011). The byproduct NO may exert some secondary biological functions. For instance, a heme NO/O_2 binding protein has been identified in a *Nostoc* species, which has ~35% sequence identity and high structural homology to the beta subunit of soluble guanylyl cyclase and may function as a NO sensor in the cyanobacterium (Tsai et al. 2010). Moreover NO alleviates oxidative damage induced by UV-B irradiation in the cyanobacterium *Spirulina platensis* 794 strain (Xue et al. 2007). The administration of NO increases superoxide dismutase, catalase and glutathione levels and activities, consequently reducing superoxide concentration in the cell (Xue et al. 2007). However, it is still unknown if NO release from deoxy-cyanoglobin would be sufficient to evoke this antioxidant effect. The elimination of excess NO is catalyzed by NORs (Busch et al. 2002).

In the cyanobacteria *Synechocystis sp*. and *Ralstonia eutropha* a putative NOR-encoding gene (*norB*) has been identified and strains deficient in *norB* are more sensitive to NO cytotoxicity (Busch et al. 2002).

Interestingly, hemoproteins (hemoglobin, neuroglobin and myoglobin) in animals also generate NO by NO_2^- reduction (Smagghe et al. 2008; Shiva et al. 2011; Tiso et al. 2011). Although these proteins were primarily considered as NO scavengers (Sharma et al. 1983), recently their NO_2^- reductase activity has been shown. Although it is long known that hemoglobins are involved in NO_2^- metabolism of muscle (Walters et al. 1967), the physiological relevance of their NO_2^- reductase activity is still undefined. A recent study shows that NO_2^-/NO reduction by myoglobin mediates NO_2^- induced vasodilation (Ormerod et al. 2011).

2.5 Oxidative NO Synthesis from L-arginine in Prokaryotes

2.5.1 Early Evidences on the Existence of Bacterial NOS Molecules

The first bacterial NOS-like activity was shown in *Nocardia sp.* strain NRRL 5646 isolated from a garden soil sample (Chen and Rosazza 1994). This species is closely related to *N. tenerifensis* and *N. brasiliensis*, and recently the name *N. iowensis* has been proposed for its taxonomic identification (Lamm et al. 2009).

This bacterium produces NO by conversion of L-arginine to L-citrulline and this NOS-like activity can be inhibited by the mammalian NOS-inhibitors (L-NMMA and L-NNA) (Chen and Rosazza 1994). The responsible 52 kDa protein is a NADPH, O_2, Ca^{2+}, FAD, FMN, and BH_4-dependent enzyme (Chen and Rosazza 1994), designated as *Nocardia*NOS (NOS_{Noc}) (Chen and Rosazza 1995). Interestingly, *Nocardia* produces NO not only from L-arginine and Nω-hydroxy-L-arginine but also L-arginine containing peptides (Son and Rosazza 2000). Consumption of small L-arginine containing peptides by NOS also occurs in a limited extent in eukaryote cells (Hecker et al. 1991; Rőszer et al. 2006). Cells of *Nocardia* also utilize thiols in the detoxification of NO, resembling the glutathione/*S*-nitrosoglutathione system of the eukaryote cells (Lee et al. 2007). This bacterium also shows guanylyl cyclase activity and synthesizes BH_4, the cofactor of NOS_{Noc} (Son and Rosazza 2000; He and Rosazza 2003). Administration of BH_4 increases, while impaired BH_4 synthesis reduces NOS_{Noc} activity, showing the direct relationship between BH_4 availability and NO synthesis (Chen and Rosazza 1994; He and Rosazza 2003). Inhibition of NOS_{Noc} or guanylyl cyclase reduces the cGMP levels in the culture media, suggesting the existence of a NO/cGMP pathway in this bacterium (Son and Rosazza 2000).

Similar reports show NOS-like activity in other bacteria, such as *Lactobacillus fermentum* (Morita et al. 1997), *Rhodococcus sp.* (Sari et al. 1998) and *Salmonella typhimurium* (Choi et al. 2000). These species show L-arginine dependent NO synthesis, which is sensitive to mammalian NOS inhibitors (Choi et al. 2000; Cohen and Yamasaki 2003). A protein of *Rhodococcus* was recognized by an antibody raised

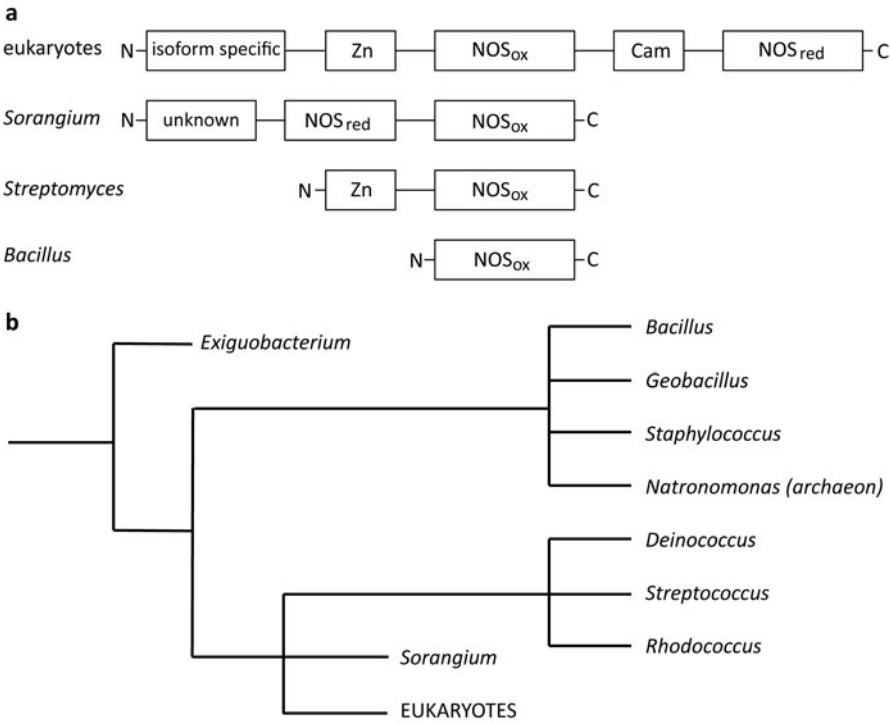

Fig. 2.7 Schematic representation of NOS domain structure and the possible phylogenic tree of prokaryote NOS-encoding genes. Recent studies have identified open reading frames encoding homologs of the mammalian oxygenase NOS domain (NOS_{ox}) in the genome of several gram-positive bacteria. These bacterial NOS molecules show similarities to the eukaryote-type NOS_{ox}. Bacterial NOS molecules lack the covalently attached reductase domain (NOS_{red}), with the exception of the *Sorangium* NOS (**a**). *Zn* Zn-containing domain, *Cam* CaM-binding domain. A simplified phylogenetic tree of various prokaryote NOSs (Sudhamsu and Crane 2009) (**b**)

against human iNOS (Cohen and Yamasaki 2003). However, the known *Nocardia* genomes do not contain any NOS-homolog genes and in the other species the genes encoding the responsible NOS molecules are similarly undefined (Crane et al. 2010).

2.5.2 Characterization of Bacterial NOS Molecules

Recent studies have identified open reading frames encoding homologs of the mammalian oxygenase NOS domain (NOS_{ox}) in the genome of several gram-positive bacteria (representatives of the Bacillales, Actinobacteria and Deinococcus orders) and an archaeon (Fig. 2.7). These bacterial NOS_{ox}-like proteins have been characterized in *Bacillus subtilis* [bsNOS], *Bacillus anthracis* [baNOS], *Bacillus cereus* [bcNOS], *Staphylococcus aureus* [saNOS], *Streptomyces turgidiscabies*

[stNOS], *Geobacillus* (*Bacillus*) *stearothermophilus* [gsNOS], *Sorangium cellulosum* [scNOS] and *Deinococcus radiodurans* [drNOS] (Sudhamsu and Crane 2009; Crane et al. 2010; Montgomery et al. 2010). These bacterial NOS molecules catalyze the oxidation of L-arginine or Nω-hydroxy-L-arginine and produce NO. Structures of these proteins show high similarities to the mammalian NOS_{ox}, and their catalytic activity resembles the mammalian-type NO synthesis (some differences exist, e.g. bacterial NOS utilizes either BH_4 or tetrahydrofolate). However, bacterial NOS molecules lack the covalently attached reductase domain (Fig. 2.7) and they receive electrons from various reductase partners (e.g. *B. subtilis* flavodoxin) (Crane et al. 2010). There is only one bacterial NOS, *Sorangium cellulosum* scNOS, which contains a reductase domain (Agapie et al. 2009; Crane et al. 2010).

2.5.3 Functions of Bacterial NOS

The available studies show that NOS is not associated with the cell membrane, thus it is supposed to be a cytosolic protein in the bacterial cell (Fig. 2.8). This cytosolic NO production is essential for oxidative stress protection: NO inhibits the generation of free radicals (OH^-, $OH^\bullet$) within the bacterial cell (Gusarov and Nudler 2005). Exposure of bacteria to hydrogen peroxide (H_2O_2) leads to the production of OH^-, $OH^\bullet$ which cause DNA damage (Sudhamsu and Crane 2009). The generation of OH^-, $OH^\bullet$ is attributable to the Fenton-reaction of H_2O_2 with free cellular Fe^{2+}. This process is shown by eq. 2.2 (Prousek 2007).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \tag{2.2}$$

The free reduced Fe^{2+}, which is required for the Fenton reaction, is depleted instantly with H_2O_2 exposure. However, the presence of cellular reductants (such as cysteine) reduces Fe^{3+} to Fe^{2+} and this recycling of Fe^{3+}/Fe^{2+} sustains the Fenton reaction and leads to cell death (Prousek 2007). Synthesis of NO suppresses the Fenton reaction by transiently inhibiting cysteine reduction (Gusarov and Nudler 2005). Moreover, NO increases the expression of catalase (CAT) in *Bacillus subtilis*, which detoxifies H_2O_2 and mitigates oxidative stress (Gusarov and Nudler 2005).

Recently, it has been shown that NOS-derived NO ensures resistance of *Bacillus subtilis* to certain antibiotics (quinolone, acridine, amynoglicoside, cephalosporin, phenothiazine and lactam-type antimicrobials) (Gusarov et al. 2009). The underlying mechanism may be a chemical reaction of NO with the antibiotics (Gusarov et al. 2009). In the plant pathogen *Streptomyces* strains, NOS activity is required for the production of thaxtomin-A, a bacterial toxin, which interferes with the plant cell wall synthesis. Bacterial NOS is essential for the nitration of the tryptophanyl motiety of thaxtomin-A. This nitro group of thaxtomin-A is derived from the terminal guanidinium nitrogen of L-arginine. To date NOS is the only known enzyme, which catalyzes the oxidation of terminal guanidinium nitrogen of L-arginine (Crane et al. 2010) (Fig. 2.8). The NOS-derived NO also affects gene expression: transcriptional changes during the recovery from radiation damage and cell proliferation in *Deinococcus* are associated with NO synthesis (Crane et al. 2010).

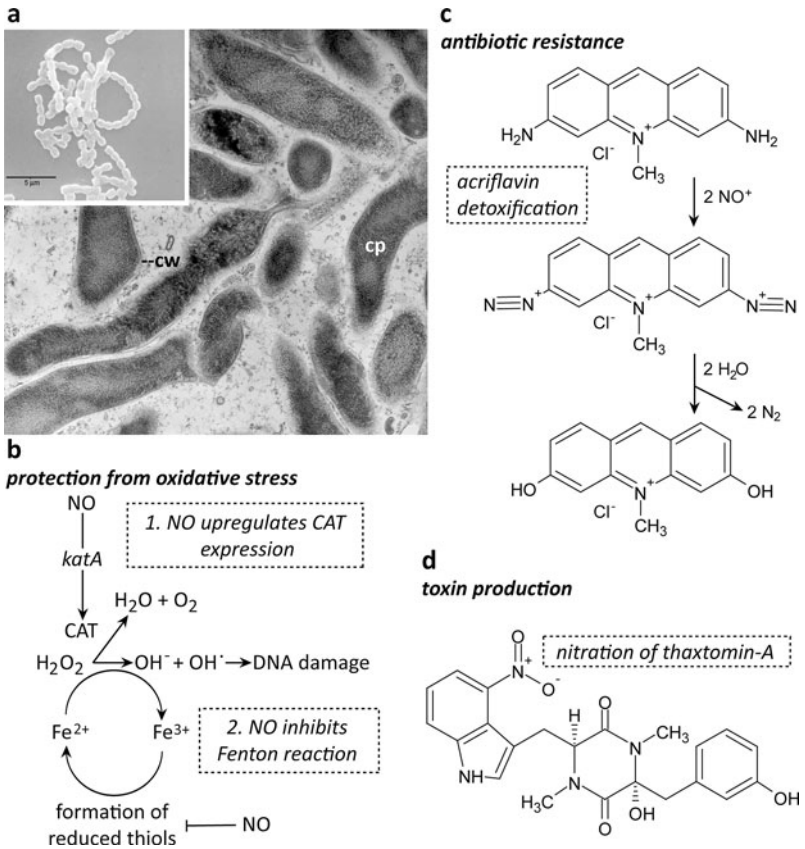

Fig. 2.8 Biological functions of NOS activity in bacteria. *Streptomyces* (**a**) and other bacteria contain NOS in their cytoplasm (SEM and TEM images with the courtesy of Dr. Iván Schmelczer; *cw* cell wall, *cp* cytoplasm). The cytosolic NO production is essential for oxidative stress protection (**b**), since NO inhibits the generation of OH^- and $OH^{\bullet}$ by interruption of the Fenton reaction. NO also increases the expression of CAT in *Bacillus subtilis*, which detoxifies H_2O_2 and mitigates oxidative stress. NOS-derived NO ensures resistance of *Bacillus subtilis* to certain antibiotics, possibly by chemical modification of the antibiotics, e.g. acriflavin (**c**). In the plant pathogen *Streptomyces* strains, NOS activity is required for the nitration of the tryptophanyl moiety in thaxtomin-A (**d**), a bacterial toxin, which interferes with the plant cell wall synthesis

2.6 Subcellular NO Synthesis: Fruit or Root in the Tree of Phylogeny?

NO synthesis is widespread among the prokaryotes so we may assume that NO generating organisms were present in the archaeal biosphere. The generation of NO requires the reduction of NO_2^- or oxidation of guanidino nitrogens of L-arginine; thereby the evolutionary driving force for the development of NO-synthesizing

pathways is the large-scale production of NO_2^- and L-arginine in the biosphere. Logically, NO production could become possible when these substances were available in the ancient Earth.

Today, it is commonly accepted that cellular life originated from a last universal common ancestor (LUCA) which occurred in the Hadean ocean of the ancient Earth, around 4.5–5 Ga (gigaannum, 10^9 years) ago (Nisbet and Sleep 2001). This hypothetical LUCA gave rise to the diversification of prokaryotic life and the evolution of all cellular organisms (Prokaryota [Archaea and Bacteria] and Eukaryota). LUCA and its immediate descendants could gain NO-synthesizing ability only if substrates were available. There is a great debate, however, regarding the chemical composition and physical characteristics of the environment in which the early diversification and proliferation of cellular life took place.

In the case of NO_2^-, the substrate of reductive NO synthesis, there are two major hypotheses (Vlaeminck et al. 2011). The so-called "ON" scenario on the timing of nitrogen metabolism hypothesizes that the primordial atmosphere contained CH_4 and less than 50% CO_2 and oxygenic photosynthesis evolved before the biological generation of nitrogen oxides. In this model, LUCA and the first prokaryotes were unable to generate NO since NO_2^- could be present only in negligible amounts. The estimated development of NO_2^- reduction could be ~3.8–2.5 Ga, when oxygenic photosynthesis was already evolved (Vlaeminck et al. 2011).

The contrary "NO" scenario implies that the ancient atmosphere had a significantly higher CO_2 concentration and various nitrogen oxides were generated abiotically (e.g. by electric discharges, volcanic activity and UV radiation, Fig. 2.9). This hypothesis postulates that NO was abundant in the primordial atmosphere and the Hadean ocean was rich in NO_2^-. Nitrogen oxides therefore were available substrates for the hypothetical LUCA and the first prokaryote organisms ~4–3.8 Ga ago (Vlaeminck et al. 2011) (Fig. 2.9). Indeed, it is also hypothesized that NO was the first strong oxidant in the archaean world (Ducluzeau et al. 2009). Recent phylogenic analysis of respiratory O_2 reductases and NORs suggests that NORs could be present in LUCA. It is possible that the most primordial prokaryotic cells could reduce NO_2^- to NO and the mechanisms similar to the recent denitrification and anammox processes developed before the advent of O_2 in the atmosphere (Ducluzeau et al. 2009). The example of *Methylomirabilis oxyfera* also suggests that NO_2^- reduction could also contribute to O_2 generation in the ancient biosphere before the occurrence of the first photosynthetic organisms. The "NO" model also postulates that decreasing atmospheric CO_2 levels led to the decline of abiotic generation of nitrogen oxides. This event evoked a "nitrogen crisis" of the Archaean life ~3.8 Ga ago. Limitation of NO, NO_2^- and NO_3^- availability might lead to the development of N_2 fixation and could also provide evolutionary force to NORs to use O_2 as an alternative substrate instead of NO (Ducluzeau et al. 2009).

Oxidative synthesis of NO from L-arginine is less widespread in the prokaryote world than the reductive NO production from NO_2^-. Abiotic generation of basic amino acids, such as arginine is a debated issue, and possibly, arginine was absent from the prebiotic Earth (McDonald and Storrie-Lombardi 2010). Although it is hypothesized that amino acids could be delivered by extraterrestrial materials (Kvenvolden et al. 1970; Barbier et al. 1998), the notable lack of arginine has been

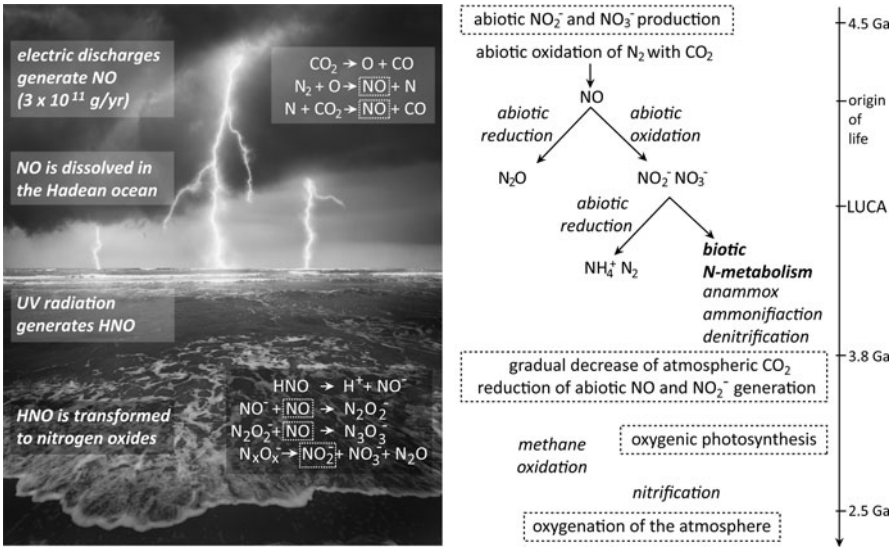

Fig. 2.9 Abiotic generation of NO and the possible development of reductive NO synthesis. The "NO" scenario implies that various nitrogen oxides—including NO—were generated abiotically (e.g. by *electric discharges*, volcanic activity and *UV radiation*) in the ancient atmosphere (Ducluzeau et al. 2009). This hypothesis postulates that NO was abundant in the primordial atmosphere and the Hadean ocean was rich in NO_2^-. It is possible that primordial prokaryotic cells could reduce NO_2^- to NO by mechanisms similar to the recent denitrification and anammox processes

found in meteorites that contain organic compounds (so-called carbonaceous chondrites) (Miller 1986; Engel and Macko 2001). Since amino acids of carbonaceous chondrites are synthesized abiotically, it is plausible that similar prebiotic synthesis took place on the primitive Earth (Miller 1986). However, the lack of arginine from the yet analyzed meteorites makes arginine's presence in the most ancient biosphere unreliable. Accordingly, several bacterial proteins, e.g. bacterial flagellar proetins do not contain basic amino acids (arginine and lysine), supporting the idea that cellular life occurred in an arginine-free environment (McDonald and Storrie-Lombardi 2010). However, most bacteria have the ability to metabolize L-arginine (Hird 1986) and *de novo* arginine biosynthesis is present in a limited number of bacterial phyla (Xu et al. 2007). L-arginine provides a positively charged guanidino group, which affects secondary protein structures and allows establishment of DNA-protein interactions, thereby it could be involved in the development of eukaryotic chromatin structures (Hird 1986). Arginine metabolism, and particularly oxidative NO synthesis from L-arginine, requires a prevailing complexity of cellular life, suggesting that NOS-catalyzed L-arginine oxidation could evolve after the reductive NO synthesis.

As a milestone in the development of the eukaryotic world, the cell architecture became more complex and distinct biochemical microniches have evolved into the cell organelles (e.g. mitochondria, cell nucleus, endoplasmic membrane systems) and specific subcellular compartments (e.g. membrane microdomains). Some of the cell organelles are derived from endosymbiotic associations of ancient prokaryote

cells. Hence, several aspects of organelle-specific NO synthesis and function resemble bacterial-type NO homeostasis. For instance, the intermembrane space of mitochondria and the thylakoid lumen of chloroplasts are evolutionary descendents of the periplasmic space of bacteria (Herrmann et al. 2009). Accordingly, these subcellular compartments display reductive NO synthesis and bacterial-type NO scavenging (Castello et al. 2006, 2008; Poyton et al. 2009; Nakanishi et al. 2010). NOS-like enzymes in the chloroplast stroma and the mitochondrial matrix may be descendents of the prokaryotic NOS molecules. Organelles with endosymbiotic origins thereby have preserved the key features of ancient prokaryotic NO biology. However, further development of subcellular NO biology required the diversification of NOS molecules and the development of various mechanisms (e.g. dynamic post-translational modifications) which ensure the correct intracellular orientation of NOS in the complex eukaryotic cell.

2.7 Chapter Summary

Mechanisms of NO generation in prokaryotes	• NO_2^-/NO reduction is catalyzed by dissimilatory NO_2^--reductases (NirK, NirS) • Deoxygenated bacterial hemoglobin is also capable of reducing NO_2^- to NO • Several bacteria contain NOSs, and synthesize NO from L-arginine • Bacterial N-metabolism also contributes to chemical NO release from nitrogen oxides
Functions of NO in the prokaryote cell	• Within the bacterial cell, NO has antioxidant effects by the inhibition of Fenton chemistry. It increases the transcription of genes involved in stress adaptation, denitrification and NO-elimination • NO synthesis is involved in bacterial toxin production and antibiotic resistance • NO may release from the bacterial cell and inhibit the growth of competitor bacteria, or facilitate the development of bacterial-plant symbiosis
Origin of spatial organization of subcellular NO synthesis	• Reductive NO synthesis is associated with the bacterial cell membrane, while NOS activity is confined to the cytosol. Similar arrangement of NO synthesis is notable in homolog structures of the eukaryote cell

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Part III
Nitric Oxide in Plant Organelles

Chapter 3
Nitric Oxide Synthesis in the Chloroplast

3.1 *Vivat, crescat et floreat!**—Overview of NO Effects in Plant Physiology

Nitric oxide production in plant cells was first described under specific stress conditions and NO emission was long considered as a non-physiological response to stressors (Harper 1981; Dean and Harper 1986; Shapiro 2005). Constitutive NO synthesis, however, has been detected in a variety of plant species ranging from unicellular algae to vascular plants (Cormophyta) and several roles are currently attributed to NO in plant physiology (Mallick et al. 1999; Sakihama et al. 2002; Shapiro 2005; Chen et al. 2010; Yordanova et al. 2010). Cellular events affected by NO occur at all major stages of plant life, such as germination (Beligni and Lamattina 2000; Bethke et al. 2004b; Zhao et al. 2009; Gniazdowska et al. 2010), pollen tube orientation (Prado et al. 2004), growth (Guo et al. 2003; Otvos et al. 2005; Shapiro 2005), symbiotic plant-bacteria interactions (Shimoda et al. 2005), flowering and senescence (He et al. 2004; Crawford and Guo 2005; Guo and Crawford 2005). Wound healing (Shapiro 2005), stress response (Sang et al. 2008; Tossi et al. 2009), defense against pathogens (Zeidler et al. 2004; Cortez et al. 2010) and heavy metal tolerance (Zeidler et al. 2004; Ma et al. 2010; Xu et al. 2010) are also linked to NO biosynthesis.

Major functions mediated or affected by NO in early plant development are involvement in seed dormancy, germination and growth of the seedlings (Fig. 3.1). Studies with NO donor compounds and NO scavengers show that NO interrupts seed dormancy in the cruciferan plant *Arabidopsis thaliana*, the barley *Hordeum sp.* (Bethke et al. 2004b) and in the apple *Malus domestica* (Gniazdowska et al. 2010) and alleviates glucose-inhibited germination in the legume *Lotus japonicus* (Zhao et al. 2009). Similarly, in the lettuce *Lactuca sativa*, which requires light for germination, NO treatment allows seeds to germinate in darkness (Beligni and Lamattina 2000). In etiolated seedlings (grown in the lack of light), NO partially restores etiolation and helps normal growth, as NO administration leads to

*Latin; meaning: Live, grow and flourish!

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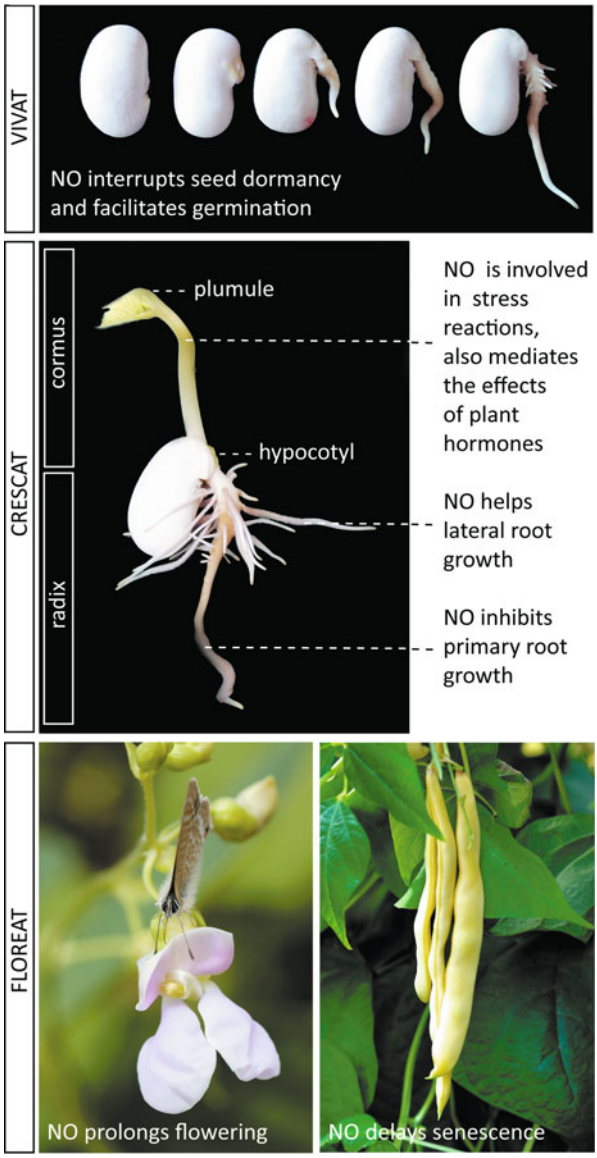

Fig. 3.1 Summary of NO functions in plant physiology. In the last decade the exploration of how NO functions in plants has been emphasized. The administration of NO evokes various physiological changes in vascular plants such as the break of seed dormancy and the promotion of germination. In growing and adult plants, NO affects respiration by modulating stoma closure and helps survival through its involvement in various forms of abiotic stress responses. Normal root development, an important determinant of plant growth is also affected by NO. And finally, NO also affects flowering and maturation: acts against senescence and delays flowering time

greening and reduced elongation of the hypocotyl (part of the primary axis of the developing embryo, Fig. 3.1) (Beligni and Lamattina 2000). Involvement of NO has also been implicated in lateral root development and suppression of primary root growth (Shapiro 2005; Kolbert et al. 2008; Jin et al. 2011) (Fig. 3.1). In legume root nodules, NO also affects the development of legume-rhizobium symbiosis (Shimoda et al. 2005) and the atmospheric nitrogen (N_2) fixation (Kato et al. 2010).

With plant maturation and senescence NO production is reduced (Shapiro 2005). Accordingly, administration of NO delays flowering time, maturation and senescence (Crawford and Guo 2005; Shapiro 2005) (Fig. 3.1). However, the underlying molecular mechanisms by which NO contributes to plant development are still debated. It is suggested, that NO may mediate the effects of other plant hormones (Otvos et al. 2005; Shapiro 2005) such as cytokinins (CKs) (Beligni and Lamattina 2000), abscisic acid (ABA) (Tun et al. 2001) and auxin (Kolbert et al. 2008; Romera et al. 2011). Cytokinins help the proliferation of hypocotyl cells and promote growth of the seedling (Beligni and Lamattina 2000). They also restore the normal growth in etiolated plants (Beligni and Lamattina 2000) and induce NOS activity in plant cell cultures (Tun et al. 2001). This finding supports the idea that NO may be a downstream signal to CKs. Similarly, it is suggested that NO mediates the ABA induced changes in water and ion transport of guard cells in the leaf epidermis (Bright et al. 2006; Zhang et al. 2010). In response to decreased soil water potential, when root epithelia are unable to absorb sufficient amounts of water, root cells produce ABA which is transported to the leaves. In the leaf epidermis, ABA induces changes in the osmotic potential of the the so-called guard cells, which consequently shrink and close the stomata (Hopkins and Hüne 2009). As a net effect of water deprivation induced ABA production, stoma closure reduces evaporation and mitigates dehydration of the plant. In guard cells, ABA effects are at least partially mediated by NO and administration of NO also induces stoma closure (Shapiro 2005; Bright et al. 2006). It is likely that in plants—similar to animal cells—NO activates cGMP synthesis (Shapiro 2005; Isner and Maathuis 2011) and also leads to S-nitrosylation (Gupta 2011) or tyrosine nitration of proteins (Moreau et al. 2010). ABA effects—at least partially—are mediated by the cGMP pathway, suggesting the involvement of NO (Shapiro 2005). ABA induces Ca^{2+} release from the endoplasmic reticulum and in turn opens Ca^{2+} sensitive ion channels of the cell membrane (Shapiro 2005; Bright et al. 2006; Sun et al. 2010). Efflux of ions and water leads to cell shrinkage and consequent stoma closure (Shapiro 2005). In various plant species NO synthesis is required for mediating the effects of auxin on cell division and root branching (Otvos et al. 2005; Kolbert et al. 2008; Jin et al. 2011; Romera et al. 2011).

Studies with NO indicator dyes show that chloroplasts, mitochondria (Fig. 3.2), leaf peroxisomes and cytoplasm are the main sites of NO production (Barroso et al. 1999; Pedroso et al. 2000; Jasid et al. 2006; Prado et al. 2006; Sang et al. 2008; Gupta and Kaiser 2010). In chloroplasts, mitochondria and leaf peroxisomes the biosynthesis of NO from L-arginine has been shown (Jasid et al. 2006). In plants, however, nitrite (NO_2^-) may also be an important source of reductive NO synthesis (Yamasaki 2000; Yamasaki and Sakihama 2000; Rockel et al. 2002). In the cytoplasm and in the chloroplasts NO may be produced from NO_2^- by nitrate reductase (NR) (Berkels et al. 2004; Kolbert et al. 2008) when NO_2^- is accumulated in the cell (Gupta et al. 2010). It happens under various conditions, including hypoxia, inhibition of the photosynthetic electron transport or excess NO_2^- uptake from the soil (Shapiro 2005). The NR-catalyzed NO synthesis has been found to mediate the effect of auxin on root branching (Kolbert et al. 2008). However, more recently the involvement of a NOS-like activity has been documented in auxin signaling in root cells (Jin et al. 2011).

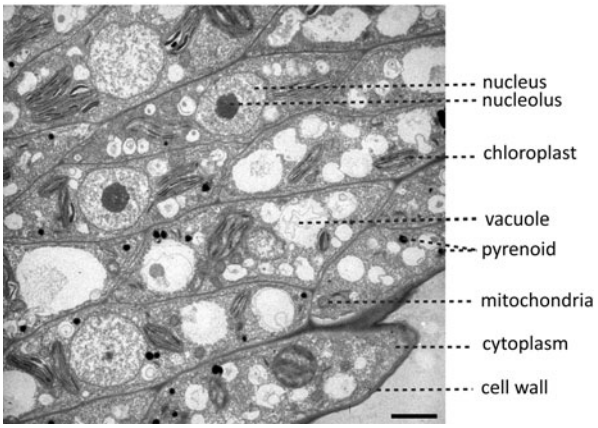

Fig. 3.2 Organelles of a typical eukaryote plant cell. A photosynthesizing parenchyme cell of *Funaria hygrometrica* is a good representative of typical plant cell architecture. Plant specific organelles are the *chloroplasts* (and other plastids), the large *vacuoles* and the rubisco enzyme containing *pyrenoids*. Cells are bordered with a *cell wall*, which is missing from animal cells. Author's TEM image, scale bar 10 μm

In plant mitochondria, NO is generated from NO_2^- by the respiratory electron transport chain (Stoimenova et al. 2007). Cytoplasmic, mitochondrial, peroxisomal or endoplasmic reticulum-associated heme-containing proteins are also able to reduce NO_2^- to NO under hypoxia (Igamberdiev et al. 2010). In the root plasma membrane of tobacco, *Nicotiana tabacum,* a putative NO_2^-/NO-reductase (NI-NOR) is also a NO producer (Stohr et al. 2001). Some data show that in plants NO may be formed in non-enzymatic processes, mainly under acidotic conditions (Bethke et al. 2004a), although the relevance of non-enzymatic NO release is as yet undefined and whether it occurs physiologically is still under discussion (Shapiro 2005; Marechal et al. 2010). With low efficacy, polyamines and hydroxylamine can also be oxidized to NO, however, its physiological impact is debated (Moreau et al. 2010).

This chapter is dedicated to the understanding of chloroplast NO synthesis and its functional impact. The next chapter will show NO synthesis in other plant organelles: leaf peroxisomes and plant-type mitochondria.

3.2 Chloroplast: A Prokaryote Heritage of Plants

Plants produce carbohydrates from inorganic elements and then use them for *de novo* synthesis of amino acids, lipids and other organic compounds (Ruhlman and Daniell 2007; Hopkins and Hüne 2009). The organelles, where the light-catalyzed autotrophic anabolism photosynthesis takes place, are the chloroplasts. These organelles are exclusively characteristic for plants (Figs. 3.2 and 3.3) and are abundant in all photosynthesizing cells. They occur in various forms from unicellular algae to leaf parenchymal cells and guard cells of vascular plants (Hopkins and Hüne 2009) (Fig. 3.3).

According to the current paradigm known as the endosymbiont theory, we consider chloroplasts as descendents of ancient chemosynthesizing prokaryotes. In this

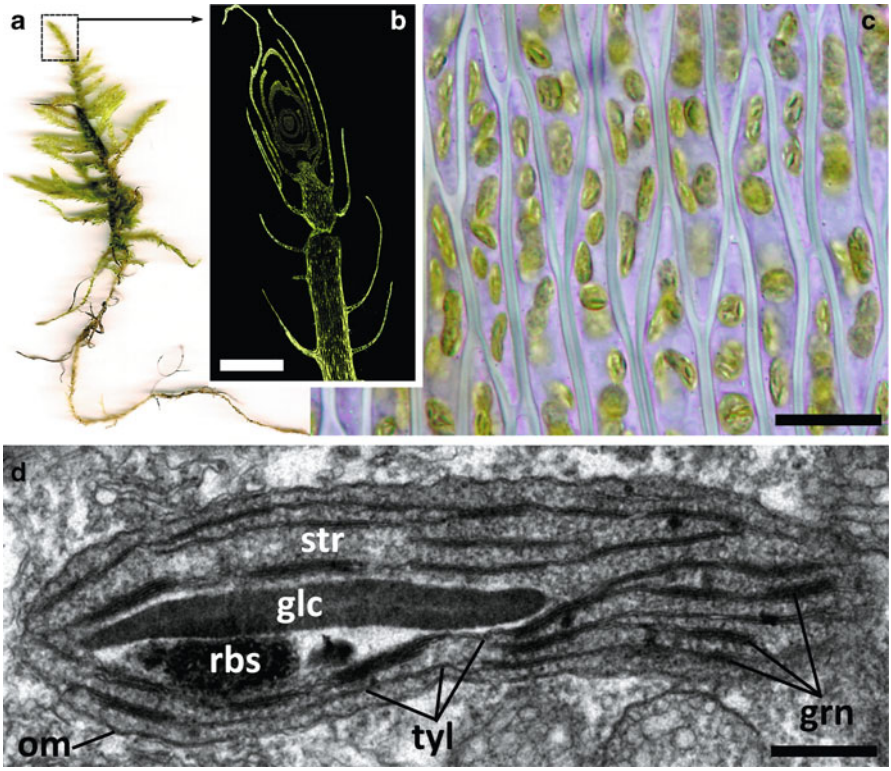

Fig. 3.3 Architecture of a chloroplast. Chloroplasts are abundant in photosynthesizing plant cells (**a**), such as parenchyme of the moss *Funaria hygrometrica*. Longitudinal section shows *green* fluorescence of the chloroplast containing cells (**b**). Scale bar 200 µm. The *rounded green* organelles are chloroplasts, scattered in the cytoplasm of fusiform parenchyme cells (**c**). Scale bar 50 µm. Ultrastructure of a chloroplast. TEM image, scale bar 10 nm. *om* outer membrane, *glc* glycogen, *str* stroma, *tyl* thylakoid membranes, *grn* grana, *rbs* rubisco complex or pyrenoid; Author's images

scenario, an ancient prokaryote has engulfed a photosynthesizing cell (an ancient cyanobacterium) and this phagocytosed endosymbiont functioned as an energy producing organelle of the host cell. This symbiotic relationship has been conserved in the evolution and led to the development of chloroplasts. One of the main arguments supporting this possibility is the striking similarity between chloroplast and cyanobacterium ultrastructure (Fig. 3.3). Chloroplasts, as highly specialized plastids display a complex suborganellar compartmentalization: the organelle is bound by a two layered envelope consisting of outer and inner membranes which enclose the chloroplast stroma (Hodge et al. 1955, 1956) (Fig. 3.3d). The outer membrane is considered the homologue of the phagosome membrane of the ancient host cell, while the inner membrane is the equivalent of the cell membrane of the engulfed prokaryote. The chloroplast stroma is homologue to the cytoplasm of the endosymbiont. As in the prokaryote cytoplasm, it contains circular DNA (residue of the prokaryote

genome), ribosomes, intermediates and cofactors of photosynthesis, lipid droplets, starch granules and pyrenoids (concentrations of the rubisco enzyme) (Fig. 3.3d). The light-independent or dark period of photosynthesis takes place in the stroma. The enzymes of the Calvin cycle which produce carbohydrate from carbon dioxide are located outside the thylakoids and dissolved in the stroma. In some algae we also find chloroplastic endoplasmic reticulum, which resemble the structure of the endoplasmic reticulum of eukaryote cells (Hodge et al. 1955, 1956; Govindjee and Yang 1966).

As a specialized form of the chloroplastic endoplasmic reticulum, the stroma also contains a multilayered membrane structure, the thylakoid membrane system (Fig. 3.3d). Thylakoid membranes contain photosystems I and II, the electron transport chain proteins, cytochromes, proton translocating proteins, ATPase proteins, and the light harvesting antenna complex. Thylakoid membranes may form column like structures, the so-called grana, in which these photosynthetic proteins are concentrated (Hopkins and Hüne 2009).

3.3 Chloroplast NO Production and Photosynthesis

3.3.1 Biochemistry of NO Production in the Chloroplast

Chloroplast biogenesis requires NO, since NO affects iron homeostasis and consequently chloroplast development (Graziano et al. 2002; Shapiro 2005). A NOS-like activity, which converts L-arginine to L-citrulline and liberates NO is confined to the chloroplast stroma, as suggested by an immunohistochemical study of NOS distribution in plant cells (Barroso et al. 1999) (Fig. 3.4). However, there is an additional NO production from NO_2^-, which is most likely catalyzed by NR (Rockel et al. 2002; Jasid et al. 2006), especially under stress conditions when the NO_2^- supply is increased (Shapiro 2005) (Fig. 3.4).

Production of NO from NO_2^- by NR may be the more ancient form of NO synthesis, since this mechanism has been found in the cyanobacterium *Anabaena doliolum* (Mallick et al. 1999). A similar function of NR has also been shown in the *Chlamydomonas reinhardtii* (Sakihama et al. 2002), *Scenedesmus* and *Synechococcus* species of green algae (Mallick et al. 1999). In the *Chlamydomonas reinhardtii* cc-2929 mutant which lacks NR, NO synthesis is not affected by NO_2^-, providing evidence that NO emission is linked to the activity of NR (Yamasaki and Sakihama 2000; Sakihama et al. 2002).

Interestingly, illumination and consequent photosynthesis inhibits NO_2^- dependent NO production in green algae (Sakihama et al. 2002). Since light dependent reactions of photosynthesis favor NO_2^-/NH_4^+ conversion and the utilization of NH_4^+ for amino acid synthesis (Krueger and Kliewer 1995), light exposure reduces the chloroplast NO_2^- source and consequently reduces the rate of NO_2^-/NO conversion (Sakihama et al. 2002). Consistently, the inhibition of photosynthetic

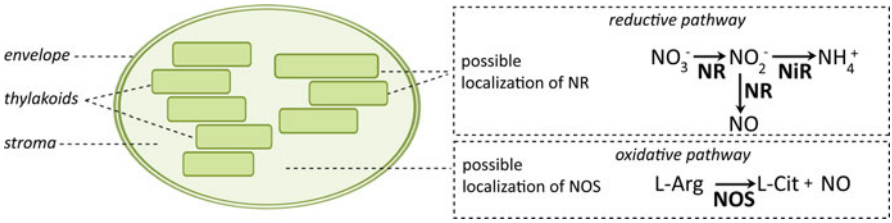

Fig. 3.4 Schematic representation of reductive and oxidative NO synthesis in the chloroplast. Reduction of NO_2^- to NO is catalyzed by nitrate reductase (NR) which is possibly localized in the thylakoid system. An additional physiological role of NR is the NO_3^-/NO_2^- reduction. NO_2^- is further converted to NH_4^+ by nitrite reductase (NiR). Oxidation of L-arginine (L-Arg) to L-citrulline (L-Cit) is associated with the stroma and catalyzed by a NO-synthase (NOS)-like enzyme

electron transport leads to the accumulation of NO_2^- since the chloroplastic assimilatory NO_2^--reductase (NiR) does not receive a sufficient ferredoxin supply from the photosynthetic electron transport chain, and is thus unable to catalyze NO_2^-/NH_4^+ conversion (Shapiro 2005). Note that chloroplastic NiR is distinct from prokaryotic dissimilatory NiRs (e.g. NirK) and fails to reduce NO_2^- to NO (Chap. 2). Inhibition of photosynthesis (e.g. by light stress or herbicides) or uncoupling factors which block the transport and detoxification of NO_2^- by the chloroplasts all evoke NO_2^-/NO reduction by NR (Shapiro 2005). Light exposure fails to mitigate NO_2^-/NO conversion by NR if the photosynthetic electron transport is inhibited (Krueger and Kliewer 1995; Sakihama et al. 2002).

In green algae, it is clear that NR is associated with the pyrenoids and the thylakoid membranes of the chloroplast (Lopez-Ruiz et al. 1985) and NR is responsible for chloroplastic NO_2^-/NO reduction (Mallick et al. 1999; Yamasaki and Sakihama 2000; Sakihama et al. 2002; Chen et al. 2010). In vascular plants, however, NR is a cytoplasmic enzyme (Shapiro 2005) and its association with chloroplast membranes is debated (Ritenour et al. 1967; Dalling et al. 1972). However, NO_2^-/NO conversion occurs in the chloroplasts of vascular plants and its biochemistry suggests that the catalyzing enzyme may be a thylakoid-associated NR (Jasid et al. 2006) (Fig. 3.4).

In chloroplasts of vascular plants, a NADPH dependent oxidation of L-arginine also produces NO (Jasid et al. 2006) (Fig. 3.4). This NOS-like activity is sensitive to various inhibitors of mammalian-type NOS isoforms, such as L-NMMA (Pedroso et al. 2000), L-NNA and L-NAME (Jasid et al. 2006; Sang et al. 2008). Although there is Ca^{2+} influx to the stroma (Sai and Johnson 2002), NOS-like activity appears to be independent from Ca^{2+} or calmoduline (Jasid et al. 2006). Chloroplasts synthesize various amino acids including the NOS substrate L-arginine (Ruhlman and Daniell 2007). L-arginine is one of the most abundant amino acids in the chloroplast stroma, it is available at nanomolar concentrations which provides sufficient quantities for an ongoing NOS activity (Jasid et al. 2006). The steps of L-arginine synthesis involve the incorporation of NH_4^+ to glutamate, which is then converted to glutamine, ornithine and finally L-arginine in transamination steps (Krueger and Kliewer 1995).

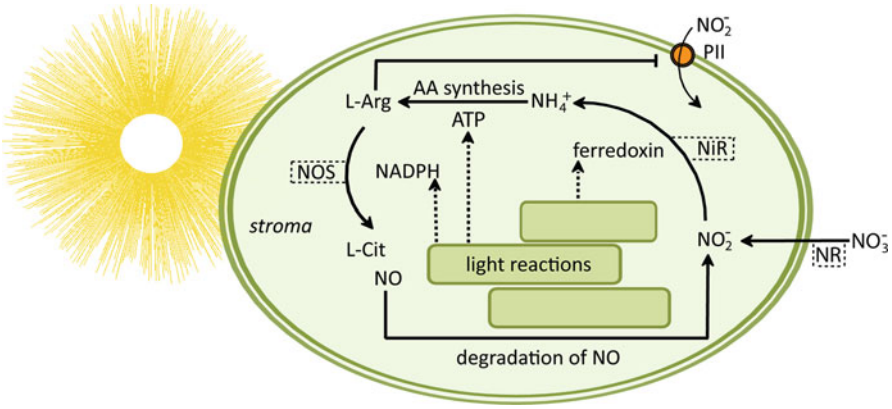

Fig. 3.5 Effects of light exposure on oxidative NO synthesis of the chloroplast. Photosynthesis provides ATP and carbohydrates for amino acid (AA) synthesis, and increases the L-arginine pool of the chloroplast. At the same time light reactions also facilitate NO_2^-/NH_4^+ conversion by nitrite reductase (NiR), and incorporation of NH_4^+ into AAs. L-arginine (L-Arg) is oxidated to L-citrulline (L-Cit) by a NO-synthase (NOS)-like activity. NO_2^- is derived from degradation of NO and also taken up from the cytosol. Interestingly, L-arginine may inhibit one transport protein (PII) which carries NO_2^- from the cytosol to the chloroplast stroma. The cytosolic nitrate reductase (NR) catalyses NO_3^-/NO_2^-reduction, and limits the accumulation of NO_2^-. Inhibition of the photosynthetic light reactions leads to NO_2^- accumulation, which is then reduced to NO by NR (reductive NO synthesis)

Key enzymes catalyzing these conversions are abundant in photosynthesizing tissues. The carbohdydrate and ATP supply of L-arginine synthesis is provided by the photosynthetic light reaction, therefore L-arginine production correlates with photosynthetic activity (Krueger and Kliewer 1995) (Fig. 3.5). Diurnal periodicity of leaf L-arginine synthesis raises the interesting question, whether NOS activity also shows a circadian pattern as a consequence of periodic changes of substrate availability.

Since light exposure and active photosynthesis increases L-arginine and decreases NO_2^- levels in the chloroplast, we may assume that sunlight increases NOS activity and concurrently inhibits NR (Fig. 3.5). This scenario is supported by the effect of light exposure, which promotes chloroplastic reduction of NO_3^- to NO_2^- (by NR) and to NH_4^+ (by NiR) and amino acid synthesis (Shapiro 2005). L-Arginine also inhibits chloroplastic NO_2^- uptake through the PII protein (Fig. 3.5), which displays a 50% similarity to prokaryote PII, a possible regulator of the NO_2^-/NO_3^-transporter in cyanobacteria (Ferrario-Mery et al. 2008). In *Arabidopsis* mutants lacking PII the chloroplastic NO_2^- uptake is increased along with the signs of NO_2^- toxicity and reduced L-arginine biosynthesis (Ferrario-Mery et al. 2008). These findings suggest that light exposure and photosynthesis increases the L-arginine pool and reduces NO_2^- levels within the chloroplast, thus favoring NOS-like activity and inhibiting the NO-forming capacity of NR. A very recent study provides evidence that inhibition of NOS increases NR-mediated NO production in wheat leaves (Rosales et al. 2010), supporting an important interplay between oxidative and reductive NO synthesis.

Further support of L-arginine dependent NOS activity of the chloroplast is provided by studies with *Arabidopsis thaliana* mutants which accumulate L-arginine in the chloroplast and consequently display increased NO synthesis (He et al. 2004). These *Arabidopsis* mutants also show delayed flowering time as a consequence of NO-overproduction. In these mutant plants a *CUE1* (chlorophyll a/b bindig protein-underexpressed-1) gene is deleted, which leads to the deficiency of a chloroplast-type phosphoenolpyruvate/phosphate translocator (PPT). PPT resides in the inner membrane of the chloroplast envelope and links the stromal metabolism with the surrounding cytosol (Streatfield et al. 1999). In mutant plants lacking chloroplastic PPT the endogenous L-arginine level is increased as a consequence of impaired stroma/cytoplasm transport (He et al. 2004). Interestingly, *CUE1* is also considered to be a key regulator of light-induced transcription changes in gene encoding chloroplast proteins (Li et al. 1995; Streatfield et al. 1999), which supports the existence of a photoperiodic pattern of chloroplast NOS activity. Moreover, in *Arabidopsis,* the involvement of NO in the circadian photoperiod pathways has also been postulated (He et al. 2004).

NADPH, an important cofactor of NOS, is also produced in the light-dependent period of photosynthesis by electron transport molecules located in the outer surface of the thylakoids (Fig. 3.5), thus NADPH is available in the stroma for NOS (Shapiro 2005). Similarly, O_2 is also generated during photosynthesis, which may also be consumed by NOS (Shapiro 2005; Jasid et al. 2006).

Theoretically, NO may diffuse throughout the plant cells, rapidly escape from the chloroplast and spread in the cytoplasm. However, its diffusion is limited by its reactions with H_2O_2, oxygen and water (Shapiro 2005). Recent findings show that intracellular spreading of NO may also be controlled by aquaporin channels (Hachez and Chaumont 2010). Since aquaporins are present in organelle membranes, NO distribution must be a more active process than it has been considered for a long time. Intensive labelling of chloroplasts by NO-indicator dyes (Pedroso et al. 2000) also supports that NO acts primarily within the organelle. The specific suborganellar distribution of NOS-like activity and the possible anchoring of NR to thylakoids (Ritenour et al. 1967; Dalling et al. 1972) (Fig. 3.4) further suggest that NO has functions within the chloroplast.

3.3.2 Iron Chelation and Photosynthesis is Affected by NO

Biosynthesis of chlorophyll requires iron (Fe^{2+}). However, plants absorb iron from the soil as Fe^{3+}, which is then reduced to Fe^{2+} and used for chlorophyll biosynthesis in the chloroplasts, the major destination for iron in plants (Murgia et al. 2002). Studies in maize *Zea mays* show that administration of NO restores the consequences of Fe^{2+} deficiency in the chloroplasts (e.g. insufficient chlorophyll synthesis, impaired chloroplast morphology) (Graziano et al. 2002). This effect may be explained by the involvement of NO in stromal iron homeostasis (Graziano et al. 2002). The key

player in chloroplastic iron bioavailability is ferritin, an already identified target of NO (Murgia et al. 2002).

Iron is chelated by stromal ferritin in chloroplasts, since unsequestrated Fe^{2+} would induce oxidative damage through the generation of hydroxyl radicals in Fenton chemistry (Chap. 2). Ferritin is responsible for the storage and delivery of iron in a non-toxic Fe^{2+} form for chlorophyll biosynthesis. In response to iron overload (Arnaud et al. 2006), ferritin gene expression is increased, leading to a subsequent iron sequestration and attenuated oxidative stress. Importantly, NO quickly accumulates in the chloroplasts after iron treatment (Arnaud et al. 2006). It has been shown that NO upregulates ferritin gene expression (Murgia et al. 2002), and iron-induced transcription changes of ferritin are also mediated—at least partially—by NO (Arnaud et al. 2006). A study has compared the effects of different NO-donor compounds on plant ferritin transcription and found that only the nitrosyl (NO^+) form of NO is effective (Murgia et al. 2004a). The binding of NO to ferritin has also been shown (Cooper 1999), although its functional impact has not yet been determined (Shapiro 2005). Collectively, NO increases iron chelation and facilitates chlorophyll synthesis through the increased transcription of ferritin.

3.4 Chloroplast NO Synthesis and Cell Death

3.4.1 The Effects of NO on the Chloroplast Membrane Systems: Thread Linking Photosynthesis and the Chloroplastic Way of Cell Death

Nitric oxide produced in the stroma also interacts with thylakoid membranes. One thylakoid membrane protein, the ascorbate peroxidase (APX1) is also a known NO target (Murgia et al. 2004b). This enzyme detoxifies peroxides produced within the chloroplast and therefore protects thylakoid membranes from reactive oxygen species (ROS). For instance NO increases APX1 transcription in water stressed maize, which mitigates H_2O_2 induced damage (Sang et al. 2008). High levels of NO, however, reduce APX1 activity and also downregulate its mRNA transcription (Murgia et al. 2004b). Stromal NO may affect the ROS eliminating capacity of APX1, and in turn, APX1 may protect thylakoids from oxidative injury caused by NO. However, the physiological impact of NO/APX1 interaction is still undefined (Shapiro 2005).

Photosynthetic electron transport chain proteins are located in the thylakoids, and from the stroma, NO may easily diffuse to reach these molecules and affect electron transport and photophosphorylation. It has been shown that NO competes with bicarbonate ions for binding to the nonheme irons in photosystem II (van Rensen 2002). By replacing bicarbonates, NO reversibly inhibits electron transport activity in photosystem II, reduces light-induced ΔpH formation across the thylakoid membrane, and consequently reduces photosynthetic ATP synthesis (Takahashi and Yamasaki

2002; Jasid et al. 2006). Chloroplast NO thus reduces the efficacy of photophosphorylation; the most important and characteristic function of chloroplasts. Reduced photosynthesis has a functional impact in the reduction of oxidative stress caused by an increased rate of photosynthetic electron transport (for details see Sect. 3.4.2).

It has also been described that inhibition of the photosystem II is involved in the initiation of plant cell apoptosis (Samuilov et al. 2003, 2008) which led to the hypothesis that increased chloroplast NO synthesis may trigger apoptosis through the inhibition of photophosphorylation. Supporting this possibility, chloroplasts may be initiators of cell death (Chen and Dickman 2004; Lum et al. 2005; Liu et al. 2007; Doyle et al. 2010; Mubarakshina and Ivanov 2010) and chloroplasts produce higher levels of NO in dying plant cells (Pedroso et al. 2000). Apoptosis or programmed cell death (PCD) is a physiological elimination process of unwanted cells (Reape and McCabe 2010). For example, nutritive aleuron cells of the germinating seeds or cells with temporary functions within the developing nucellus are deleted with PCD. Selective cell death is also required for organ morphogenesis; senescence, pathogen infections and response to various abiotic stressors (Pennell and Lamb 1997). Recently PCD has also been shown in unicellular algae, where PCD occurs as a response to environmental stress (Yordanova et al. 2010). The principle mechanisms of apoptosis are very similar in plants and animals: cell suicide requires transcription and synthesis of apoptotic proteins, activation of serine proteases (caspases in animals and caspase-like proteins in plants) and enzymatic DNA fragmentation (Reape and McCabe 2010). Morphological changes, such as cell shrinkage and nuclear condensation are also common features of animal and plant apoptotic events. However, there are striking differences in PCD in plants and animals; for example cell corpses are not removed by phagocytosing cells in plants, and some of the known key apoptotic factors from animal cells are missing or are not related to PCD in plants (Pennell and Lamb 1997).

Although several details in the molecular background of plant PCD are still undefined, increased NO synthesis is involved in PCD of distinct plant species, ranging from algae (*Chlamydomonas reinhardtii*) to vascular plants (e.g. *Arabidopsis thaliana,* tobacco *Nicotiana tabacum*, chayote *Sechium edule*, sunflower *Helianthus annuus,* ricinus *Ricinus communis,* wheat *Triticum sp.,* rice *Oryza sativa*) (Rockel et al. 2002; Lombardi et al. 2010; Ma et al. 2010; Rosales et al. 2010; Yordanova et al. 2010). Various stress conditions, e.g. light stress, UV-B irradiation, dehydration, excess NO_2^- absorption, are all known factors leading to PCD and increased NO production (Liu et al. 2007; Samuilov et al. 2008; Sang et al. 2008; Chen et al. 2010). NO-mediated PCD also occurs under physiological conditions, e.g. during the elimination of aleuron cells in germinating seeds (Lombardi et al. 2010).

Since chloroplasts are vital organelles of plant cells, they may limit cell survival (Samuilov et al. 2003). Increased NO synthesis in chloroplasts may lead to the inhibition of photosynthesis and evoke oxidative damage of the thylakoid membranes leading to consequent PCD (Chen and Dickman 2004; Jasid et al. 2006). Competition of NO with bicarbonate ions is a reversible inhibition of photosystem II, which may be beneficial under extreme light intensity and lead to photoinhibition, protecting chloroplasts from oxidative damage (Yamasaki 2000; Shapiro 2005). As previously

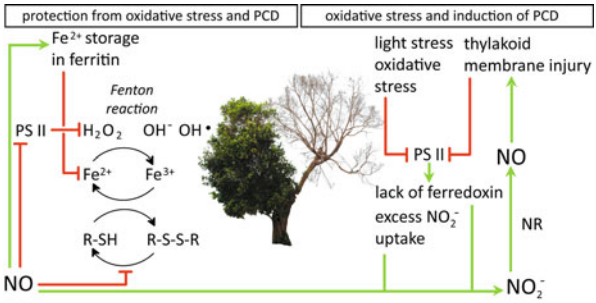

Fig. 3.6 Two faces of chloroplast NO. Chloroplast NO synthesis may reduce H_2O_2 and free radical (OH^-, $OH^\bullet$) production, since NO inhibits PS II (NO competes with bicarbonate ions), Fenton reaction and helps scavenging of iron in the stroma. However, increased NO production causes irreversible damage of the thylakoid membranes and the PS II, thus induces PCD. *R-SH* reduced thiol groups of stromal proteins; *NR* nitrate reductase

discussed, there is a light dependent interplay between chloroplast L-arginine/NO conversion and photosynthesis (Fig. 3.4), which makes it unlikely that NOS-like activity would trigger PCD through a declined photosynthesis.

PCD-inducing stressors also increase NR-mediated NO release in the chloroplast. The amount of NO liberated by NR (12–32 nmol mg^{-1} protein NO liberation by NR, compared to 7 nmol mg^{-1} NO production by NOS-like activity in a 10 min period) may be sufficient for damaging the chloroplast thylakoids (Jasid et al. 2006). NO may form peroxynitrite ($ONOO^-$) and cause lipid peroxidation and protein nitration in the thylakoid system (Jasid et al. 2006). This reductive NO production and NO-mediated injury may easily evoke an irreversible photosynthesis blockade and lead to PCD (Fig. 3.6).

3.4.2 Similar Roles of NO in Prokaryotes and the Chloroplast

Under certain physiological conditions chloroplast NO generation may protect thylakoid membranes from free radical injury, since NO prevents the oxidation of chloroplast proteins and decreases lipid radical content of the plastids (Fig. 3.6) (Jasid et al. 2006). Similar effects of NO have been reported in the cyanobacterium *Spirulina platensis* (Xue et al. 2007). In this prokaryote, NO administration alleviates UV-B irradiation induced oxidative damage, possibly as a consequence of increased activities of superoxide dismutase (SOD) and catalase (CAT) which break down superoxide and hydrogen peroxide and alleviate oxidative stress (Xue et al. 2007). Although SOD and CAT expression may be enhanced by NO in vascular plants also (Sang et al. 2008; Lombardi et al. 2010), in chloroplasts the underlying mechanism behind the antioxidant profile of NO is more likely an interference with the so-called Fenton chemistry (Shapiro 2005).

Oxidative stress results in a Fe^{2+} mediated production of hydroxyl radicals (Fenton reaction). The Fe^{2+} recycling from Fe^{3+} may be blocked by NO, since NO interrupts the formation of reduced thiols, required for the reduction of Fe^{3+} (Fig. 3.6) (Shapiro 2005). Of note, in chloroplasts NO also promotes chelation and storage of Fe^{2+} by ferritin (Murgia et al. 2002). In many prokaryote cells NO plays analogue roles; inhibits Fenton reaction and upregulates genes alleviating oxidative stress (Sudhamsu and Crane 2009; Crane et al. 2010) (Chap. 2), which let us speculate on a homology of NOS functions in prokaryotes and chloroplasts.

In the cynobacter *Anabaena* a family of flavoprotein reductases is known, which includes NRs and NOS. To date, NOS has been found in many prokaryotes (bacteria and archaea) and these prokaryote NOS molecules show sequence similarities to the oxidase domain of mammalian NOS (Sudhamsu and Crane 2009; Crane et al. 2010). The secondary structure of the known bacterial NOSs also resemble the mammalian inducible NOS (iNOS) (Sudhamsu and Crane 2009). An immunogold electron microscopy study using an antibody raised against iNOS has localized iNOS-like immunoreactive material to the chloroplast stroma in pea (*Pisum sativum*) leaves (Barroso et al. 1999). Structural similarities of mammalian iNOS and bacterial NOS molecules make it likely that a bacterial NOS would crossreact with iNOS-antibodies. According to the current paradigm, chloroplasts are descendents of ancient endosymbiotic cyanobacteria, and based on this argument we can assume the iNOS immunoreactive protein of the chloroplast stroma may be a cognate of prokaryote NOS molecules.

3.5 Open Debates and Perspectives

Chloroplast NO plays pivotal roles in iron homeostasis, photosynthetic light reactions and protection against oxidative stress, thus NO occupies a niche in light dependent regulatory networks of photosynthesis. Moreover, chloroplastic NO production impacts plant cell death. Under stress conditions a NO burst of the chloroplasts may initiate or execute PCD.

Although knowledge about plant type NO homeostasis is increasing rapidly, the bottle neck of this precise area is the lack of information on the amino acid sequence and protein structure of chloroplast NOS. Consequently, there is no data available on the transcriptional control of the gene encoding plant NOS. Indirect data support that light dependent reactions of photosynthesis govern roles in the regulation of chloroplast NO synthesis. Accordingly, circadian pattern of light exposure may be a key factor in the balance between oxidative and reductive NO generation in the chloroplast. In chloroplasts, as light harvesting organelles, light is a master transcription regulator of a gene network involved in photosynthesis and related chloroplast pathways (Li et al. 1995; Puthiyaveetil et al. 2008, 2010). Functional genomics of light dependent changes in chloroplast NO synthesis, however, is still an unexplored field. Merely an outline of the role of NO in the transcriptional control of genes activated in repsonse to oxidative stress has been defined (Shapiro 2005). Since chloroplasts are potential targets of metabolic engineering and improvement of crop nutritive

value (Ruhlman and Daniell 2007), understanding the molecular biology of NO on photosynthesis and chloroplast metabolism deserves growing interest.

3.6 Chapter Summary

Biochemistry of chloroplast NO synthesis	• Oxidative NO production is mediated by a stromal NOS-like activity. Reductive NO production is possibly catalzyed by NR • The oxidative NO synthesis is increased in light exposure and may inhibit the reductive NO production
Function of NO within the chloroplast	• NO affects ferritin expression, chloroplastic iron homeostasis, secondarily helps photosynthesis • NO reversibly inhibits thylakoid electron transport and protects chloroplasts from excess activation of photosynthesis and consequent oxidative stress • NO exerts direct and indirect antioxidant effects, by the inhibition of Fenton chemistry and gene regulation of CAT and SOD • A chloroplast NO burst can initiate PCD
Functional similarities shared with prokaryotes	• The role of NO in the attenuation of oxidative damage is similar in prokaryotes and the chloroplasts

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Chapter 4
Nitric Oxide Synthesis in Leaf Peroxisomes and in Plant-Type Mitochondria

4.1 Leaf Peroxisomes are Sites of Oxidative NO Synthesis

Peroxisomes are single-membrane bound organelles that are present in almost all types of eukaryote cells (Mano and Nishimura 2005). Plant peroxisomes may be specialized to perform certain functions, such as the glyoxysomes of oilseeds, root nodule peroxisomes of tropical legumes or the leaf peroxisomes of photosynthesizing cells (Mano and Nishimura 2005; Nyathi and Baker 2006). Glyoxysomes are responsible for the β-oxidation of fatty acids and also contain the glyoxalate cycle enzymes which convert lipids to carbohydrates, representing a plant-specific metabolite transition. Root nodule peroxisomes are sites of allantoin (the major transportable nitrogen form) biosynthesis (Mano and Nishimura 2005). The leaf peroxisomes are organelles of photorespiration and are usually present in close vicinity of the chloroplasts and the mitochondria since photorespiration establishes a metabolic interlace between these three plant organelles (Fukao et al. 2002). Peroxisomes are also sites of hydrogen peroxide (H_2O_2) generation, reactive oxygen species (ROS) detoxification and involved in the biosynthesis of vitamins and plant hormones (Mano and Nishimura 2005; Nyathi and Baker 2006; Babujee et al. 2010).

Peroxisomal NO synthesis has been shown in germinating pollen tubes of *Lilium longiflorum* (Prado et al. 2004), in the leaf of pea (*Pisum sativum*) plants (Barroso et al. 1999; Corpas et al. 2006) and in *Arabidopsis thaliana* (Corpas et al. 2009). Synthesis of NO in the pollen tube and leaf peroxisomes has been confirmed by spectrofluorimetric analysis (using the NO-indicator DAF-2 dye), ozone chemiluminescence detection and electron paramagnetic resonance spectroscopy (Corpas et al. 2004; Prado et al. 2004; Del Rio 2011). Leaf peroxisomes show L-arginine/L-citrulline conversion, which requires Ca^{2+}, calmodulin, FAD, FMN, and NADPH (Del Rio 2011). Peroxisomal NOS activity has been assayed in the presence of BH_4, although it is not synthesized by plants (Basset et al. 2002) and other studies have shown that BH_4 is not required for oxidative NO synthesis in plants (Guo et al. 2003; Shapiro 2005). Peroxisomal NOS-like activity produces 5.6 nmol mg^{-1} protein min^{-1} L-citrulline and 5 μmol mg^{-1} protein min^{-1} NO. The presence of NADPH is pivotal for maintaining this NOS-like activity (Barroso et al. 1999; Del Rio et al.

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2003; Del Rio 2011). It is possible that peroxisomal NADP-dehydrogenases provide the necessary NADPH supply of the L-arginine oxidation (Barroso et al. 1999). Peroxisomal NOS-like activity is sensitive to mammalian-type NOS inhibitors: its activity can be reduced by L-NAME (by 90%), L-NMMA (by 88%), thiocitrulline (by 80%), 7-nitroindazole (by 59%), diphenyliodonium (by 60%), L-N5-(1-iminoethyl)-ornithine (by 59%). Administration of the mammalian iNOS-inhibitor aminoguanidine totally abolishes the peroxisomal NOS-like activity (Barroso et al. 1999). An antibody reacting with mammalian iNOS has also been found to reduce the NOS activity of the peroxisomal fractions in a dose dependent manner (Barroso et al. 1999).

The leaf peroxisomes contain a protein which is recognized by an antibody raised against mammalian iNOS suggesting that a NOS-cognate enzyme may be responsible for NO synthesis (Barroso et al. 1999). It has been shown that the peroxisome marker catalase (CAT) is colocalized with this iNOS-like protein and immunogold electron microscopy has also confirmed the presence of the iNOS-immunoreactive material in the peroxisome matrix (Barroso et al. 1999; Corpas et al. 2001; Del Rio et al. 2003). Peroxisomal protein transport molecules, the peroxin Pex12 and Pex13 can be responsible for peroxisomal entry of this putative NOS protein, since pex12 and pex13 mutant *Arabidopsis* plants show reduced peroxisomal NO levels (Corpas et al. 2009). However, the plant peroxisomal NOS protein has not been isolated and characterized yet.

Recently it has also been shown that NO in plant peroxisomes may be generated by NO_2^- reduction under hypoxic or anoxic conditions (Igamberdiev et al. 2010). The responsible mechanism may be the NO_2^-/NO reducing ability of deoxygenated heme-containing proteins within the peroxisomes (Igamberdiev et al. 2010; Sturms et al. 2011). Similar reductive NO generation has been shown in the plant mitochondria, plasma membrane, cytosol and endoplasmic reticulum (Igamberdiev et al. 2010). Reduction of NO_2^- to NO by heme-proteins (e.g. hemoglobins) also occurs in cyanobacteria (Sturms et al. 2011) and mammalian tissues under O_2 limitation (Shiva et al. 2011; Tiso et al. 2011).

4.2 Possible Roles of Peroxisomal NO Synthesis

In pollen tubes NO may release from the peroxisomes and activate cGMP synthesis (Prado et al. 2004). Subcellular distribution of peroxisomes possibly generates a NO-gradient within the cell, which determines the orientation of pollen tube growth (Prado et al. 2004). In the peroxisomal matrix NO can bind to heme-proteins, such as CAT or ascorbate peroxidase, and such an interaction may affect the enzyme activities (Del Rio 2011). It is also possible that NO might lead to S-nitrosylation of peroxisomal proteins, such as CAT, glycolate oxidase, hydroxypyruvate reductase and malate dehydrogenase (Del Rio 2011). Although S-nitrosylation of several plant proteins has already been described in the cruciferan plant *Arabidopsis thaliana* (Romero-Puertas et al. 2008), how S-nitrosylation would affect protein function in plants is still incompletely understood (Shapiro 2005; Holzmeister et al. 2011).

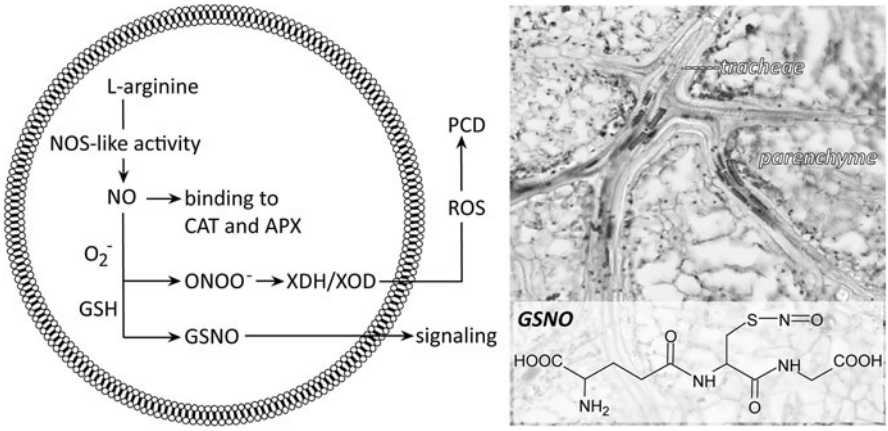

Fig. 4.1 Putative model explaining the roles of peroxisomal NO synthesis. In the peroxisomal matrix a NOS-like activity generates NO by L-arginine oxidation. NO can bind to heme-proteins, such as catalase (*CAT*) or ascorbate peroxidase (*APX*), possibly affecting their activity. NO also reacts with O_2^- and forms $ONOO^-$, which changes the activity of xanthine dehydrogenase (*XDH*) to xanthine oxidase (*XDO*), leading to increased ROS production and evoking programmed cell death (*PCD*). Both NO and $ONOO^-$ react with glutathione (*GSH*), forming S-nitrosoglutathione (*GSNO*). GSNO is a NO-carrier which may be distributed by the plant circulation

The peroxisome matrix is rich in O_2^- produced by xanthine oxidase (Del Rio et al. 2003), thereby NO can react with O_2^- and generate $ONOO^-$ (Sakuma et al. 1997). This reactive nitrogen species (RNS) changes the activity of xanthine dehydrogenase to xanthine oxidase, leading to increased O_2^- production (Sakuma et al. 1997; Barroso et al. 2006) (Fig. 4.1). In the prevailing oxidative environment of the peroxiomes both NO and $ONOO^-$ react with glutathione (GSH), the major intracellular antioxidant, forming S-nitrosoglutathione (GSNO) (Del Rio 2011). It was long debated that GSNO can be formed physiologically in plant tissues (Shapiro 2005), however the accumulation of this RNS has been shown by immune-electron microscopy and immunocytochemistry in the leaf peroxisome matrix (Barroso et al. 2006). It is possible that hypoxia is required for GSNO formation (Shapiro 2005). GSNO generation interrupts the ascorbate-glutathione cycle, a major antioxidant pathway of the peroxisome (Moro et al. 1994; Wink et al. 1996; Del Rio 2011), and peroxisomal NO thereby compromises the major antioxidant defense of the plant cell. GSNO-reductase (GSNOR) is also present in the peroxisome matrix, and possibly antagonizes the effect of NO on the ascorbate-glutathione cycle (Barroso et al. 2006) by converting GSNO to NH_4^+ and oxidized glutathione (Holzmeister et al. 2011). Accordingly, plants accumulate various RNSs in the lack of GSNOR (Lee et al. 2008).

Collectively, peroxisomal NO synthesis increases ROS and RNS production, which in response to certain abiotic stressors (Corpas et al. 2009) or pathogen infections may support the effective host defense or lead to apoptotic cell death (Shapiro 2005; Del Rio 2011) (Fig. 4.1). Moreover, GSNO may be released from the peroxisome by diffusion or possibly through porin channels and may be distributed

within the plant tissues (Barroso et al. 2006) (Fig. 4.1). GSNO is a NO-donor compound which may elaborate NO spontaneously (Floryszak-Wieczorek et al. 2006). The NO release can be catalyzed by ambient light ($h\nu$) exposure (4.1) or transition metals (4.2).

$$\mathrm{GSNO} \xrightarrow{h\nu} \mathrm{GS}^{\cdot} + \mathrm{NO} \tag{4.1}$$

$$\mathrm{GSNO} + \mathrm{Cu}^{+} + \mathrm{H}^{+} \rightarrow \mathrm{GSH} + \mathrm{NO} + \mathrm{Cu}^{2+} \tag{4.2}$$

The leaf peroxisome-derived GSNO may thereby be a NO-carrier molecule which is transported and distributed by the vascular system of the plant (Shapiro 2005). However, future studies should define the biological role of NO release from GSNO in plant tissues.

4.3 Plant-Type Mitochondria: Oxidative or Reductive NO Synthesis?

NO synthesis has been reported in mitochondria of vascular plants (Gupta et al. 2005; Planchet et al. 2005; Planchet and Kaiser 2006; Stoimenova et al. 2007; Gupta and Kaiser 2010) and the green alga *Chlorella sorokiniana* (Tischner et al. 2004). Mitochondrial NO synthesis becomes evident under O_2 deprivation, when NO_2^- is being reduced in the mitochondrial electron transport chain (Planchet and Kaiser 2006; Stoimenova et al. 2007; Gupta and Igamberdiev 2011). The mechanism of mitochondrial reductive NO synthesis resembles the bacterial NO_2^--respiration, which allows the anoxic mitochondria to oxidize NADH and NADPH and retain a limited ATP synthesis by using NO_2^- as an alternative electron acceptor (Stoimenova et al. 2007). Reductive NO synthesis in the plant mitochondria is associated with the cytochrome bc1 (or complex III) and cytochrome-c oxidase (CcO or complex IV) (Igamberdiev et al. 2010; Gupta and Igamberdiev 2011) (Fig. 4.2). CcO reacts with various nitrogen oxides and is capable of reducing NO_2^- to NO (Cooper 2002; Gupta and Igamberdiev 2011). Note, that a similar role of CcO has been documented in fungus and animal cell mitochondria (Castello et al. 2006). Moreover, CcO may also generate $ONOO^-$, oxidize NO to NO_2^- or reduce NO to N_2O. The underlying mechanisms have yet to be completely explored (Cooper 2002; Gupta and Igamberdiev 2011). Reductive NO synthesis by CcO is increased in hypoxia and the decrease of pH—a common condition observed under O_2 limitation—also favors NO_2^-/NO reduction by CcO (Gupta and Igamberdiev 2011). Plant tissues may suffer from hypoxia under physiological conditions, due to the limitation of their O_2 transporting system. Under hypoxic and acidic conditions plant tissues accumulate NO_2^- and they increase the activity and transcript level of NR, thereby NO_3^- is converted to NO_2^- and accumulated in the cytoplasm (Botrel and Kaiser 1997) (Fig. 4.3). Especially in hypoxic root cells the further reduction of NO_2^- is also mitigated (Kaiser and Huber 2001). Hypoxia therefore increases NO_2^- availability (Rockel et al. 2002), favoring reductive NO synthesis. Since roots grow into hypoxic soil, mitochondrial

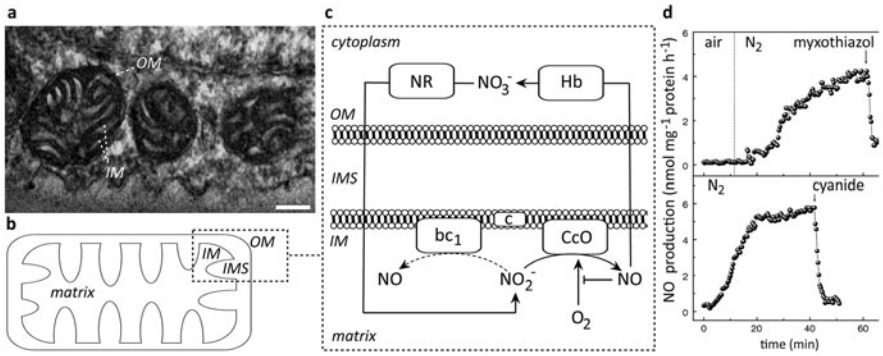

Fig. 4.2 Reductive NO synthesis in the plant mitochondria. TEM image shows bean leaf mitochondria; scale bar 0.2 μm; Author's image (**a**). Major compartments of the plant mitochondrion (**b**). *OM* outer mitochondrial membrane, *IM* inner mitochondrial membrane, *IMS* intermembrane space, *matrix*-mitochondrial matrix; Plant mitochondria generate NO by NO_2^- reduction (**c, d**). The responsible reductases are the mitochondrial respiratory enzymes cytochrome bc_1 (*bc_1*) and cytochrome oxidase (*CcO*). *c*-cytochrome c; NO inhibits electron transport to O_2 at the site of CcO, thereby reduces O_2 consumption. NO also releases to the cytosol, where it undergoes oxidation to NO_3^- by class 1 non-symbiotic hemoglobin (*Hb*). Cytoplasmic NO_3^- reductase (*NR*) further reduces NO_3^- to NO_2^-, which recycles to the mitochondria and maintains the NO_2^- supply for the anoxic ATP synthesis. The lack of O_2 (replacement of air with N_2) allows plant mitochondria to produce NO from NO_2^- in the presence of NADH (**d**). Inhibition of cytochrome bc_1 by myxothiazol or CcO by cyanide abolishes NO generation. Panel **d** is reprinted with permission. (Stoimenova et al. 2007)

NO synthesis from NO_2^- is more prominent in root cells than in leaf tissues (Gupta et al. 2005) (Fig. 4.3). Mitochondrial NO generation is associated therefore with anaerobiosis, and NO occurs as a side product of anoxic, NO_2^- driven ATP synthesis (Stoimenova et al. 2007). Of note, hypoxic and acidic plant cells possibly also generate NO by NR, which reduces NO_2^- to NO with low efficacy (Rockel et al. 2002) and deoxygenated mitochondrial heme-proteins can also reduce NO_2^- to NO (Igamberdiev et al. 2010). A protonated form of NO_2^- (HNO_2) may also release NO chemically in an acidic environment (Yamasaki 2000).

A recent model suggests that the NO/NO_2^-/NO recycling between the anoxic mitochondria and the cytoplasm improves the redox and energy status of cells suffering from O_2 limitation. Within the mitochondria NO inhibits electron transport to O_2 at the CcO site, thereby reducing O_2 consumption when O_2 availability is limited (Igamberdiev and Hill 2004; Stoimenova et al. 2007; Igamberdiev et al. 2010; Gupta and Igamberdiev 2011). The reductive NO generation in the mitochondria also leads to the S-nitrosylation of glycine decarboxylase and inhibition of the photorespiratory cycle (Gupta and Igamberdiev 2011). Administration of NO also increases the expression of alternative oxidase (AOX) (Igamberdiev et al. 2010), which is implicated in protection from programmed cell death (Hachiya and Noguchi 2011). NO also releases from the mitochondria to the cytosol, where it undergoes oxidation to NO_3^-. The responsible molecule for NO/NO_3^- oxidation may be the hypoxically induced

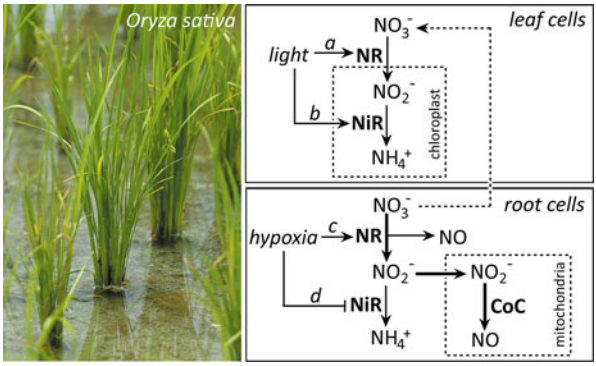

Fig. 4.3 Hypoxia favors, while light irradiation antagonizes reductive NO synthesis form NO_2^-. The example of rice, *Oryza sativa* illustrates that metabolism of NO_2^- is affected by light exposure or hypoxic conditions. In the leaf tissues NO_2^- is used for ammonification and amino acid anabolism, since light exposure increases NR (**a**) and NiR (**b**) activity. In the anoxic root cells NR level is increased which can form NO (**c**), NiR is inhibited (**d**) and mitochondria reduce the accumulated NO_2^- to NO

plant hemoglobin (class 1 non-symbiotic hemoglobin) (Igamberdiev and Hill 2004). As a next step, cytoplasmic NR reduces NO_3^- to NO_2^-, which recycles to the mitochondria and is being reduced to NO (Gupta and Igamberdiev 2011) (Fig. 4.2). This NO/NO_2^- exchange between the mitochondria and cytoplasm maintains the NO_2^- supply for the anoxic ATP synthesis (Gupta and Igamberdiev 2011). The cytoplasmic NO/NO_2^- conversion also keeps NADH/NAD^+ and NADPH/$NADP^+$ ratios low, ensuring a low redox level during the adaptation to anoxia (Igamberdiev et al. 2010).

The ability of NO to inhibit CcO is also implicated in seed germination (Gniazdowska et al. 2010). In several plants the seed dormancy is interrupted by imbibition, a process in which water penetrates the seeds, generating a temporal hypoxic condition and initiating germination. Imbibition is associated with rapid increase of NO synthesis (Liu and Zhang 2009), and the low O_2 levels in seeds possibly also favor mitochondrial reductive NO synthesis (Gupta and Igamberdiev 2011). Inhibition of CcO by NO stimulates germination (Gniazdowska et al. 2010), suggesting that hypoxic NO synthesis is an important player in breaking the dormancy of seeds.

It has been shown that plant mitochondria oxidize L-arginine to NO and the responsible NOS-like molecule may reside in the mitochondrial matrix or the intermembrane space (Guo and Crawford 2005). However, antibodies raised against mammalian iNOS fail to recognize the plant-mitochondrial NOS (Barroso et al. 1999), suggesting that this isoform could be distinct from the putative NOS molecules of the chloroplasts and the peroxisomes (Del Rio 2011). The candidate mitochondrial NOS is the *Arabidopsis thaliana* NOS1 (AtNOS1) (Guo et al. 2003; Guo and Crawford 2005). However, more recent works have shown that this protein is a cognate of membrane-bound small GTPases and lacks the ability of L-arginine oxidation (Moreau et al. 2008; Sudhamsu et al. 2008). Oxidative NO synthesis of the plant mitochondria is thereby still debated.

4.4 Hunting for a Plant-Type NOS

4.4.1 The First Pitfall in Finding Plant NOS

Many attempts have been made to isolate and characterize a plant-type NOS during the last decade (Shapiro 2005). Despite strong evidence which supports that enzymatic oxidation of L-arginine takes place in the chloroplast and the leaf peroxisomes (Foissner et al. 2000; Pedroso et al. 2000; Jasid et al. 2006; Sang et al. 2008; Vitecek et al. 2008; Del Rio 2011), and that the hypothetical plant NOS has gained a strong interest (Crawford and Guo 2005), the responsible NOS molecule in higher plants is still unknown (Moreau et al. 2010). This gap in our knowledge needs some explanation, since the number of NOS isoforms characterized in various organisms contrasts the lack of defined NOS isoforms in plants. The pioneer works describing plant-type NOS were found to be scientific misconducts and have been retracted shortly after their publication (Travis 2004). This negative history of plant NOS is likely responsible for the delay in the development of the field.

In 2003 and 2004, works later retracted reported that plant NOS is a variant of the *Arabidopsis* P-protein (AtvarP) of the glycine decarboxylase complex and it functions as an inducible-NOS upon pathogen infection (Klessig et al. 2004a, b; Travis 2004). This finding was challenged by a report showing that following infections with various bacteria, *Arabidopsis* plants do not respond with altered activity of AtvarP (Zeidler et al. 2004).

4.4.2 The Arabidopsis thaliana NOS-1

While the publications on NOS-like activity of AtvarP were erased from the literature (Travis 2004; Shapiro 2005), the first real candidate has emerged for the role of plant-specific NOS in *Arabidopsis thaliana* (Guo et al. 2003). This putative NOS molecule has been identified based on its sequence homology (23% identity, 39.5% similarity) to the *Helix*NOS (its alternative name is br-1 protein) characterized previously in neurons of the snail *Helix pomatia* (Huang et al. 1997) (Fig. 4.4). This 561-amino acid protein has been annotated as *Arabidopsis thaliana* NOS-1 (AtNOS1), later renamed as *Arabidopsis thaliana* NOS-associated protein-1 (AtNOA1) (Moreau et al. 2010). AtNOS1 does not show sequence similarities to any mammalian NOS isoform (Guo et al. 2003), however it has been shown that AtNOS1 produces NO by using L-arginine as a substrate and requires NADPH and Ca^{2+}, and is sensitive to known inhibitors of mammalian NOS (Guo et al. 2003). Importantly, an *Arabidopsis* mutant lacking AtNOS1 (*Atnos1*) shows impaired NO production (Guo et al. 2003). Moreover, the *Atnos1* mutant plants are defective in some functions attributed to NO-signaling, such as organ growth and ABA-induced stomatal movements (Bright et al. 2006). Supporting this scenario, expression of AtNOS1 with a viral promoter in *Atnos1* mutant plants resulted in overproduction of NO (Guo et al. 2003). Accordingly, plant mitochondria lacking AtNOS1 do not show NOS-like activity (Crawford

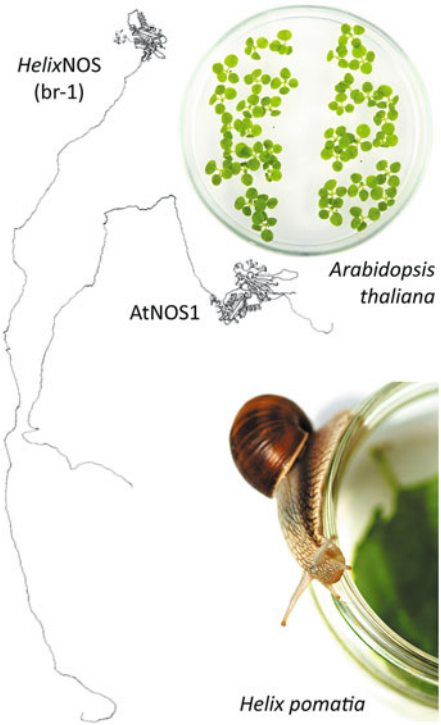

Fig. 4.4 Putative NOS-associated molecules in plants and snails. *Arabidopsis thaliana* NOS-1 (AtNOS1, NCBI Reference Sequence: NP_190329.2, residues 1–561) (Salanoubat et al. 2000) shares common sequences with *Helix*NOS (br-1, GenBank accession number: CAA65719.1, residues 1–433) (Huang et al. 1997), a putative NOS-associated protein of snail (*Helix pomatia*) neurons. These molecules contain long hydrophobic chains and myristoylated sites, which allow them to anchor cell membranes. Possibly they are capable of controlling NOS catalytic activity

and Guo 2005; Guo and Crawford 2005). AtNOS1 is also upregulated in *Arabidopsis thaliana* by bacterial lipopolysaccharides and this effect is associated with a NO burst, which suggests that the inducible NOS-activity upon bacterial infections is due to the increased AtNOS1 activity (Zeidler et al. 2004). In accordance with this possibility, AtNOS1 mutants show increased susceptibility to the pathogen *Pseudomonas syringae pv. tomato* DC3000, which further confirms that AtNOS1 is involved in pathogen-induced NOS activity (Zeidler et al. 2004). However, in these pathological conditions reductive NO synthesis also increases (Vitecek et al. 2008), which makes it difficult to find the link between AtNOS1 expression changes and increased NO emission.

Importantly, recent studies have challenged that AtNOS1 would be a functional NOS (Moreau et al. 2008; Sudhamsu et al. 2008). The AtNOS1 and a deletion variant of AtNOS1 were expressed in *E. coli* and the lack of L-arginine/L-citrulline conversion and NO production has been shown (Moreau et al. 2008). AtNOS1 shares sequence homologies with small GTPase proteins, similar to the *Helix*NOS (Huang et al. 1997). Evidence has been provided that AtNOS1 functions as a GTPase and not as a NOS-like enzyme (Moreau et al. 2008). Although *Helix*NOS also fails to synthesize NO, it is necessary for NO synthesis in snail neurons and shows immunological similarities to mammalian nNOS (Huang et al. 1997). *Helix*NOS is considered as a putative NOS-associated molecule, which determines NOS catalytic activity (Huang et al. 1997; Rőszer et al. 2010). Possibly due to its myristoylated membrane binding sequence it provides membrane anchoring ability to NOS (Huang et al. 1997). It is

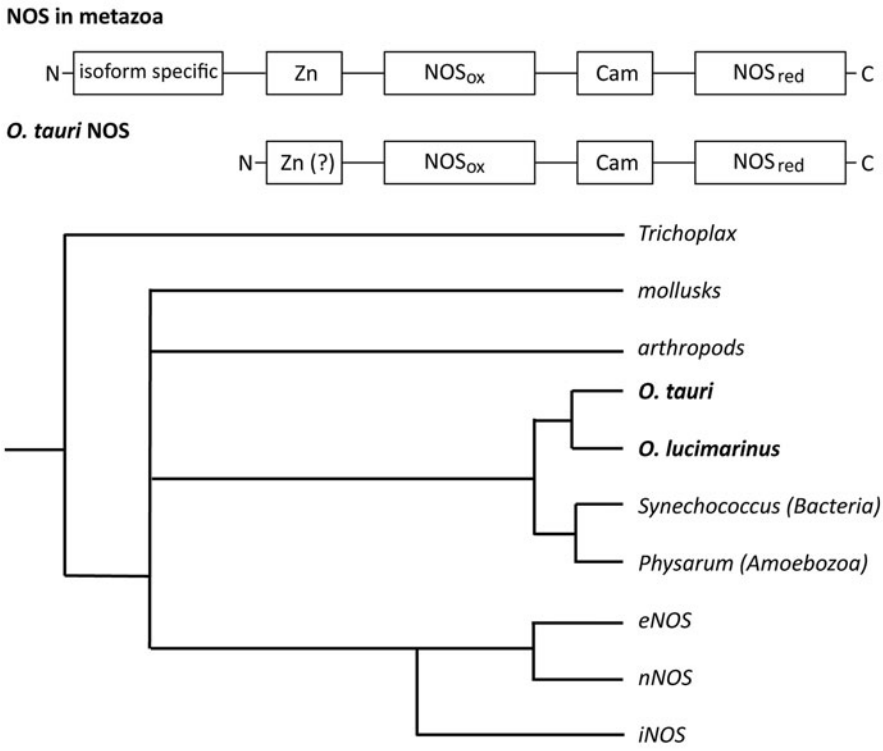

Fig. 4.5 Domain structure and possible phylogenic relations of metazoa and plant-type NOS. Both metazoa and *O. tauri* NOS contain oxygenase (*NOSoxy*) and reductase (*NOSred*) domains and bind CaM. Phylogenetic analysis of NOS-encoding sequences has revealed that *O. tauri* NOS clusters together with putative NOS sequences of a *Synechoccocus sp.* (*Bacteria*) strain and *Physarum polycephalum* (*Amoebozoa*). This cluster appears as an outgroup of NOS representatives from metazoa. (Foresi et al. 2010)

logical to assume that this protein is required for dissociation of NOS from membranes, which is an important determinant of NOS catalytic activity (Rőszer et al. 2010).

Although we can conclude that AtNOS1 itself is not a functional NOS, it may be required for NOS-associated functions in plants or may regulate NO levels indirectly. For instance AtNOS1 is required for salicylic acid-induced NO accumulation in guard cells and consequent stomatal closure in *Arabidopsis* plants (Sun et al. 2010). However, the ablation of AtNOS1 increases the salicylic acid level (Majlath et al. 2011), which is a potent inducer of stomatal closure (Lee 1998). Thus, AtNOS1 may affect guard cells in two antagonistic ways, which makes the interpretation of AtNOS1 function difficult. Similarly, the lack of MtNoa1, an AtNOS1 orthologue in *Medicago truncatula* reduces root NO levels (Pauly et al. 2011). This AtNOS1-cognate protein is also required for the establishment of symbiotic plant-rhizobium interaction (Pauly et al. 2011), a process in which NO is involved as a signal molecule (Del Giudice et al. 2011; Pauly et al. 2011). However in root nodules MtNoa1 fails to affect NO levels (Pauly et al. 2011). These findings show that AtNOS1 and its

orthologues contribute to the control of intracellular NO homeostasis, although their effects are often controversial. There is still no evidence however, that AtNOS1 or related proteins would be present in the chloroplast or the leaf peroxisome, the major organelles where oxidative NO synthesis takes place in plants.

4.4.3 The End of a Story?

The search for the plant-type NOS seems to end with the identification of mammalian NOS-homolog genes in the genome of the green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi et al. 2010). These marine organisms were first identified in the 1990's and today they are considered the smallest free-living eukaryotes (Robbens et al. 2007). They represent an ancient group of the green algae possibly evolved early after the endosymbiotic event and their ascendents could give rise to the photosynthetic eukaryotes (Keeling 2007). Based on the genomic sequences (Derelle et al. 2006; Robbens et al. 2007) a recombinant *O. tauri* NOS has been characterized (Foresi et al. 2010) (Fig. 4.5). This protein shows structural similarities to the mammalian eNOS and catalyzes L-arginine/NO oxidation in a CaM and BH_4 dependent manner (Foresi et al. 2010). Its NO synthesis is sensitive to L-NAME. Cultures of *O. tauri* also show NOS-dependent NO emission (Foresi et al. 2010). Light irradiation—resembling the chloroplastic NO synthesis—is a strong inducer of the L-arginine-dependent NO synthesis in this alga species (Foresi et al. 2010).

4.5 Chapter Summary

Peroxisomal NOS-like activity	• *Inducer of cell death* NOS-like activity increases ROS and RNS emission from the peroxisomes. This may lead to cell death or may be involved in plant response to pathogen infection • *Source of intercellular NO-signaling* NO reacts with peroxisomal glutathtione and forms GSNO. GSNO is a transportable NO-carrier, which may spread by the plant circulation. NO can be released from GSNO, allowing NO to act at long distances from its cellular source
Mitochondria produce NO from NO_2^-	• Anaerobiosis favors mitochondrial reductive NO synthesis • Reduction of NO_2^- to NO is catalyzed by the electron transport chain in the mitochondrial inner membrane. Activity of NR and chemical NO release may also contribute to NO generation under hypoxia • Mitochondrial NO production is a consequence of NO_2^- driven anoxic ATP synthesis. NO inhibits CcO, upregulates AOX, thus controls O_2 consumption • Recycling of NO/NO_2^-/NO between the mitochondria and the cytoplasm maintains anoxic energy production, thus helps plant survival under O_2-deprivation
Does the plant-type NOS exist?	• AtNOS1 may regulate cellular NO levels indirectly, however it fails to synthesize NO • To date *Ostreococcus tauri* NOS is the only known plant-specific NOS isoform • In vascular plants the enzyme responsible for NOS-like activity is still unknown

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Part IV
At the Edge of the Plant and Animal Kingdom

Chapter 5
NO Synthesis in Subcellular Compartments of Fungi

5.1 Introduction to the NO Biology in Fungi

Fungi are capable of synthesizing NO, which is involved in the control of development, morphogenesis, reproduction and apoptosis from primitive (Chytridiomycota, Zygomycota) to higher (Ascomycota, Basidiomycota) phyla of fungi (Ninnemann and Maier 1996; Kanadia et al. 1998; Song et al. 2000; Almeida et al. 2007; Kig and Temizkan 2009; Vieira et al. 2009). In some species NO activates cGMP synthesis, indicating the existence of a NO/cGMP signalling pathway (Vieira et al. 2009; Li et al. 2010). It has been shown, that NO acts directly at the transcriptional control level of certain genes (Horan et al. 2006; Chiranand et al. 2008; Kim et al. 2008; Kig and Temizkan 2009; Lushchak et al. 2010). Mitigation or aggravation of oxidative stress (Tillmann et al. 2011) and protein modification by S-nitrosylation (Lee et al. 2010) or tyrosine nitration (Castello et al. 2006) are also key mechanisms, by which NO occupies a niche in fungal cell homeostasis.

5.2 Be Fruitful and Multiply: The NO/cGMP Pathway and Sporulation

5.2.1 Asexual Spore Formation Requires NO

An important role of NO in fungal physiology is the control of sporulation and spore germination, the main pillars of asexual reproduction and spreading of fungi (Fig. 5.1). In *Blastocladiella emersoni*, a representative of primitive fungi, a putative NO/cGMP pathway controls zoospore biogenesis (Vieira et al. 2009). *Blastocladiella emersoni* is an aquatic fungus and belongs to the phyla of Chytridiomycota, one of the most ancient groups of fungi (James et al. 2006). Chytrids live in aquatic or moist habitats as saprobes or parasites. In their asexual sporulation stage, chytrids generate and release motile zoospores, which disperse in the water. Since zoospores

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are flagellate cells, chytrids are long considered to be protists (animal-type unicellular organisms) (James et al. 2006).

Fluorescent indicators have detected increased levels of NO and NO-derived products (NO_3^-, NO_2^-, nitrosothiol, nitrosamines and iron-nitrosyl complexes) in sporulating *Blastocladiella emersonii* (Vieira et al. 2009). Accordingly, sporulation and the late phase of zoospore biogenesis is linked to an increased cGMP synthesis and the upregulation of mRNA levels of guanylyl cyclases and a cGMP-phosphodiesterase (Silverman and Epstein 1975; Vieira et al. 2009). Germination can be initiated by cGMP administration (Gomes et al. 1980; Gottschalk and Sonneborn 1982), while the inhibition of cGMP synthesis completely prevents sporulation (Vieira et al. 2009), showing the pivotal role of the cGMP-mediated signal pathway in *Blastocladiealla* zoospore development. An L-argentine/L-citrulline oxidizing NOS-like activity has been assayed in *Blastocladiella* cells, which increases during sporulation (Vieira et al. 2009). Inhibitors of mammalian NOS (L-NAME and *1*-[*2*-(trifluoromethyl)-phenyl]-imidazole) reduce NOS-like activity, intracellular NO and cGMP levels, which confirms that a NOS-like enzyme is responsible for NO synthesis and activation of guanylyl cyclase during zoospore generation (Vieira et al. 2009).

5.2.2 *Fungal Photoperiod and Sporulation: NO is Involved in Light Signalling*

Sporulation of algal fungi Zygomycota, another ancient group of fungi, also requires NO synthesis (Fig. 5.1). Light is an important determinant of life cycle in fungi, since light signals activate morphogenetic pathways and help hyphal growth and reproduction (Rodriguez-Romero et al. 2010). In the zygomycote *Phycomyces blakesleeanus*, light exposure induces NO emission of developing cells (Maier et al. 2001). *Phycomyces* displays a light-regulated sporulation and the NO donor sodium nitroprusside (SNP) can replace the light effect on sporangiophore generation (Maier et al. 2001). These findings suggest that NO is involved in the light signaling which triggers sporulation (Maier et al. 2001).

Similarly, NO also controls a photomorphogenetic event, the asexual spore formation or conidiation in the phylum Ascomycota, one major group of higher fungi. Conidiation is the mitotic spore formation process of filamentous fungi, which in some species shows a light-dependent circadian pattern. In mycelia of *Coniothyrium minitans* the early stage of conidiation is linked to increased NO generation (Gong et al. 2007; Li et al. 2010). Staining with a NO-sensitive fluorescent probe suggests that primordia and young pycnidia (in which conidia are forming) are the main sites of NO generation (Gong et al. 2007). The dynamics of NO synthesis is closely mirrored by the changes in cGMP levels during pycnidial development. Accordingly, the NO donor sodium nitroprusside (SNP), stimulates the accumulation of cGMP almost instantly in mycelium during the hyphal growth stage, suggesting that a NO/cGMP

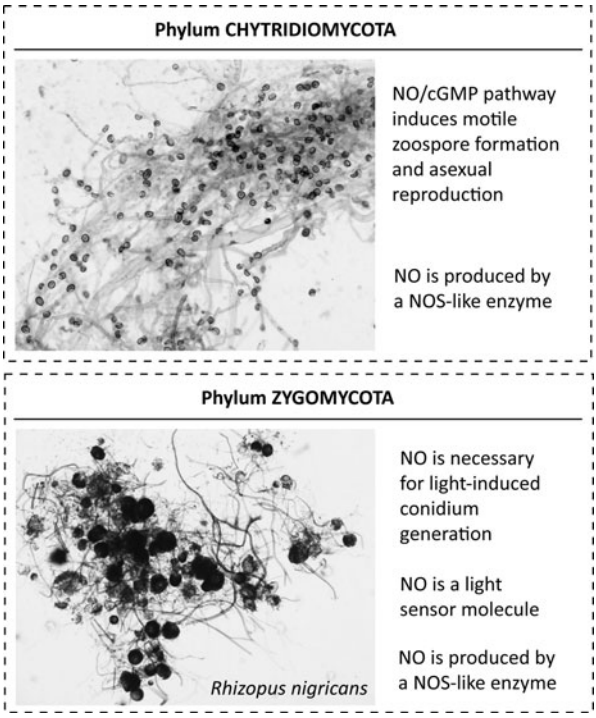

Fig. 5.1 Physiological effects of NO in representative of lower fungi. The existence of a NOS-like enzyme is postulated in these species and the NO synthesis is sensitive to light, thus NO is required for light-dependent processes, such as conidiation

pathway is involved in hyphal growth and mitospore generation (Gong et al. 2007; Li et al. 2010).

Endogenous production of NO and the inhibitory effect of NO on light-induced conidiation has been demonstrated in another sporulating ascomycote, *Neurospora crassa* (Ninnemann and Maier 1996). Whether this photomorphogenic role of NO in *Neurospora* requires cGMP or not is not yet established. The circadian conidiation rhythm of *Neurospora* is mainly determined by a photoperiodic pattern of calcium/calmodulin and cAMP-dependent protein phosphorylation (Techel et al. 1990). In blue-light irradiated *Neurospora* mycelia cGMP levels are sustained (Shaw and Harding 1987), and light dependent changes do not affect cGMP synthesis (Sokolovskii et al. 1983), which do not imply the involvement of cGMP in light-dependent conidiation. However, cGMP administration evokes elongation of *Neurospora* mycelia and thus promotes fungal growth (Rosenberg and Pall 1979). A *Neurospora* mutant lacking cGMP shows growth retardation and reduced lifespan, which can be reversed by exogenous cGMP administration or inhibition of cyclic nucleotide phosphodiesterase, the main cGMP degrading enzyme (Munkres 1990). A set of antioxidant enzymes are upregulated at the transcription level by cGMP, thus it helps fungal growth and delays senescence, and secondarily may counteract spore formation (Munkres 1990). Although intracellular cGMP concentration is less than 1% of the cAMP levels in *Neurospora* (Shaw and Harding 1987), cGMP is pivotal for its normal growth (Munkres 1990). Interestingly, adenylyl cyclase may form not only

cAMP but also cGMP in *Neurospora* (Shaw and Harding 1987). To date, however, the possible interaction of NO with cAMP and cGMP levels in photoconidiation of *Neurospora* is undefined.

5.2.3 *A Putative NO/cGMP Pathway in the Sporulation of Unicellular Fungi*

A recent study has pointed out that NO may regulate the sporulation in the fission yeast *Schizosaccharomyces pombe* (Kig and Temizkan 2009). Administration of NO upregulates a set of genes required for sporulation and guanylyl cyclase inhibitors reduce spore formation (Kig and Temizkan 2009), however the mechanism by which NO acts as a transcriptional regulator is still uncertain. Guanylyl cyclase activity which can be activated by the NO donor SNP (Kuo et al. 1998) and a particulate guanylyl cyclase activity with a possible role in ascospore conjugation has also been proposed in yeast (Eckstein and Schlobohm 1997). The yeast genome (including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans*) lacks homologues to guanylyl cyclase genes (Schaap 2005), which makes the interpretation of a NO/cGMP signaling pathway difficult in yeasts. Studies with various inhibitors and activators of guanylyl cyclase in *Candida albicans* have also yielded controversial results. While the guanylyl cyclase activator YC-1 inhibits a morphogenetic event, the budding-to-hyphal transition, other guanylyl cyclase inhibitors or activators (LY-83583 and furoxan, respectively) are cytotoxic in *Candida albicans* (Toenjes et al. 2009). Some guanylyl cyclase activators (LY-83583, minoxidil) and cGMP phosphodiesterase inhibitors (MY-5445, MBCQ, 8-bromo-cGMP) do not affect *Candida albicans* morphogenesis or survival (Toenjes et al. 2009). These findings suggest that effects of these ligands do not reflect a NO/cGMP axis in yeasts.

5.2.4 *Photomorphogenesis and Light Dependent NO Synthesis in Basidiomycotes*

Involvement of NO in photomorphogenesis of Basidiomycetes has been established (Fig. 5.2). In these fungi, light exposure is required for the normal development of fruiting bodies in which sexual spore generation takes place. In the cultivated golden needle mushroom or enokitake *Flammulina velutipes*, NO stimulates fungus growth and the formation of fruiting bodies (Song et al. 2000). A NADPH, FAD, BH_4 and FMN dependent L-arginine/L-citrulline converting NOS-like activity has been detected in *Flammulina* cells. Exposure to light gradually increases this NOS-like activity, which implies that NO plays a role in light-dependent development of reproductive fruiting bodies (Song et al. 2000). This putative basidiomycete-type NOS does not require calcium-calmodulin and its catalytic activity can be inhibited by aminoguanidine and L-NAME (Song et al. 2000).

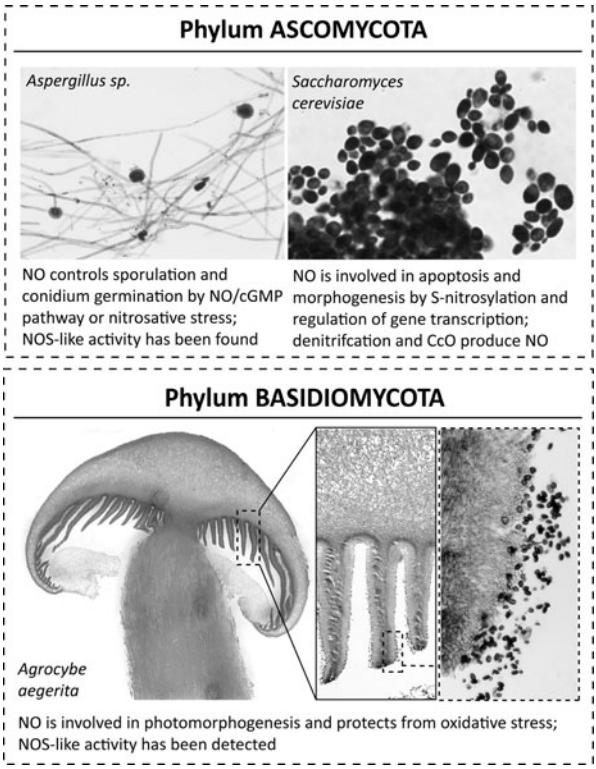

Fig. 5.2 Physiology of NO in higher fungi. In higher fungi both oxidative and reductive NO synthesis occur and affect spore germination, morphogenesis and stress response. In the presence of O_2 the NO is generated by a NOS-like enzyme; under O_2-limitation the mitochondrial denitrification system or the cytochrome-c oxidase (CcO) produce NO

5.3 Destructive and Protective Faces of NO in Fungi: Nitrosative Stress, Apoptosis and the Antioxidant Nature of NO

5.3.1 Delaying Spore Germination by Mean of Nitrosative Stress

Studies on germinating conidia of ascomycetes conclude that NO may evoke nitrosative or oxidative stress in fungal cells. Conidia disseminate in a temporarily dormant stage, characterized by anaerobic fermentation and accumulation of enzymes to protect from oxidative stress (Teutschbein et al. 2010) which ensures the effective spread and colonization of spores. When conidia reach a suitable substrate, they shift to respiratory metabolism, reactivate protein synthesis and start to germinate and generate a germ tube, which gives rise to the consequent hyphal growth (Lamarre et al. 2008; Teutschbein et al. 2010).

In the ascomycote *Colletotrichum coccodes*, NO synthesis occurs during the germination process (Wang and Higgins 2005). Exogenous NO administration delays germination in this fungus (Wang and Higgins 2005), suggesting that NO may be necessary to control the appropriate time of exit from spore dormancy. Decrease of cGMP levels has been shown during conidium germination in *Aspergillus*

(Kunkel and Romer 1980), which suggests that a NO/cGMP pathway may be responsible for delaying germination. However, it has not been shown, that NO synthesis would affect cGMP levels in conidia and this increase in cGMP synthesis would delay germination.

A more likely explanation of the negative effect of NO on conidial germination is cellular damage evoked by NO. In *Penicillium expansum*, NO increases the level of intracellular reactive oxygen species (ROS) and enhances carbonylation damage, and thus secondarily leads to growth retardation and injury of spores (Lai et al. 2011). The exogenous administration of NO also reduces the activities of superoxide dismutase (SOD) and catalase (CAT), as well as ATP content in the spores (Lai et al. 2011).

Similar effects of NO have been described in plant pathogens *Aspergillus niger*, *Monilinia fructicola* and *Penicillium italicum* under *in vitro* conditions (Lazar et al. 2008). Based on these effects of NO, the possible treatment of harvested crops with NO as an antifungal agent has been proposed in horticulture (Lazar et al. 2008).

In the human pathogen *Candida albicans*, NO administration also reduces cell viability, since NO decreases ATP synthesis through inhibition of the mitochondrial electron transport chain and the plasma membrane H^+-ATPase (Haque et al. 2005). Conidia are more sensitive to NO than hyphae (Abaitua et al. 1999). The immune response against invading *Candida albicans* cells and conidia involves the increased generation of NO by macrophages and neutrophils, thus host-induced nitrosative stress is an important factor limiting *Candida albicans* colonization (Tillmann et al. 2011).

5.3.2 Mechanisms to Escape Nitrosative Stress: Flavohemoglobins and Antioxidants

In germinating conidia of the ascomycote *Botrytis cinerea*, the transcription level of *Bcfhg1* encoding a flavohemoglobin is increased (Turrion-Gomez et al. 2010). Flavohemoglobins are NO-oxidoreductases (also known as NO-dioxygenases, EC 1.14.12.17) and capable of converting NO to NO_3^-, in a NADPH, FAD and O_2 dependent manner, and thus constitute important enzymes involved in NO detoxification (Turrion-Gomez et al. 2010; Zhou et al. 2011). The elimination of NO by flavohemoglobins has also been described in bacteria (Gardner et al. 1998), thus it may be a prokaryote heritage of fungi. The exposure of conidia to NO enhances *Bcfhg1* transcription, indicating that cells respond to a NO challenge with increased flavohemoglobin levels, which protect germinating conidia from nitrosative stress (Turrion-Gomez et al. 2010).

Flavohemoglobins play a similar role in other fungus species, such as *Aspergillus oryzae* (Zhou et al. 2011) and the yeasts *Candida albicans* (Tillmann et al. 2011) and *Saccharomyces cerevisiae* (Cassanova et al. 2005). Similar to NO-exposed *Botrytis cinerea* conidia, nitrosative stress upregulates the transcription of *CaYhb1* in *Candida albicans*, encoding yeast flavohemoglobin (Hromatka et al. 2005). Yeast flavohemoglobin gene homologs are known in yeasts *Schizosaccharomyces pombe*,

Saccharomyces cerevisiae, *Kluyveromyces lactis* and the ascomycotes *Magnaporthe grisea* and *Neurospora crassa* (Liu et al. 2000; Cassanova et al. 2005; Tillmann et al. 2011). Flavohemoglobins therefore, govern a key role in limiting endogenous NO overproduction and protection from nitrosative stress in fungi.

Pathogenic fungi have developed a range of other detoxification mechanisms against NO (Tillmann et al. 2011), such as the production of antioxidants and NO-scavenging molecules (e.g. trehalose, metalloporphyrins, glutathione) and antioxidant systems (e.g. S-nitrosoglutathione reductase and thioredoxin peroxidase-1). The tolerance of NO-stress (Kunert 2000) and the response evoked by NO (Tillmann et al. 2011) determine the virulence of the pathogenic species. For example *Candida albicans* is relatively susceptible to nitrosative stress (Kunert 2000), while in *Aspergillus fumigatus* high levels of antioxidant enzymes accumulated in the resting conidia ensures a strong resistance to oxidative and nitrosative stress (Kunert 1995).

Nitrosative stress also upregulates the transcription of genes encoding antioxidant enzymes such as CAT and SOD (Lushchak et al. 2010), and the NO-scavenging glutathione in *Saccharomyces cerevisiae* (Horan et al. 2006) are subjected to exogenous NO. Administration of NO increases peroxisomal but not cytoplasmic CAT activity (Lushchak and Lushchak 2008a), and the lack of cytoplasmic CAT fails to affect NO-tolerance in yeasts (Lushchak and Lushchak 2008b), suggesting that ROS elimination in the peroxisomes is upregulated by nitrosative stress[1]. The transcription of genes encoding respiratory electron transport chains decreases in response to NO, which ensures an effective way of reducing ROS generation in *Saccharomyces cerevisiae* (Horan et al. 2006).

5.3.3 How Gene Expression Machinery Senses NO in Fungi

The possible mechanism behind NO-regulated transcription involves the nuclear translocation of a transcription factor, Yap1 (Yes associated protein-1) and the consequent transcription of antioxidant genes in *Saccharomyces cerevisiae* (Lushchak et al. 2010). In *Candida albicans* a NO-responsive element (NORE) has been identified, which is necessary for transcription of the yeast flavohemoglobin gene *CaYhb1* (Chiranand et al. 2008). A transcription factor (CaCta4), belonging to the family of $Zn(II)_2$-Cys_6 transcription factors has been identified, which may bind to a NORE within the promoter of target genes (Chiranand et al. 2008). In *Schizosaccharomyces*, the NO-responsive transcription factor is a zinc-finger transcription factor (ScFzf1) (Sarver and DeRisi 2005). In *Saccharomyces* an activator protein-1 (AP-1)-like basic leucine zipper transcription factor (SpPap1) is the candidate for mediating gene expression changes upon the NO signal (Kim et al. 2008).

[1] An association of NOS with peroxisomes has been shown in plant and animal cells. In fungal cells however, peroxisomal localization of NOS is yet undefined.

5.3.4 S-nitrosylation and Induction of Apoptotic Cell Death

Protein nitrosylation by NO may initiate apoptosis in fungi. In yeasts a classical glycolytic enzyme, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an important mediator of apoptosis (Magherini et al. 2007) and its S-nitrosylation promotes apoptosis in *Saccharomyces cerevisiae* (Almeida et al. 2007). GAPDH interacts with glutathione peroxidase 3 (Gpx3), an antioxidant enzyme involved in cellular stress protection which secondarily affects cell survival and apoptosis under oxidative stress. In yeast cells, Gpx3 modulates the activities of proteins involved in signal transduction pathways and protein translocation, it may protect proteins from inactivation and degradation (Lee et al. 2007), and affect protein repair activity (Kho et al. 2006). Gpx-3 also helps to reduce the S-nitrosylation of GAPDH, thus increases cell viability in NO stress (Lee et al. 2010). A growing body of evidence shows that S-nitrosylation provides a NO-dependent mechanism to control interactions between cellular proteins (Matsumoto et al. 2003). To date, large sets of yeast proteins have been identified as potential targets of S-nitrosylation, showing that NO may gain a strong functional impact in post-translation protein modification and protein–protein interactions in fungi (Foster et al. 2009).

5.3.5 The Antioxidant Nature of NO in Basidiomycetes

In basidiomycetes NO may protect against oxidative stress. In the button mushroom *Agaricus bisporus*, a representative species of Basidiomycota, administration of the NO-donor compound [*2*,*2′*-(hydroxynitrosohydrazino)-bisethanamine] reduces oxidative damage in harvested fruiting bodies. NO reduces both the superoxide production rate and H_2O_2 content, inhibits the activity of polyphenol oxidase (PPO) and increases the antioxidant enzyme activities of CAT, SOD and ascorbate peroxidase (APX) in *Agaricus* cells. In the saprophytic basidiomycete *Phanerochaete chrysosporium*, NO may interfere with a glutathione transferase-like system in the cytosol and the microsomal fraction (Servent et al. 1991, 1992) thus may inhibit hydroxyl radical generation by Fenton reaction (Chap. 2).

5.3.6 Social Fungi and the Antioxidant NO: Stress Resistance of Lichens

Lichens are symbiotic associations of filamentous fungi (ascomycotes or rarely basidiomycotes) and green algae or cyanobacteria. The plant-fungus association forms a common thallus, although the symbiotic fungus and plant cells preserve their structural integrity. In the organization of the thallus structure, the fungus (often called the mycobiont) plays the leading role by forming a woven hyphal network in which the algal cells or cyanobacteria are anchored (Figs. 5.3, 5.5).

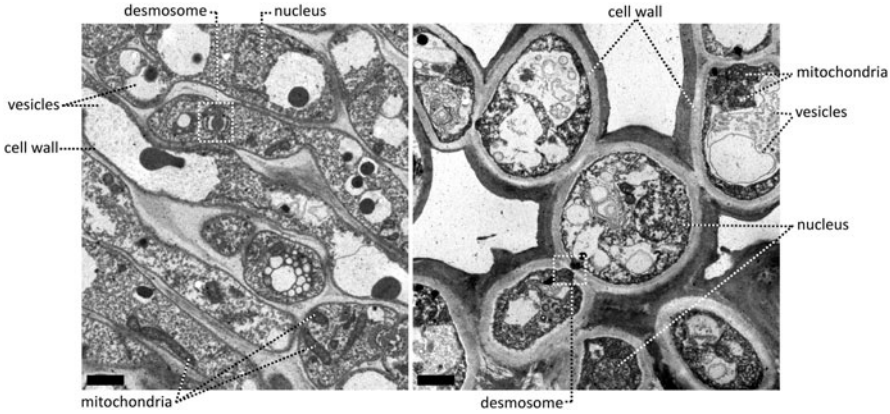

Fig. 5.3 Subcellular structure of fungus cells. TEM images of fungal cells. *On the left:* longitudinal section of *Penicillium camemberti* mycelia; *on the right:* cross-section of a lichen, showing fungal cells. Scale bars 1 μm, Author's images. Oxidative NO synthesis is associated with the cytoplasm and possibly with the particulate fraction; while reductive NO generation is detectable in the mitochondria

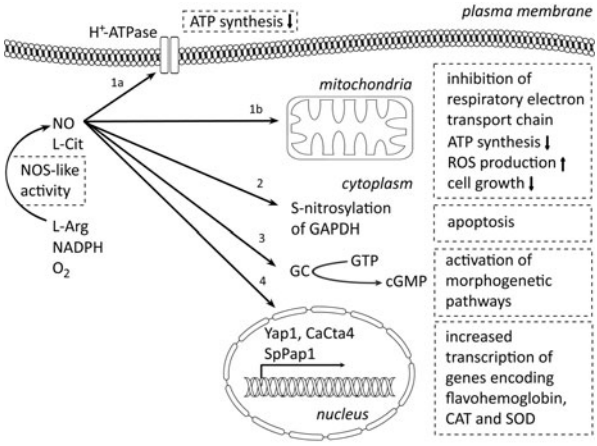

Fig. 5.4 Oxidative NO synthesis in the cytoplasm. The NOS-like activity generates NO in the cytoplasm, which affects various targets: cell membrane proteins (*1a*), mitochondria (*1b*), cytoplasmic- (*2, 3*) or nuclear proteins (*4*)

Lacking defined water resorbing organs such as roots and fluid transporting systems, lichens do not have controlled water homeostasis and their hydration stage highly depends on environmental conditions. Alternating periods of desiccation and rehydration thus determine the metabolic rate and life cycle of lichens. Desiccation-rehydration transition in the lichen *Ramalina lacera* results in a rapid increase in photosynthesis and consequently evokes a burst of intracellular production of ROSs. The use of NO-indicator fluorescent probes has shown that rehydration induces NO synthesis in the mycobiont fungus (Weissman et al. 2005a, b; Catala et al. 2010). Scavenging of NO increases both ROS production and lipid peroxidation

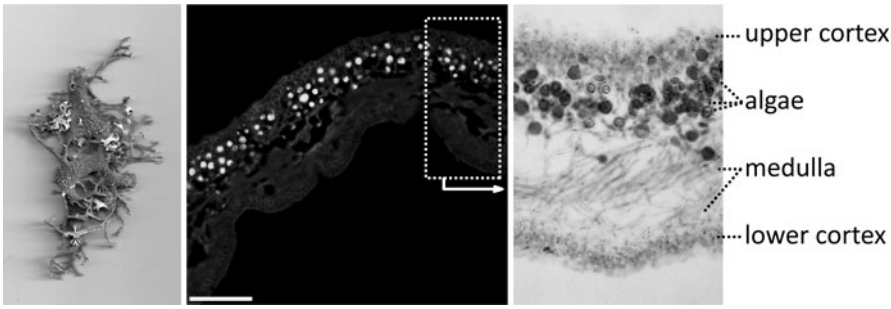

Fig. 5.5 Fungal-plant association: structure of a lichen. The lichen thallus (*on the left*) is built up from fungal cells (*upper cortex, medulla, lower cortex*) which embed several alga cells (indicated by the chlorophyll atofluorescence; *in the middle*). Fungal cells elaborate NO and reduce oxidative injury in the alga cells. Scale bar 65 μm

in the lichen (Catala et al. 2010), suggesting that the mycobiont derived NO mitigates oxidative stress and protects plant cells within the thallus. The NO emitted by the fungus may target the chloroplasts of the algal cells and mitigate photooxidative damage caused by the reactivated photosynthesis. Similar beneficial effects of chloroplast NO synthesis has been shown in chloroplasts of higher plants (Chap. 3). These recent findings point to the impact of fungal NO synthesis in the adaptive success of fungus-plant symbiotic association.

5.4 Biosynthesis of NO in the Fungal Cell

5.4.1 The Oxidative and Reductive Ways of NO Synthesis in Fungi

In representatives of chytridiomycetes, ascomycetes and zygomycetes, NO may be produced from L-arginine by NOS-like activity and this oxidative NO synthesis may be the dominant way of NO production in the cytoplasm under aerobic conditions (Vieira et al. 2009; Li et al. 2010) (Figs. 5.3, 5.4). Various forms of reductive NO synthesis have also been shown in fungi (Fig. 5.6). For instance, under hypoxic or anoxic conditions, *Saccharomyces* generates NO from NO_2^- by mitochondrial cytochrome-c oxidase (Castello et al. 2006, 2008). Similarly, mitochondrial nitrite reductase of denitrifying fungi may also produce NO by reduction of NO_2^- (Zhou et al. 2011). As a unique way of NO generation, the saprophytic basidomycote *Phanerochaete chrysosporium* may also elaborate NO by degrading glyceryl trinitrate (Servent et al. 1991). This fungus is important in the biodegradation of various organic chemicals by means of extracellular enzymes, thus its ability to decompose the explosive glyceryl trinitrate to NO and NO_2^- has a biotechnological impact. It is suggested, that NO may be involved in the glutathione transferase-like system of this fungus, thus it may control cellular oxidative stress and may interact with hemoproteins

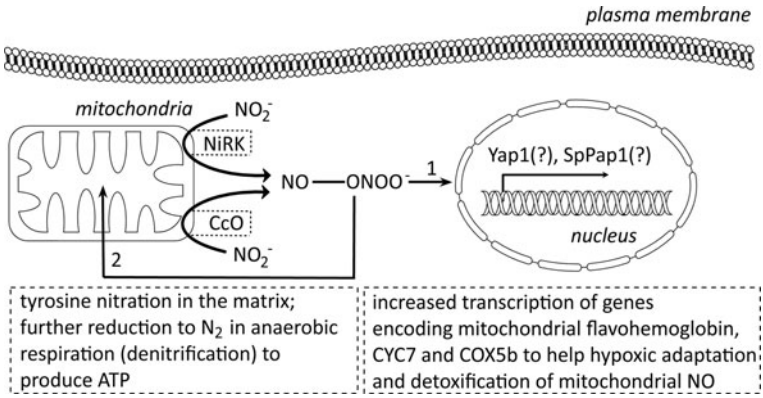

Fig. 5.6 Reductive NO synthesis in the mitochondria. Under O_2-limitation the inner membrane layer of fungal mitochondria generates NO by bacterial nitrite reductase (*NirK*) or cytochrome-c oxidase (*CcO*). The NO or the NO-derived $ONOO^-$ target the mitochondrial matrix or nuclear proteins

(Servent et al. 1992). However, the biological impact of glyceryl trinitrate conversion to NO is yet to be defined.

5.5 Oxidative NO Synthesis from L-arginine in Fungi: Biochemistry and Compartmentalization of a Putative Fungal NOS

5.5.1 Evidences Suggesting the Existence of a Fungus-Type NOS

Conversion of radiolabeled L-[^{3}H]arginine to L-[^{3}H]citrulline has been shown in the chytrid *Blastocladiella emersoni* (Vieira et al. 2009), the ascomycote *Neurospora crassa* and the zygomycote *Phycomyes blakesleeanus* (Ninnemann and Maier 1996). Inhibitors of mammalian NOS (L-NAME and N L-NMMA) inhibit *Blastocladiella* zoospore generation, enhance *Neurospora* conidiation and reverse the effects of NO donor compounds on sporulation and photomorphogenesis, respectively (Ninnemann and Maier 1996; Vieira et al. 2009). This finding suggests that the putative *Blastocladiella* and *Neurospora* NOS may display similar catalytic activity to mammalian NOS. Similarly, in the zygomycote *Phycomyes blakesleeanus* NO synthesis is pivotal for photomorphogenesis and spore formation (Maier et al. 2001). Accordingly, sporangiophores show higher production of L-citrulline than mycelia and light induces increase in L-arginine/L-citrulline conversion in the mycelium and in sporangiophores (Ninnemann and Maier 1996; Maier et al. 2001). Inhibition of BH_4 biosynthesis or BH_4 depletetion from the mycelia also inhibits light-induced morphogenesis, supporting the notion that a putative NOS molecule may be responsible for NO release. Catalytic activity of this putative NOS depends on NADPH, does not

require calcium, and is sensitive to inhibitors of mammalian NOS isoforms (Maier et al. 2001).

The existence of an L-arginine dependent NO synthesis in ascomycotes is further supported in the L-arginine auxotroph *Coniothyrium minitans* ZS-1T2029 mutant. This mutant ascomycote is deficient in the L-arginine-specific carbamoyl-phosphate synthase and thus displays compromised L-arginine biosynthesis and shows impaired conidiation (Li et al. 2010). Altered conidiation (a NO-controlled event) may be restored either by complementation with L-arginine or administration of NO (Gong et al. 2007). The reversal of the phenotype by L-arginine or NO suggests that oxidation of L-arginine to NO is required for conidiation. In wild type strains, inhibitors of mammalian NOS evoke delayed conidiation and reduce cellular NO levels. These findings collectively show that *Coniothyrium* synthesizes NO from L-arginine, possibly by a NOS-like molecule. Chelation of calcium reduces NOS-like activity in *Coniothyrium* (Gong et al. 2007; Li et al. 2010).

5.5.2 Yeast NOS: A Debated Enzyme

NOS-like activity has been documented in *Saccharomyves cerevisiae* (Kanadia et al. 1998) and most recently in *Schizosaccharomyces pombe* (Kig and Temizkan 2009). *Saccharomyces* NOS-like activity converts L-arginine to L-citrulline in the presence of calmodulin and may be inhibited by L-NAME (Kanadia et al. 1998). S*chizosaccharomyces* NOS-like activity produces NO in a calmodulin and BH_4 dependent manner and it is also sensitive to L-NAME (Kig and Temizkan 2009). However, the conversion of L-arginine to L-citrulline by S*chizosaccharomyces* NOS-like activity has not yet been shown (Kig and Temizkan 2009). Studies using an antibody against mammalian NOS have detected NOS-like immunoreactive material in the yeast cell (Kuo et al. 1996; Kanadia et al. 1998), however the identity of NOS-like activity and this NOS-immunoreactive substance is uncertain. Although their genome lacks mammalian NOS orthologues making the enzyme displaying NOS-like activity in yeasts an enigma (Toenjes et al. 2009), various yeast species are capable of producing NO. Moreover, various NOS inhibitors (diphenyleneiodonium chloride and thiocitrulline) are cytotoxic in *Candida albicans*, although the observed effects are unrelated to the modulation of NO synthesis (Toenjes et al. 2009).

5.5.3 NOS-Like Activity Occurs in the Cytoplasm

The fluorescent NO indicator *4,5*-diaminofluoroscein diacetate intensively labels *Blastocladiella* cytoplasm, and the responsible NOS-like activity occurs in the crude cell extracts, which suggests that cytoplasm may be abundant in the NOS-like protein (Vieira et al. 2009). In light-exposed *Phycomyces* sporangiophores, the soluble fraction shows high, 26 pmol $min^{-1}mg^{-1}$ NOS activity, while the NOS activity linked to

the particulate fraction is not relevant (Ninnemann and Maier 1996). In sporangiophore cells therefore, a cytosolic protein may be responsible for NOS-like activity. In *Schizosaccharomyces* the NO-forming enzyme activity was assayed in crude cell extracts (Kig and Temizkan 2009), which also suggests the abundance of the putative NOS-like enzyme in the cytosol. In extracts of *Neurospora crassa* mycelia however, the NOS-like activity occurs in both the soluble and particulate fractions. In a soluble fraction, which represents the cytoplasm, L-arginine/L-citrulline conversion is around 13 pmol $min^{-1}mg^{-1}$. The particulate fraction shows higher, 18 pmol $min^{-1}mg^{-1}$ NOS activity (Ninnemann and Maier 1996). The putative NOS may either reside in the cytoplasm, or may be linked to intracellular membranes that occur in the particulate fraction.

A recent study has described a specific subcellular distribution pattern for NO-scavenging flavohemoglobin proteins in *Aspergillus oryzae* cells (Zhou et al. 2011). The cytosol is abundant in flavohemoglobin-1 (FHb1) and its gene (*fhb1*) is being upregulated in response to administration of NO. Accordingly, disruption of *fhb1* evokes hypersensitivity to NO stress (Zhou et al. 2011). The cytoplasm of *Saccharomyces cerevisiae* is also abundant in yeast flavohemoglobin, reflecting NO synthesis in the cytosol (Cassanova et al. 2005). These findings confirm that cytoplasm is a major site of NO generation in fungi. The main pool of the NOS substrate L-arginine is also the cytoplasm. Mycelia take up L-arginine through an ATP-dependent plasma membrane transport system (Piotrowska et al. 1976), and additionally to the mitochondrial L-arginine biosynthesis (Moat et al. 2002) L-citrulline may be converted to L-arginine within the cytosol (Yu and Weiss 1992)). The cytoplasmic pool of L-arginine thus provides substrate availability for a putative cytosolic NOS in fungi.

5.6 Reductive NO Synthesis in the Fungal Mitochondria

5.6.1 A Novel Mechanism Behind Mitochondrial NO Synthesis: Cytochrome-c Oxidase

Although mitochondrial NOS-like activity has not been detected in fungi, NO-converting flavohemoglobins are present in the mitochondria of distinct fungus species (Cassanova et al. 2005; Zhou et al. 2011), indicating that mitochondria are also sites of NO synthesis (Fig. 5.6). Interestingly, oxygen availability determines the subcellular distribution of yeast flavohemoglobin (Cassanova et al. 2005). In normoxic *Saccharomyces cerevisiae* cells, flavohemoglobin is present in the cytosol and within the mitochondria, while its distribution is restricted to the proto-mitochondria in anoxic cells (Cassanova et al. 2005). This finding points out that a low oxygen level diminishes the cytoplasmic O_2-dependent (oxidative) NO synthesis, while mitochondria display an anaerobic NO forming activity.

Recent evidences suggest that the responsible enzyme for anaerobic NO production is identical with cytochrome-c oxidase (CcO) (E.C. 1.9.3.1.) in *Saccharomyces*

cerevisiae (Castello et al. 2006, 2008). CcO is the terminal oxidase (complex IV) in the respiratory electron transport chain and resides in the mitochondrial inner membrane system (Joseph-Horne et al. 2001). In hypoxic or anoxic cells, CcO catalyzes NO_2^-/NO reduction, allowing the mitochondrial electron transport chain to use NO_2^- as an alternative terminal electron acceptor instead of O_2. The utilization of NO_2^- in the lack of O_2 as a terminal electron acceptor is the so-called nitrite-respiration, which ensures anaerobic ATP synthesis and adaptation of cells to hypoxia (Figs. 5.6, 5.7). The reductive NO synthesis by CcO increases proportionally with NO_2^- levels and decreasing pH (a hallmark of hypoxia). The catalysis is optimal under hypoxic conditions but also detectable in anoxic cells (Castello et al. 2006, 2008). The activity of CcO is inhibited by yeast flavohemoglobin (Castello et al. 2006), which makes it possible for flavohemoglobins to participate actively in the control of NO synthesis. A recent finding , that low intensity broad-spectrum light also increases NO_2^-/NO reducing activity of CcO (Ball et al. 2011) suggests that light controls not only the oxidative, but also the reductive NO synthesis in fungi.

The CcO-catalyzed NO synthesis plays a role in hypoxic adaptation of yeast cells, since NO induces the transcription of genes which enable the survival of hypoxia (Castello et al. 2006). One target of NO is the hypoxia response-gene *CYC7*, which encodes *iso*-2-cytochrome-c, a protein required for oxidative phosphorylation (Kwast et al. 1999). NO also induces the transcription of *COX5b*, encoding a CcO subunit, which is a characteristic hypoxia gene in *Saccharomyces cerevisiae* (Kwast et al. 1999) and required for NO-synthesizing activity of CcO under hypoxic or anoxic conditions (Castello et al. 2008) (Fig. 5.7).

Under low oxygen levels the respiratory electron transport chain releases O_2^- radicals, which combine with NO giving $ONOO^-$(Poyton et al. 2009)). Under hypoxia $ONOO^-$ and NO become the major free radicals accumulated in the mitochondria which may account for the increased protein tyrosine nitration in hypoxic cells (Poyton et al. 2009)) (Fig. 5.6). It is suggested that $ONOO^-$ promotes tyrosine nitration of specific proteins involved in mitochondria-to-nucleus signal transmission and gene regulation in yeast (Castello et al. 2006). It has been shown that mitochondrial proteins are subjected to tyrosine nitration in *Saccharomyces cerevisiae*, including enzymes of the Krebs cycle (Bhattacharjee et al. 2009), which may lead to their inhibition. In oxidative stress, Krebs cycle inhibition is responsible for limiting the availability of NADH to the respiratory chain (Tretter and Adam-Vizi 2000) thus affecting mitochondrial damage. The activity of the Krebs cycle is also a determinant of fungal development (Khouw and McCurdy 1969) and senescence (Samokhvalov et al. 2004).

5.6.2 Nitrite Reductase of Denitrifying Fungi Also Produces NO

Nitrite respiration occurs in mmitochondria of various fungi, such as *Fusarium oxysporum*, *Aspergillus oryzae* and *Cylindrocarpon tonkinense* (Takaya and Shoun 2000; Kim et al. 2010; Nakanishi et al. 2010)). These species display denitrifying activity and reduce $NO_3^-$and NO_2^- to gaseous nitrogen forms (N_2 and N_2O) (Shoun et al.

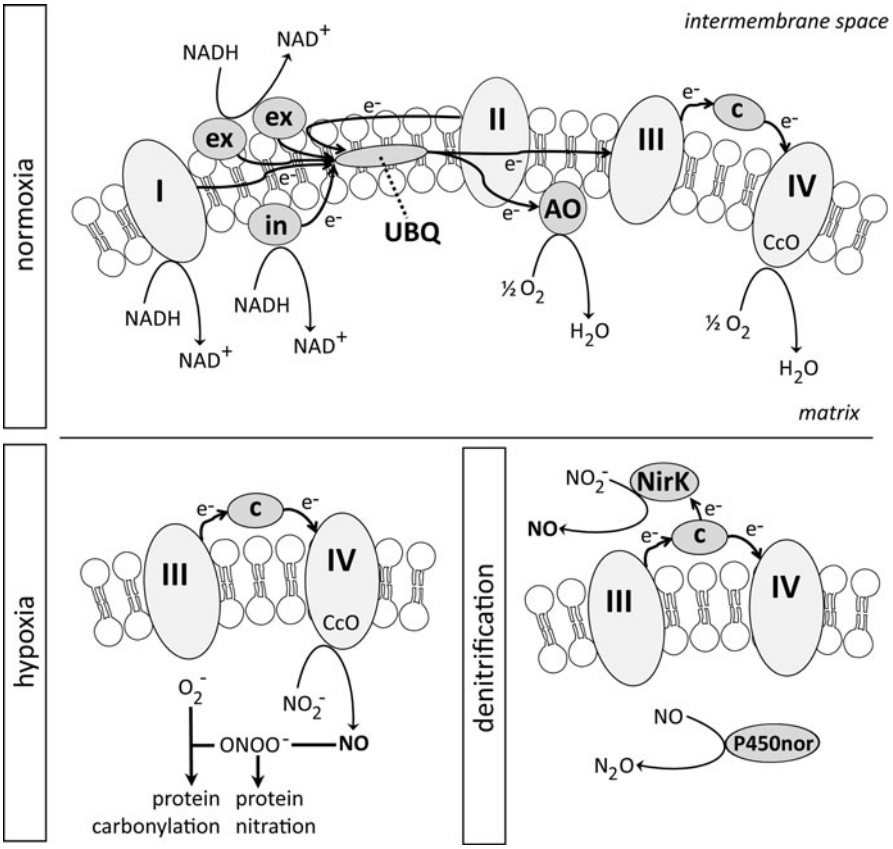

Fig. 5.7 Reductive NO generation in the inner mitochondrial membrane. *Top:* The respiratory electron transport chain under normoxia. *I–IV:* complex I–IV, *c:* peripheral cytochrome-c, *AO:* alternative oxidase, *UBQ:* ubiquinone/ubiquinol pool, *ex:* external NADH/ubiquinone oxidoreductase, in: internal NADH/ubiquinone oxidoreductase (Joseph-Horne et al. 2001); *Bottom:* NO-generation under hypoxia by the Complex IV (*CcO*); or by bacterial nitrite reductases (*NirK*). *P450nor:* p450 NO-oxidoreductase

1992; Nakanishi et al. 2010)). Flavohemoglobin gene transcription is upregulated in fungi assimilating NO_2^- (Kim et al. 2010; Schinko et al. 2010), which reflects increased mitochondrial NO generation under denitrifying conditions.

The fungal denitrifying system, coupled with the mitochondrial electron transport chain allows cells to respire anaerobically and produce ATP under hypoxia. A copper-containing dissimilatory nitrite reductase (NirK), which resides in the intermembrane space of the fungal mitochondria, catalyzes NO_2^-/NO reduction in a NADP dependent manner. The fungal NirK protein shows high sequence similarities to nitrite reductase of denitrifying bacteria (Nakanishi et al. 2010)). Transcription of mitochondrial-specific flavohemoglobin is upregulated in NirK overexpressing *Aspergillus* cells, confirming that increased NirK levels are accompanied with higher

mitochondrial NO production (Zhou et al. 2011). As a last step of denitrification, NO is further converted to N_2O by the NO-reductase enzyme cytochrome P450nor, which ensures that NO may not be accumulated in the mitochondria of denitrifying fungi (Takaya and Shoun 2000) (Fig. 5.7).

5.7 Chapter Summary

Oxidative NO synthesis	• Indirect evidences show that enzymatic conversion of L-arginine to L-citrulline and NO takes place in distinct fungus species and the responsible enzyme resides in the cytoplasm or anchors to endoplasmic membranes • Catalytic activity of the putative NOS is often stimulated by light, thus NO may be involved in light signaling during morphogenetic events
Reductive NO synthesis of the mitochondria	• Hypoxia favors the mitochondrial NO_2^-/NO reduction by CcO and NirK
NO-induced signal pathways	• In many fungi NO increases cGMP levels and a NO/cGMP pathway is possibly involved in morphogenesis and light sensing. In yeasts, the existence of a NO/cGMP pathway is debated and the transcriptional control of sporulation genes by NO has been suggested. S-nitrosylation of proteins also occurs in fungal cells and affects apoptosis and mitochondria-to-nucleus signaling

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Part V
Nitric Oxide Synthesis in Animal Cells

Chapter 6
Harboring of NOS to the Cell Membrane

6.1 Threads Linking NOS to the Cell Membrane: Acylation and Adaptor Proteins

The cell membrane establishes the physical barrier of the cell and separates the inner cell volume from the extracellular environment. This single phospholipid bilayer harbors membrane proteins, which serve a plethora of membrane functions and are essential for the life of a cell: transport mechanisms, signal transduction, cell adhesion, organization of the cytoskeleton, regulation of cell volume, and determination of the landscape of the cell surface. Cell membranes are major sites of NO synthesis in various cell types: the sarcolemma of skeletal muscle fibers (Brenman et al. 1995; Stamler and Meissner 2001; Kubisch et al. 2003) and cardiomyocytes (Gazzerro et al. 2011), the specialized membrane rafts of endothelial cells (Dessy et al. 2010; Fleming 2010; Michel and Vanhoutte 2010), and membranes of the postsynaptic densities (Yang et al. 1997) all bind distinct NOS isoforms.

Target molecules of NO (e.g. ion channels, adhesion molecules, guanylyl cyclase) are enriched in the proximity of membrane bound NOS, allowing NO to act locally (Fleming 2010; Qian et al. 2010). The cell membrane NOS pool thereby modulates membrane conductance (Stamler and Meissner 2001; Straub et al. 2011), cell–cell adhesion (Govers et al. 2002) or ensures intercellular communication between adjacent cells either by chemical neurotransmission or gap junctional coupling (Sladek et al. 1999; McKinnon et al. 2009; Tyml 2011). Membrane targeting of NOS not only ensures spatial organization of NO release but also determines its catalytic activity (Oess et al. 2006; Michel and Vanhoutte 2010).

Principally, there are two possible mechanisms, which allow NOS to bind cell membranes: fatty acylation of the NOS protein and association of NOS with distinct membrane-anchoring proteins (Fleming 2010). Fatty acylation is a covalent attachment of fatty acids to proteins (Resh 1999). This process takes place in the cytoplasm, the cytoplasmic surface of membranes or within secretory vesicles. Fatty acylation is a widespread posttranslational modification, which provides intermediate

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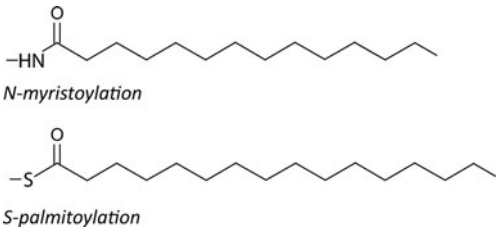

Fig. 6.1 Fatty acylated motifs enable membrane binding of NOS molecules. Covalent addition of tatty acids is a post-translational modification, which allows membrane targeting of proteins. N-myristoylation is the addition of myristate to an N-terminal glycine residue, while S-palmitoylation is the attachment of palmitate to a cysteine residue. These acylated sequences occur dually on eNOS and iNOS ensuring intracellular trafficking. Of note, nNOS lacks fatty acylated sequences, with the exception of the mitochondrial variant of nNOS (mtNOS)

hydrophobicity of distinct protein regions and thus allows their reversible membrane docking (Peitzsch and McLaughlin 1993; Resh 1999). Lipidation of proteins also affects their stability, half-life and intracellular traffic. For instance, eNOS and iNOS are modified by dual acylation and they bear N-terminal myristoylated and S-palmitoylated sequences (Busconi and Michel 1995; Liu et al. 1995, 1996) (Fig. 6.1). N-myristoylation is the covalent addition of myristate (myristic acid, $C_{14}H_{28}O_2$) to an N-terminal glycine residue through an amide linkage. S-palmitoylation is the covalent attachment of the long-chain fatty acid palmitate (palmitic acid, $C_{16}H_{32}O_2$) to a cysteine residue via a thioester bond (Resh 1999). Acylation of eNOS and iNOS is required for their proper membrane targeting and endoplasmic reticulum—Golgi-system—plasma membrane trafficking (Garcia-Cardena et al. 1996, 1997; Sowa et al. 1999; Navarro-Lerida et al. 2006; Villanueva and Giulivi 2010) (Fig. 6.2).

Palmitoylation of NOS is a highly dynamic process, illustrated by the short (45 min) palmitate turnover of eNOS (Liu et al. 1995). Protein lipidation may take place nonenzymatically in the presence of palmitoyl-CoA or can be catalyzed by protein acyltransferases (Nadolski and Linder 2007). In the case of eNOS, its palmitoylation is catalyzed by members of the DHHC acyl transferase family (DHHC2, DHHC3, DHHC7, DHHC8 and DHHC21) and it takes place at the cytoplasmic region of the Golgi-system (Fernandez-Hernando et al. 2006). The DHCC protein acyltransferases contain an aspartate-histidine-histidine-cysteine (DHHC) motif embedded in a cysteine-rich domain, which is essential for their activity (Fukata et al. 2006; Mitchell et al. 2006; Nadolski and Linder 2007). Extracellular signals may regulate the palmitoylation of proteins, thereby affecting their distribution in distinct subcellular membrane compartments (Nadolski and Linder 2007). To date, however, the possible signals regulating the palmitoylation state of NOS have not been understood, although dynamic changes in the acylation pattern may allow redistribution of the intracellular NOS pool under certain conditions (Chap. 12).

Myristoylation of eNOS is permanent and myristate turnover of eNOS is the same range as of eNOS protein itself (around 20 h) (Liu et al. 1995). Increased N-terminal myristoylation enhances the membrane association of NOS while deficient

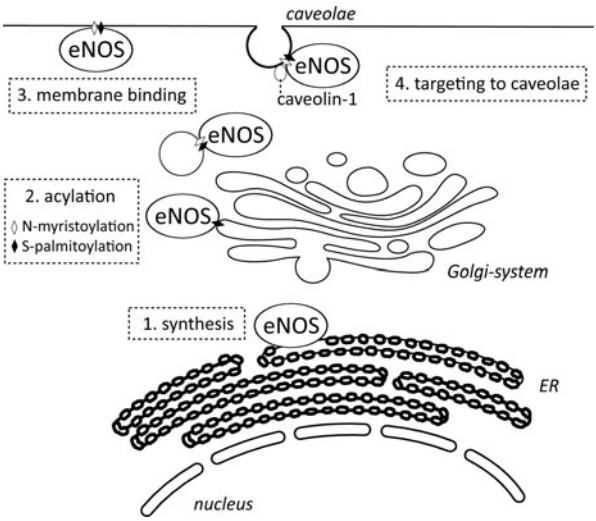

Fig. 6.2 Anterograde eNOS transport. Following the synthesis of eNOS in the endoplasmic reticulum (ER), the eNOS protein is allocated to the Golgi-system where it undergoes dual acylation. The acylated (*N-myristoylated* and *S-palmitoylated*) eNOS is targeted to the cell membrane by vesicular transport. Acylation allows eNOS to anchor the vesicular membranes. Following the fusion of the transport vesicle with the cell membrane, eNOS is being concentrated in specific membrane microdomains, the caveolae. Caveolar targeting is facilitated by caveolin-1, which in this process, functions as an eNOS-chaperone protein

palmitoylation has no effect on membrane binding although it decreases NOS activity (Liu et al. 1996; Navarro-Lerida et al. 2006). Myristoylation and palmitoylation together determine the proper intracellular sorting and activity of NOSs (Fig. 6.2).

Various adaptor proteins (Fig. 6.3) also ensure recruitment of NOSs to specific membrane compartments. For example, eNOS may be anchored to the plasma membrane by caveolin-1, caveolin-3 or various adhesion molecules (Bucci et al. 2000; Dessy et al. 2010; Gazzerro et al. 2011). These protein–protein interactions are established by consensus binding sequences between eNOS and the corresponding adaptor proteins (Villanueva and Giulivi 2010). Membrane binding of eNOS and iNOS allows their interaction with various regulatory proteins, which modulate NO synthesis. Acylation also increases the effective binding of NOSs to their membrane-associated partners and this is why the deficient NOS palmitoylation may reduce NO synthesis (Liu et al. 1996).

Although we only have a limited insight into the evolution of NOS compartmentalization to date, it seems that acylation of NOS may be an ancient mechanism which ensures its reversible membrane-association. In snail neurons a putative partner of NOS (the so-called *Helix*NOS; a GTPase-like protein) bears myristoylated sequences (Huang et al. 1997), which may determine the distribution pattern and activity of NOS (Rőszer et al. 2010).

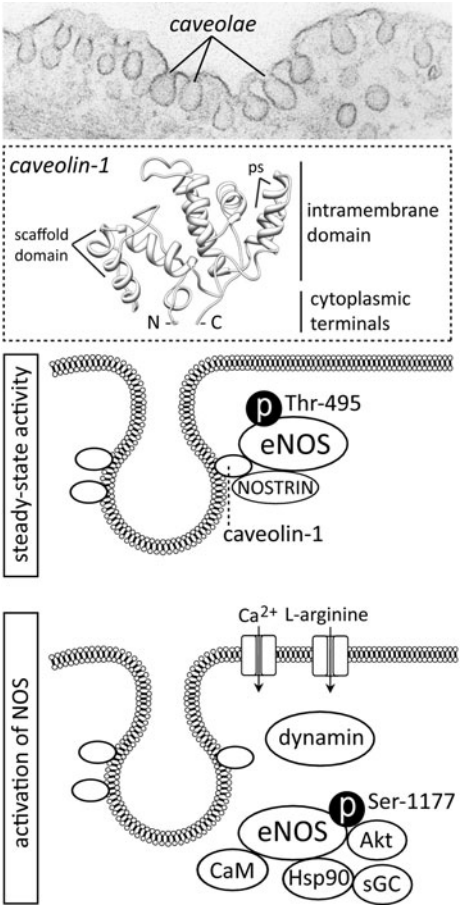

Fig. 6.3 Association of eNOS with caveolae of the endothelial cells. The caveolar membrane concentrates eNOS molecules by eNOS/caveolin-1 binding. *Top:* TEM image showing cell membrane caveolae. Reprinted with permission (Sandvig et al. 2008). *Middle:* Caveolin-1 is a structural hairpin-loop protein of the caveolae. Its hydrophobic domain is embedded into the cytoplasmic surface of the cell membrane, and its N- and C-terminal ends both reach the cytosol, where they establish bindings with other proteins, including eNOS. It also bears palmitoylated sequences (ps). *Bottom:* The eNOS/caveolin-1 complex is stabilized by NOSTRIN and Thr-495 phosphorylation of eNOS. This membrane-associated eNOS shows steady-state NO synthesis. When intracellular Ca^{2+} is increased, CaM displaces caveolin-1 and eNOS dissociates from the cell membrane. At the same time, eNOS undergoes Ser-1177 phosphorylation and forms complexes with its downstream target sGC. Hsp90 and dynamin facilitates the membrane release and the consequent association of eNOS with Akt (the protein kinase responsible for Ser-1177 phosphorylation) and soluble guanylyl cyclase (sGC). As a result, NO synthesis by eNOS is increased

Table 6.1 Protein–protein interactions of nNOS

nNOS-interacting protein	Localization	Function
PSD-95	Postsynaptic membrane	Anchors nNOS to the cell membrane and to the NMDA-R (Chaudhury et al. 2009)
CAMK-IIα	Associated with the nNOS/PSD95/NMDA-R complex	Ser-847 phosphorylation, diminishes nNOS activity (Yan et al. 2004)
6-phosphofructokinase, muscle type (PFK-M)	Occurs at synaptic membranes	May protect neurons from NO-induced neurotoxicity (Firestein and Bredt 1999)
CAPON	Binds to nNOS at the postsynaptic membranes	Inhibits the coupling between nNOS and PSD95 (Jaffrey et al. 1998)
α1-syntrophin	Member of the DGC, anchors nNOS to the sarcolemma	Required for controlled nNOS activation (Lai et al. 2009)
PIN	Binds directly to nNOS and colocalizes with nNOS in certain brain regions	May be involved in axonal transport of nNOS (Jaffrey and Snyder 1996)
Dexras1	Binds to CAPON	S-nitrosylated by NO and may be a downstream effector of nNOS (Nguyen and Watts 2005; Cheah et al. 2006)
NIDD (nNOS-interacting DHHC domain-containing protein with dendritic mRNA)	nNOS binding through PDZ domain	Increases nNOS enzyme activity by targeting the nNOS to the synaptic plasma membrane (Saitoh et al. 2004)

However, nNOS lacks acylated motifs and its membrane association depends on protein–protein interactions (Table 6.1). Its unique N-terminal PDZ (PSD-95/discs-large/ZO-1) domain, which is absent from the other NOS isoforms, can bind nNOS to various adaptor molecules (e.g. α1-syntrophin) (Lai et al. 2009). These protein–protein interactions ensure membrane targeting and modulate the activity of nNOS. Comparative studies indicate the presence of a PDZ domain in *Trichoplax adhaerens* NOSA, molluscan, echinoderm and ascidian NOSs (*Lehmannia valentiana* NOS [molluscan], *Strongylocentrotus purpuratus* NOSA [echinoderm], *Ciona intestinalis* NOS [ascidian]), while arthropod-type NOS molecules lack PDZ domains (Andreakis et al. 2011).

Membrane-bound NOS molecules are often associated with other proteins (e.g. protein kinases, heat shock protein 90), which regulate their catalytic activity through site-specific phosphorylation (Table 6.2) or steric modulation (Brouet et al. 2001; Dessy et al. 2010; Fleming 2010; Michel and Vanhoutte 2010). The following examples show the mechanisms behind membrane association of distinct NOS isoforms.

Table 6.2 Phosphorylation sites of NOS isoforms and their effects on NOS activity

	Phosphorylation site	Protein kinase	Upstream signal	Effect on eNOS
eNOS	Ser-615	PKA, Akt	VEGF, hypoxia, adiponectin	Increases CaM sensitivity and eNOS assembly to signalosomes (Fleming 2010)
	Ser-633	PKA	Fluid shear stress, VEGF, bradykinin, ATP	Enhances eNOS stimulation (Fleming 2010; Xiao et al. 2011)
	Ser-114		Constitutively phosphorylated	Negative regulator of eNOS activity (Brouet et al. 2001; Fleming 2010)
	Ser-847	CAM-KIIα CaM-KIα CaM-KIV	Okadaic acid, glutamate	Decreases activity, possibly suppresses CaM binding. Antagonist of protein phosphatase 2A, which restores activity by Ser-847 dephosphorylation. (Komeima et al. 2000; Rameau et al. 2004)
	Ser-1177	Akt, PKA, AMPK, CaM-KII	VEGF, insulin, estrogen, fluid shear stress, bradykinin, increased Ca^{2+}-levels	Increases NO synthesis and allows eNOS activation at lower Ca^{2+}-concentrations (Harris et al. 2001) (Fulton et al. 2002; Fleming 2010)
	Thr-495	PKC	Constitutively phosphorylated, histamine, bradykinin	Inhibits CaM binding (Fleming et al. 2001; Fleming 2010)
	Tyr-81	v-Src	Oxidative stress, acetylcholine, angiopoetin, ATP, estrogen, bradykinin, VEGF	Allows eNOS activation at lower Ca^{2+}-concentrations (Fleming 2010)
	Tyr-657	PYK2	Fluid shear stress, insulin, angiotensin II	Inhibits activity (Loot et al. 2009)
nNOS	Ser-1412	Akt	Phosphatidylinositol-3 kinase, glutamate	In steady-state speeds electron transfer out of the reductase domain, fosters heme reduction and reduces NO synthesis. Following NMDA-R activation increases activity by lowering its Ca^{2+}-dependence (Rameau et al. 2004; Rameau et al. 2007)
iNOS	Tyr-1055	Src		Stabilizes iNOS (Tyryshkin et al. 2010)

Akt Akt protein kinase, *AMPK* AMP activated protein kinase, *CaM* Ca^{2+}/calmodulin, *CAMK* Ca^{2+}/calmodulin dependent protein kinase, *NMDA-R* NMDA-receptor, *PKA* protein kinase A, *PKC* protein kinase C, *PYK2* nonreceptor tyrosine kinase, *v-Src* viral sarcoma protein kinase, *VEGF* vascular endothelial growth factor

6.2 Association of eNOS with Caveolae of the Cell Membrane

In endothelial cells, eNOS is associated with the caveolae (caveolae intracellularis, intracellular caveoles) of the cell membrane (Atochin and Huang 2010; Fleming 2010) (Figs. 6.2, 6.3). Caveolae are specialized membrane rafts formed by cave-like 50–100 mm invaginations of the plasma membrane (Anderson 1998). These membrane regions are enriched in sphingolipids and cholesterol and are involved in endocytosis and formation of clathrin-coated vesicles (Li et al. 2005). Caveolae contain various ion channels, G-proteins, tyrosine-kinase- and G-protein-linked membrane receptors, therefore they integrate distinct signal transduction pathways, and are considered microdomains or "signalosomes" (Atochin and Huang 2010).

The structural proteins of caveolae are oligomerized caveolin-1, -2, and -3; hairpin-loop proteins with a membrane-bound hydrophobic domain and cytoplasmic N- and C-terminals (Williams and Lisanti 2004) (Fig. 6.3). Caveolin-1 and -3 share more than 85% sequence similarity and both of them are capable of binding eNOS (Feron et al. 1996; Garcia-Cardena et al. 1997; Bucci et al. 2000; Drab et al. 2001; Felley-Bosco et al. 2002; Wang et al. 2009; Dessy et al. 2010; Fernandez-Hernando et al. 2010). In endothelial cell caveolae, the most important scaffolding protein for eNOS is caveolin-1, which binds to its Phe-350–Try-358 consensus sequence and anchors it to the cell membrane (Fleming 2010). Caveolin-3 plays a similar role in ciliated epithelial cells (Krasteva et al. 2007), binding eNOS to the apical cell membrane (Chap. 9). In cardiomyocytes, caveolin-3 anchors eNOS to caveolae of the sarcolemma (Gazzerro et al. 2011). In skeletal muscle fibers, caveolin-3 has no mechanical anchoring function; however, it binds to nNOS at the sarcolemma and inhibits NO synthesis (Kubisch et al. 2003).

The association of eNOS with caveolin-1 inhibits NO synthesis, possibly by antagonizing the binding of calcium-calmodulin (CaM) (Garcia-Cardena et al. 1997). The caveolin-binding motif of eNOS is located adjacent to its CaM-, L-arginine-binding and heme domains (Garcia-Cardena et al. 1997). Accordingly, coexpression of eNOS and caveolin-1 in COS-7 (transformed monkey kidney fibroblast) cells leads to inhibition of eNOS catalytic activity (Michel et al. 1997). Caveolin-1 overexpression also reduces endothelial NO synthesis (Fernandez-Hernando et al. 2010). Inhibition of eNOS/caveolin-1 binding impairs vasodilator responses *in vivo* and mice lacking caveolin-1 show persistent eNOS activation, exaggerated vascular response to acetylcholine and higher endothelial cGMP levels than wild-type mice (Bucci et al. 2000; Drab et al. 2001; Zhao and Malik 2009). The association of eNOS with the cell membrane through caveolin-1 therefore mitigates NO synthesis, while dissociation of eNOS from the cell membrane increases its catalytic activity (Brouet et al. 2001; Fleming 2010) (Fig. 6.3).

In addition to the steric inhibition of eNOS activity, caveolin-1 facilitates the interaction of eNOS with various modulatory proteins, which are required for activation of NO synthesis (Feron et al. 1996; Feron and Balligand 2006; Atochin and Huang 2010). The "resting" eNOS is attached to the cell membrane through caveolin-1 which favors threonine phosphorylation (Thr-495) of eNOS and compromises its association with CaM (Fleming et al. 2001; Fleming 2010) (Fig. 6.3). The

activation and dimerization of eNOS is initiated by its dissociation from caveolin-1 allowing successive association with CaM. This early phase of activation is triggered by an increase of intracellular Ca^{2+}-levels (Marletta 1994) which allow CaM to displace caveolin-1 and reverse its inhibitory interaction with eNOS (Fleming 2010). Importantly, CaM also activates acyl-protein thioesterase 1 (APT1) which catalyzes depalmitoylation of eNOS (Yeh et al. 1999). Reduced acylation of eNOS favors its dissociation from the caveolae, consequently facilitating the assembly of active eNOS/CaM complexes. The transition from this early Ca^{2+}-dependent activation to the late phosphorylation dependent activation requires the binding of heat shock protein 90 (Hsp90) to eNOS (Brouet et al. 2001). The association of Hsp90 with eNOS recruits protein kinases (e.g. Akt) leading to serine phosphorylation (e.g. on Ser-1177 in human, Ser-1176 in mouse and Ser-1179 in bovine) and sustained eNOS activity (Atochin and Huang 2010; Dessy et al. 2010; Fleming 2010) (Table 6.2). Phosphorylation at Ser-1177 results in a negative charge, favoring electron transfer through the reductase domain and diminishing CaM dissociation, thus increasing NO synthesis. Substitution of Ser-1177 to aspartate mimics the negative charge of the phosphate group—due to the negatively charged carboxyl group of the aspartate side chain—thereby increasing NOS activity. Alanine substitution of Ser-1177, however, cannot be phosphorylated—since it lacks a hydroxyl group and cannot be a substrate of Akt—and eNOS cannot be activated (Atochin and Huang 2010).

Under physiological conditions of shear stress, vascular endothelial growth factor, insulin and estrogens increase Ser-1177 phosphorylation of eNOS by Akt and increase NO synthesis (Table 6.2). Reduced Ser-1177 phosphorylation impairs NO generation leading to endothelial dysfunction e.g. in diabetes, hypertension, obesity and in response to the inflammatory mediators TNFα and IL-6. Additional serine, threonine and tyrosine phosphorylation of eNOS further modulates its activity (Komeima et al. 2000; Adak et al. 2001; Fleming 2010; Xiao et al. 2011) (Table 6.2). The phosphatases that regulate the dephosphorylation of eNOS have not been identified (Atochin and Huang 2010), although protein phosphatase 2A has been implicated in Ser-847 dephosphorylation of nNOS (Komeima et al. 2000).

Other proteins of the NO signaling pathway are also associated with the caveolae: arginine succinate synthase and lyase, arginine-channels, Ca^{2+}-channels, bradykinin and acetylcholine receptors are present in the vicinity of membrane-anchored eNOS (Fleming 2010) (Fig. 6.3). These proteins ensure substrate availability and activation of eNOS. Association of Hsp90 with the caveolae facilitates not only eNOS activation, but also its folding and interaction with various upstream eNOS activators and the NO target soluble guanylyl cyclase (Fleming 2010) (Fig. 6.3).

6.3 Association of eNOS with Cell–Cell Junctions

6.3.1 Endothelial Cell–Cell Adhesions Bind eNOS: More than Mechanical Anchoring

Confluent monolayers of cultured endothelial cells show increased eNOS activity compared with subconfluent cells, although the expression level of eNOS is sus-

tained in confluent or subconfluent endothelia (Govers et al. 2002). This correlation between cell confluence and eNOS activity raises the interesting possibility that the establishment of endothelial lateral border contacts increases eNOS activation. Supporting this idea, the enrichment of eNOS has been detected in cell–cell adhesions of neighboring endothelial cells (Andries et al. 1998) colocalizing with PECAM-1 (platelet-endothelial adhesion molecule 1 or CD31) (Govers et al. 2002).

PECAM-1 is a type I transmembrane glycoprotein and belongs to the immunoglobulin superfamily of cell adhesion molecules (Dejana 2004). It has six extracellular immunoglobulin-like homology (Ig) domains: two of them bind extracellular matrix components and leukocyte cell surface molecules, while the N-terminal Ig domain establishes homophilic PECAM-1/PECAM-1 interactions. Its transmembrane domain bears a palmitoylated sequence (the Cys-595 residue), which allows PECAM-1 enrichment in membrane microdomains (Fig. 6.4). The cytoplasmic tail contains residues for palmitoylation and phosphorylation and harbors various cytosolic signal molecules (Privratsky et al. 2010) (Fig. 6.4). In endothelial cells, PECAM-1 is the major constituent of intercellular junctions. It ensures mechanical stabilization of cell contacts (Dejana 2004) and impacts the cell-matrix interactions (Crockett et al. 2010; Park et al. 2010) required for capillary morphogenesis (Dimaio et al. 2008; Park et al. 2010) and leukocyte transmigration through the endothelial layer (Ensminger et al. 2002; Kamei and Carman 2010; Privratsky et al. 2010).

Associating eNOS with the endothelial cell–cell contacts is a consequence of the physical interaction between PECAM-1 and eNOS (Fig. 6.4), which is stabilized by the binding of eNOS to the cytoskeleton (Govers et al. 2002). The regulatory role of PECAM-1/eNOS association on NO synthesis has been implicated in response to endothelial shear stress (Fleming 2010). Enhanced association of PECAM-1 and eNOS occurs in the secondary (so-called Ca^{2+}-independent) phase of shear stress (Fleming et al. 2005). However, the Ca^{2+}-dependent phase of fluid shear stress induces dissociation of eNOS from PECAM-1 and increases NO synthesis (Dusserre et al. 2004). Accordingly, in PECAM-1 deficient cells, eNOS is mislocated from the cell contacts and the endothelial cells display increased basal eNOS activity and NO production (McCormick et al. 2011). Lacking PECAM-1, however, other studies show attenuated eNOS phosphorylation and NO synthesis and reduced expression of eNOS (Dimaio et al. 2008; Fleming 2010). These conflicting findings consistently show that PECAM-1 enables membrane harboring of eNOS, thereby modulating its activity and couples shear stress to changes in NO synthesis (Bagi et al. 2005).

Moreover, a recent study suggests that PECAM-1 is also involved in the transcriptional control of NOSTRIN (eNOS traffic inducer), a recently identified eNOS-associated human protein (Zimmermann et al. 2002; Choi et al. 2005; Icking et al. 2005; McCormick et al. 2011) (Fig. 6.5). NOSTRIN mRNA is abundant in highly vascularized tissues such as the placenta, kidneys, lung, and heart and the 58 kDa NOSTRIN protein is expressed in vascular endothelial cells (Zimmermann et al. 2002; Xiang et al. 2005). Its murine orthologue has also been identified (Choi et al. 2005). NOSTRIN belongs to the PCH (Pombe Cdc15 homology) family of

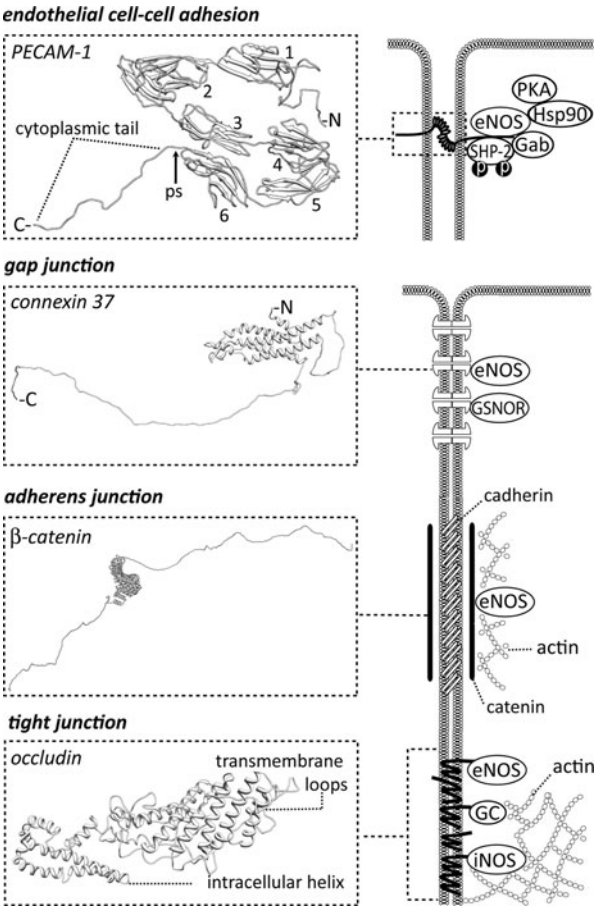

Fig. 6.4 Membrane-bound NOS at various intercellular junctions. At endothelial cell–cell adhesions, the cytoplasmic tails of PECAM-1 bind eNOS while Ig-like domains (1–6) serve heterologous or homologous PCEAM-1/PECAM-1 associations. The palmitoylated sequence (ps) of PECAM-1 facilitates its membrane binding. Adaptor proteins (e.g. SHP-2, Gab) stabilize PECAM-1/eNOS binding. PKA and Hsp90 are involved in the activation of eNOS. At gap junctions, eNOS is possibly anchored by caveolin-1. S-nitrosylation of gap junction proteins (*connexins*) is determined by eNOS in concert with GSNOR. Catenin and the cytoskeletal actin filaments bind eNOS to adherens junctions. NO affects assembly of these junctional complexes. Tight junctions anchor eNOS, iNOS and guanylyl cyclase (GC), possibly with the help of the actin cytoskeleton. Occludin, the main structural component of tight junctions is under the transcriptional control of NO

proteins (Icking et al. 2006). This protein family involves phospholipid-binding proteins affecting membrane dynamics and cytoskeletal assembly (Aspenstrom et al. 2006). Through its FCH (Fes/CIP homology) region, NOSTRIN is associated with the cell membrane, whereas its Cdc15 (cell division control protein 15) domain allows binding to diverse subcellular compartments (Icking et al. 2006). The SH3

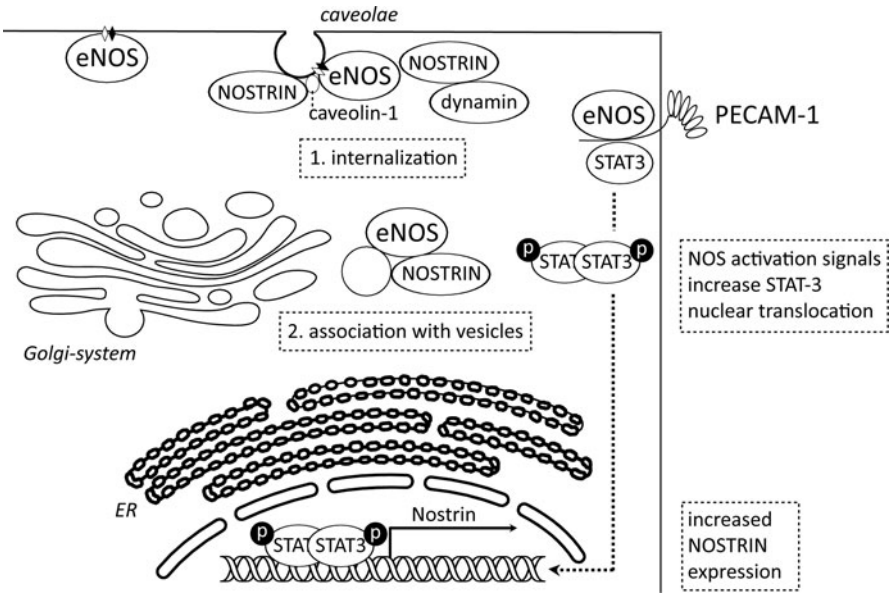

Fig. 6.5 Internalization of eNOS: the role of NOSTRIN. Endocytosis-like internalization of activated eNOS is facilitated by dynamin (which undergoes S-nitrosylation) and NOSTRIN. Interestingly, PECAM-1 at the endothelial cell–cell junctions harbors STAT3, which upon eNOS activation is internalized to the nucleus where it activates the transcription of N. Increased NOSTRIN levels may augment this activation-dependent eNOS internalization

(SRC homology 3) domain of NOSTRIN binds to eNOS and anchors it to the plasma membrane (Zimmermann et al. 2002).

NOSTRIN overexpression induces a profound redistribution of eNOS from the plasma membrane to intracellular vesicle-like structures and inhibits NO release (Zimmermann et al. 2002) (Fig. 6.5). The central domain of NOSTRIN directly binds to caveolin-1 allowing the assembly of NOSTRIN/caveolin-1/eNOS complexes at the endothelial plasma membrane (Schilling et al. 2006). Confluence and thrombin activation activates the formation of NOSTRIN/caveolin-1/eNOS complexes (Schilling et al. 2006).

NOSTRIN also functions as an adaptor to recruit dynamin-2, which is essential for endocytosis-like internalization of the NOSTRIN/eNOS complexes and also dissociation of eNOS from the cell membrane (Schilling et al. 2006; Fleming 2010) (Fig. 6.5). Endocytotic fission from the cell membrane depends on GTPase dynamin proteins, such as dynamin-2, a multidomain protein which catalyzes the formation of endocytotic vesicles and their internalization into the cell (Kasai et al. 1999). Increased NO production by eNOS enhances the dynamin-2 mediated endocytosis, possibly through the S-nitrosylation of dynamin-2 (Wang et al. 2011). This mechanism is implied in the NOS-associated endosome functions (engulfment pathogens) in response to bacterial stimuli (Wang et al. 2011) (Chap. 8). A shortened variant

of NOSTRIN, the so-called NOSTRIN-β mainly localizes to the cell nucleus suggesting a possible function in transcriptional control (Wiesenthal et al. 2009). It has been shown that the nuclear NOSTRIN-β may negatively regulate transcription of the NOSTRIN gene (Wiesenthal et al. 2009).

The cytoplasmic tail of PECAM-1 serves as a scaffolding partner for STAT3 (signal transducers and activators of transcription 3) (McCormick et al. 2011). Following activation, STAT3 translocates to the nucleus and increases NOSTRIN expression, which helps eNOS targeting to the caveolae (Fig. 6.5). Lack of PECAM-1 decreases NOSTRIN expression, which may also account for mislocalization and increased basal activity of eNOS (McCormick et al. 2011). Consequently, PECAM-1 affects eNOS compartmentalization and activity by an additional indirect mechanism through the regulation of NOSTRIN transcription (McCormick et al. 2011). Interestingly, pharmacological inhibition of eNOS reduces endothelial PECAM-1 expression (Hebeda et al. 2008), although its implication in NOSTRIN gene regulation is yet unexplored.

Assembly of the eNOS/PECAM-1 complex is more than a mechanical link between eNOS and the cell membrane. PECAM-1 is required for activation dependent NO synthesis and also affects the expression level of NOSTRIN, thus secondarily, it may change the dynamics of NOSTRIN/caveolin-1/eNOS complexes and the subcellular redistribution of eNOS.

6.3.2 Association of NOS with Gap Junctions: Dynamic S-nitrosylation/denitrosylation

Recent findings show that gap junction opening is also modulated by NO synthesis (McKinnon et al. 2009; Jia et al. 2011; Straub et al. 2011). Colocalization and coimmunoprecipitation of eNOS and the gap junction protein connexin 37, have been shown in mouse and human endothelial cells (Pfenniger et al. 2010). Heterocellular gap junctions interconnecting endothelia and vascular smooth muscle cells are also associated with eNOS in the mouse aortic wall (Straub et al. 2011). Moreover, caveolins are also present at gap junctions which facilitate connexin 43 trafficking to the gap junctions and also provide an optimal binding site and microsignaling domain for eNOS (Rath et al. 2009; Liu et al. 2010) (Fig. 6.4).

These findings suggest a possible regulatory role of locally produced NO in gap junction assembly or function. Supporting this notion, electric coupling is reduced by exogenous NO administration in cultured rat and mouse microvascular endothelial cells (McKinnon et al. 2009; Tyml 2011). Similarly, in adipose tissue-derived mesenchymal stem cells, the inhibition or downregulation of nNOS improves gap junction conductance and synchronized intracellular Ca^{2+}-oscillations between individual cells (Sauer et al. 2011). The NO-induced reduction of electrical coupling between microvascular endothelial cells depends on connexin 37, suggesting that connexins are candidate NO-target proteins in the tight junctions

(McKinnon et al. 2009; Tyml 2011). Accordingly, a recent study shows a mechanical link between local NO synthesis and intercellular conductance, since dynamic S-nitrosylation/denitrosylation of connexin 43 regulates gap junction permeability (Straub et al. 2011). Compartmentalized S-nitrosoglutathione reductase (GSNOR) regulates the levels of S-nitrosylated proteins at the gap junctions, thus the balance between NOS and GSNOR modulates the exchange of signaling molecules between interconnected cells (Straub et al. 2011) (Fig. 6.4). Interestingly, the lack of connexin 37 or connexin 40 decreases eNOS expression and NO release in mice (Alonso et al. 2010). In turn, ablation of NOS impairs the establishment of gap junction between astrocytes (Bechade et al. 2011). Inhibition of eNOS evokes arterial hypertension, which is accompanied with reduced expression of connexin 43 (Haefliger et al. 1999). Reduced connexin 43 expression impairs electrical coupling of vascular cells and cardiomyocytes (Radosinska et al. 2011). In the myometrium, NO reduces connexin 43 expression (Sladek et al. 1999) and in spontaneously hypertensive rats, increased myocardial NOS activity mitigates connexin 43 phosphorylation (Radosinska et al. 2011).

To date, the possible role of NOS in gap junction dynamics has not been explored outside the vertebrates. However, association of stress-induced NOS expression and gap junction plasticity has been shown in mussel (*Crenomytilus grayanus*) neurons (Kotsyuba and Vaschenko 2010). In snail ganglia, the involvement of NO has been implicated in electric coupling of neurons (Ermentrout et al. 2004), although the direct colocalization of NOS with gap junction proteins has not been studied.

6.3.3 Tight Junctions and Adherens Junctions

In Sertoli cells of the testicular seminiferous epithelium, iNOS and eNOS are structurally associated with tight junctions[1] (Lee and Cheng 2003; Dejana 2004). A putative binding site of NOSs is occludin, a structural protein of the tight junctions (Furuse et al. 1998). Cytoskeletal components such as actin, vimentin and α-tubulin may also bind NOS molecules to tight junctions (Fig. 6.4). Moreover, soluble guanylyl cyclase is also associated with tight junctions in the seminiferous epithelium, indicating a local production of NO and cGMP (Lee and Cheng 2003).

Inhibition of NOS or guanylyl cyclase activity facilitates the assembly and maintenance of tight junctions between Sertoli cells *in vitro*. Reduced NO synthesis is accompanied by increased amounts of occludin in the Sertoli cells, suggesting that the NO/cGMP pathway reduces the steady-state expression of this tight junction protein. Increased NO synthesis (e.g. due to cytokine activation of iNOS) impairs the integrity of tight junctions and compromises the barrier function of the seminiferous epithelia (Lee and Cheng 2003). Increased NO synthesis within the testis

[1] Various cells of the testis express eNOS, iNOS and nNOS, and one testis-specific truncated nNOS (TnNOS). To date only eNOS and iNOS have been implicated in the control of tight junction regulation.

negatively affects gametogenesis and male fertility (Balercia et al. 2004; Buldreghini et al. 2010), at least partially, as a consequence of impaired junctional dynamics of the Sertoli cells.

Increased NO levels compromise the expression of tight junction structural components in other epithelial cell types also. For instance, NO may be responsible for destruction of tight junctions in the intestinal mucosal epithelia in ischemia-reperfusion injury (Takizawa et al. 2011). Expression of claudins and P-glycoprotein (permeability glycoprotein, also known as ABCB1) is reduced in ischemia-reperfusion, which may be reversed by the pharmacological inhibition of iNOS. In brain capillary endothelia, increased NO synthesis reduces occludin expression and impairs tight junctions (Yamagata et al. 2004) which may therefore account for hypoxia-induced endothelial permeability changes and may compromise epithelial barrier function.

Activation of eNOS may also increase vascular permeability by altering endothelial adherens junctions (Thibeault et al. 2010) (Fig. 6.4). The candidate NO-target protein is β-catenin in the adherens junctions. Increased NO synthesis (e.g. vascular endothelial growth factor activates eNOS) leads to S-nitrosylation of β-catenin. S-nitrosylated β-catenin dissociates from vascular endothelial-cadherin (VE-cadherin) and results in the disassembly of adherens junction complexes (Thibeault et al. 2010) (Fig. 6.4).

6.4 Sarcolemmal and Sarcoplasmic Reticulum Association of NOS

The expression of nNOS has been detected in various skeletal muscles including human gastrocnemius, omohyoideus, quadriceps, sternocleidomastoideus, urethral sphincter and vastus lateralis muscle; rat and mouse diaphragm, deltoideus, extensor digitorum longus, gastrocnemius, levator labii, soleus, quadriceps and tibialis anterior muscle; and in the skeletal muscles in a variety of other species including gerbils, guinea pigs, hamsters, turtles, chicken, pigeons, and goldfish (Stamler and Meissner 2001). In mice, a skeletal muscle-specific splice variant of nNOS (nNOSμ) has also been described (Heydemann and McNally 2009; Percival et al. 2010). Under specific conditions, smooth muscle cells also express iNOS, which is confined to the cytoplasm (Hung et al. 1995; Ravalli et al. 1998). In non-vertebrate muscle cells NO synthesis has also been shown (Martinez 1995) and NO affects ion currents (Hermann and Erxleben 2001) and contractility of muscles (Rőszer et al. 2006), although the subcellular compartmentalization of the responsible enzyme is yet unknown.

In adult skeletal muscle, nNOS (including nNOSμ) is largely targeted to the sarcolemma of fast twitch fibers (Percival et al. 2010). Although nNOS lacks acylated sequences, its unique N-terminal PDZ domain can bind to various adaptor molecules (Table 6.1). These protein–protein interactions ensure membrane targeting of nNOS (Fig. 6.6). In the sarcolemma, nNOS binds to α1-syntrophin, a member of the dystrophin-glycoprotein complex (DGC) (Brennan et al. 2004; Lai et al. 2009;

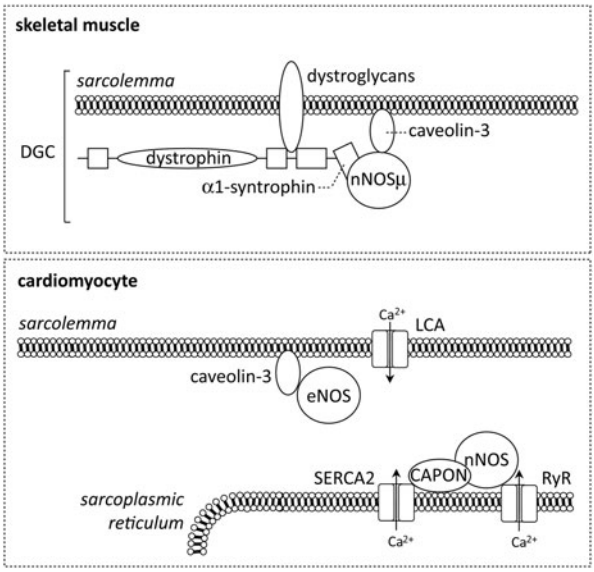

Fig. 6.6 The sarcolemma and the sarcoplasmic reticulum bind NOS. The sarcolemma of the skeletal muscle fibers binds nNOS (muscle-specific isoform, nNOSμ) with α1 syntrophin, a component of the DGC. Caveolin-3 has an inhibitory effect on NO synthesis, but it is not required for mechanical stabilization of nNOS binding. Cardiomyocyte sarcolemma (mainly at caveolar membrane invaginations) binds eNOS with the help of caveolin-3. The locally produced NO inhibits L-type Ca^{2+} channels (LCA). The sarcoplasmic reticulum harbors nNOS and the NO target ion channels are SERCA2 (in *cardiomyocytes*) and RyR (both in *skeletal* and *cardiac muscles*). RyR may bind nNOS directly, while SERCA2/nNOS association may be facilitated by CAPON. The NO released at the sarcoplasmic reticulum leads to the opening of the Ca^{2+} channels

Suzuki et al. 2010). This association is required for the sustained NOS activity and the compartmentalization of NO liberation (Lapidos et al. 2004). Caveolin-3, another component of the DGC, also associates with nNOS (Kubisch et al. 2003; Lapidos et al. 2004; Gazzerro et al. 2011) and displaces CaM by analogy to the effect of caveolin-1 on eNOS (Oess et al. 2006) (Fig. 6.6).

Sarcolemmal NO synthesis is required for the proper blood flow in arteries of the contracting muscles (Heydemann and McNally 2009). In working muscles the adrenergic activation leads to vasoconstriction and hypoxia (the so-called functional hyperemia), which is antagonized by the vasodilator effect of the NO emitted from the sarcolemmal nNOS pool (Grange et al. 2001). Interestingly, another nNOS splice variant (nNOSβ) is targeted to the Golgi-complex in the muscle fibers and is not required for this vasodilator function (Percival et al. 2010). This difference between the intracellular distribution of two variants of the same nNOS isoform highlights the compartment-specific effects of NO synthesis.

In cardiomyocytes, caveolin-3 ensures sarcolemmal compartmentalization of eNOS which is enriched in the caveolae of the T-tubules (invaginations of the sarcolemma) (Feron et al. 1996). Activators of NO synthesis such as muscarinic

acetylcholine, β-adrenergic receptors and NO-target L-type Ca^{2+} channels are all concentrated in the sarcolemma (Lapidos et al. 2004), in close proximity to the membrane-bound eNOS (Fig. 6.6). Caveolin-3 may be a molecular chaperone for eNOS, which anchors it to the sarcolemma and temporarily inactivates NO synthesis (Garcia-Cardena et al. 1997). Interestingly, although caveolin-3 also inhibits nNOS and iNOS activity (Garcia-Cardena et al. 1997) and both nNOS and iNOS are associated with caveolin-3 in the sarcolemma of skeletal muscles (Gossrau 1998; Gath et al. 1999), its functional implication is unclear (Stamler and Meissner 2001).

The sarcoplasmic reticulum also harbors nNOS. In this intracellular membrane compartment, the sarcoplasmic Ca^{2+}-channel ryanodine receptor (RyR) (Wang et al. 2010) and the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) (Oess et al. 2006) are the main targets of local NO synthesis. Direct association of nNOS with RyR has been shown although the mechanism of their binding is unknown. The RyR subunit 1 contains several cysteine residues, which undergo S-nitrosylation, leading to increased Ca^{2+} sensitivity of RyR. Since RyR is a Ca^{2+}-dependent Ca^{2+}-channel (its opening is triggered by increasing cytosolic Ca^{2+}), S-nitrosylation increases Ca^{2+} release through RyR at lower cytoplasmic Ca^{2+} levels. The most important NO-target is Cys-3635 (located in the CaM-binding domain of RyR). The S-nitrosylation of Cys-3635 is required for increased binding of CaM and RyR opening (Sun et al. 2001). In the absence of nNOS or in the presence of pharmacological NOS-inhibitors, the S-nitrosylation of RyR-2 is reduced and the myocytes show impaired Ca^{2+} release from the sarcoplasmic reticulum (Wang et al. 2010). Cardiomyocyte sarcoplasmic nNOS is also required for potentiation of the cardiac force-frequency response (Kunert 2000), a mechanism associated with the functioning of SERCA2 (Huke et al. 2003). Similarly, nNOS-deficient cardiomyocytes show reduced RyR opening probability (Wang et al. 2010). However, the physiological stimuli affecting S-nitrosylation of RyR are still unknown (Donoso et al. 2011).

Sarcolemmal NO synthesis increases Ca^{2+} release and contributes to muscle contractility. Of note, by inhibiting sarcoplasmic ion currents, the sarcolemmal NOS pool of cardiomyocytes has an antagonistic effect (Stamler and Meissner 2001; Khan et al. 2003).

6.5 The Neuronal Cell Membrane and the Anchoring of NOS

The association of various NOS isoforms with the cell membrane and synaptic vesicles has been shown in invertebrate and vertebrate neurons (Domoto et al. 1994; Yang et al. 1997) (Fig. 6.7). Possibly, invertebrate NOS isoforms bind the neuronal cell membrane through acylated motifs or PDZ-domains. In the vertebrate-type neurons, nNOS is targeted to the post-synaptic membrane (Yang et al. 1997) by association of the nNOS PDZ-domain with the scaffolding protein PSD-95 (post-synaptic density protein 95) and this interaction is obligatory for controlled nNOS activity (Stathakis et al. 1997; Yan et al. 2004; Chaudhury et al. 2009; Florio et al. 2009) (Fig. 6.7). PSD-95 is a palmitoylated protein (Fukata et al. 2006) which is anchored

to the synaptic membrane and also binds nNOS and the N-methyl-D-aspartate receptor (NMDA-R), the upstream activator of neuronal NO release (Villanueva and Giulivi 2010). NMDA-R activation triggers NO synthesis (Rameau et al. 2004; Yan et al. 2004) and also increases nNOS targeting to the cell membrane (Arundine et al. 2003). NMDA-R activation also enhances simultaneous depalmitoylation of PSD-95 (Fukata et al. 2006), which may lead to the dissociation of nNOS from the neuronal cell membrane and abolish its activity (Chaudhury et al. 2009). This mechanism may offer protection from cytotoxic NO production.

The association of nNOS/PSD-95/NMDA-R is required for nNOS phosphorylation, which determines its catalytic activity (Rameau et al. 2004). The entry of Ca^{2+} through NMDA-R triggers a phosphorylation cascade leading to CaM/nNOS association and NO synthesis (Fig. 6.7) (Rameau et al. 2004, 2007). The nNOS/PSD-90/NMDA-R complex also harbors calcium-calmodulin-dependent kinase II alpha (CaM-KIIα), which phosphorylates Ser-847 through its binding to the C-terminal domain of nNOS (Komeima et al. 2000; Yan et al. 2004). Interestingly, NMDA-R activated NO synthesis increases the expression of CaM-KIIα in neurons. In response to Ca^{2+}-influx, PI3 kinase activates Akt to phosphorylate nNOS at Ser-1412, activating NO synthesis (Adak et al. 2001). However, Ser-1412 dephosphorylation may also be induced by NMDA-R (Adak et al. 2001). NMDA-R stimulation with low glutamate levels increases phosphorylation of nNOS at Ser-847, while high glutamate treatment reverses Ser-847 phosphorylation leading to a production of cytotoxic NO levels (Komeima et al. 2000). This effect is mediated by other ion channels (L-type voltage-gated calcium channels and AMPA-type glutamate receptors; AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid) is a structural analogue of glutamate) near the nNOS/PSD-90/NMDA-R microdomains (Rameau et al. 2004, 2007). Strikingly, blocking the activity of L-type voltage-gated calcium channels and AMPA-type glutamate receptors significantly increases the basal levels of phosphorylation at Ser-847 and Ser-1412. NMDA-R and neighboring receptors thereby converge to provide regulatory control over the phosphorylation state of nNOS at Ser-847 and Ser-1412 (Rameau et al. 2007).

The PDZ domain of the membrane-associated nNOS binds a set of adaptor proteins which regulate NO synthesis: CAPON (C-terminal PDZ ligand of nNOS) (Jaffrey et al. 1998), PIN (protein inhibitor of nNOS) (Rodriguez-Crespo et al. 1998; McCauley et al. 2007), NOSIP (nitric oxide synthase-interacting protein) (Dedio et al. 2001) and phosphofructokinase-M (Luo and Zhu 2011), (Fig. 6.7).

CAPON (also known as NOS1AP) establishes interaction between nNOS and members of the synapsin family, which are associated with synaptic vesicles and involved in their exocytosis. Moreover, CAPON competes with PSD95 for interaction with nNOS (Jaffrey et al. 1998). Overexpression of CAPON results in a loss of PSD95/nNOS association showing that CAPON inhibits the coupling between nNOS and PSD95. It has also been shown that CAPON is expressed in non-neuronal cells (e.g. cardiomyocytes) and binds to nNOS (Beigi et al. 2009). (Fig. 6.8)

CAPON also links nNOS to Dexras1, a brain-enriched member of the Ras subfamily of GTPases (Nguyen and Watts 2005) (Fig. 6.7). Expression of Dexras1 is

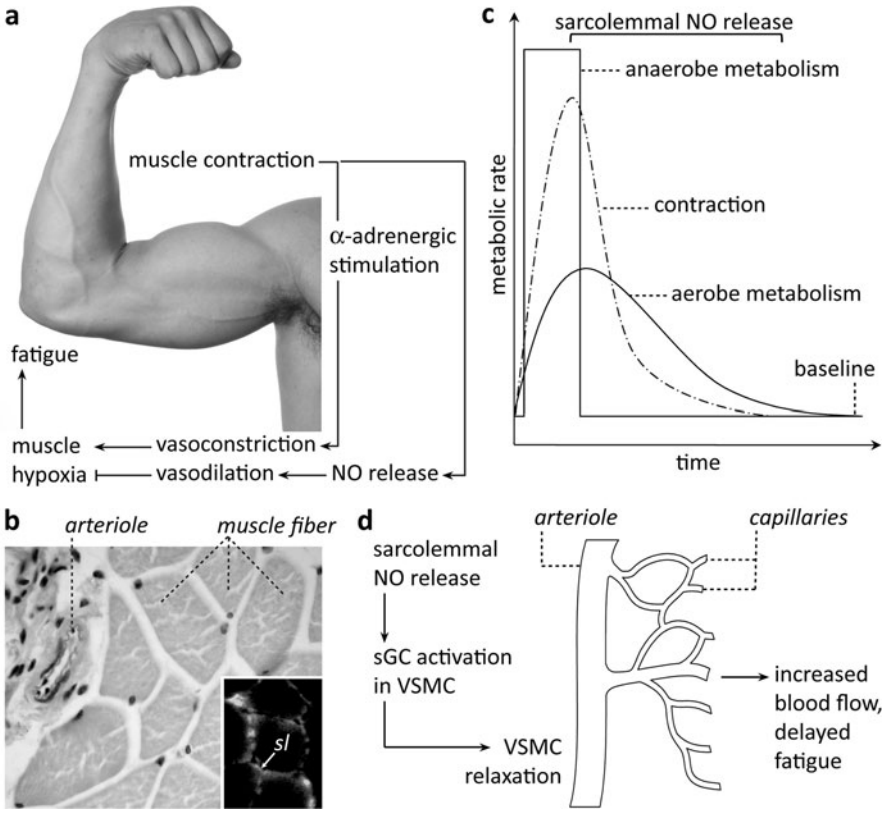

Fig. 6.7 The function of sarcolemmal NO synthesis. The α-adrenergic activation in the working muscles leads to local vasoconstriction, functional hypoxia and fatigue (**a**). In fast-twitch muscle fibers, the sarcolemma (*sl*) contains nNOS (insert) and the released NO reaches the regional arterioles (**b**). The muscle contraction is associated with anaerobe glycolysis. Parallelly with the activation of sarcolemmal NO release, the aerobe metabolism is the dominant form of ATP production (**c**). The sarcolemmal NO activates the soluble guanylyl cyclase (sGC) in the vascular smooth muscles (VSMC), leading to arteriolar dilatation and increased blood flow and increased aerobe muscle metabolism (**d**)

induced by the glucocorticoid dexamethasone (Li et al. 2008). Following NMDA-R stimulation, the assembly of ternary Dexras1/CAPON/nNOS complexes elicits S-nitrosylation and activation of Dexras1 (Jaffrey et al. 1998). Activated Dexras1 binds to a benzodiazepine receptor-associated protein, which controls an iron transport channel, the divalent metal transporter 1. This mechanism induces iron uptake in response to increased NO synthesis and may contribute to NO scavenging and neuroprotection (Cheah et al. 2006). It is also known that Dexras1 regulates receptor-mediated $G_{\beta/\gamma}$ signaling pathways; therefore, it may be a novel downstream target of nNOS (Nguyen and Watts 2005).

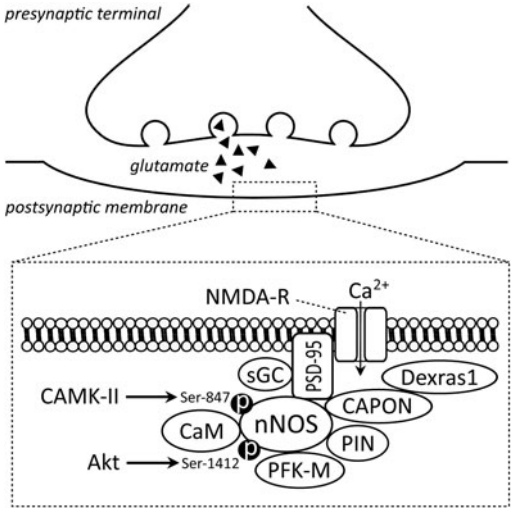

Fig. 6.8 Postsynaptic localization of nNOS and its main adaptor proteins. The acylated PSD-95 is embedded in the postsynaptic membrane where it anchors nNOS and the NO-target sGC. Dual phosphorylation of nNOS (*Ser-847* by *CAMK-II* and *Ser-1412* by *Akt*) controls its activation. The association of CaM increases nNOS activity. CAPON stabilizes nNOS anchoring to PSD-95, and PIN is possibly required for intracellular (axonal) nNOS transport. PFK-M and Dexras1 are implicated in the protection from overactivation of nNOS and cytotoxic NO synthesis

PIN, an 89-amino acid protein, is constitutively expressed in various brain regions (Greenwood et al. 1997) and interacts specifically with nNOS (residues 163-245) (Fan et al. 1998; Rodriguez-Crespo et al. 1998). It has been suggested that PIN inhibits nNOS dimerization and catalytic activity (Jaffrey and Snyder 1996) although this function of PIN has been challenged (Rodriguez-Crespo et al. 1998). PIN is identical with dynein light chain 8, and possibly involved in nNOS axonal transport in neurons (King et al. 1996; Rodriguez-Crespo et al. 1998) and intracellular delivery in non-neuronal cells (McCauley et al. 2007). Originally identified as an eNOS interacting protein, NOSIP is another nNOS inhibitor. NOSIP binds the eNOS oxygenase domain and translocates it from the cell membranes (Dedio et al. 2001). NOS/NOSIP interaction is known from various non-neuronal cell types (Konig et al. 2002, 2005). It has also been shown that NOSIP interacts and colocalizes with nNOS in synaptic membranes and inhibits nNOS activity (Dreyer et al. 2004) (Fig. 6.7).

The muscle specific 6-phosphofructokinase (phosphofructokinase-M, PFK-M) binds to the PDZ domain of nNOS in brain and skeletal muscle (Firestein and Bredt 1999; Wehling-Henricks et al. 2009). Many cortical neurons that are enriched in nNOS also contain PFK-M and they colocalize in synaptosomes (Fig. 6.7). It has been suggested that fructose-1, 6-bisphosphate, the glycolytic intermediate produced via the reaction catalyzed by PFK-M, may be neuroprotective, thereby the association of PFK-M with nNOS may protect neurons from NO-induced neurotoxicity (Firestein and Bredt 1999).

6.6 Chapter Summary

Fatty acylation and various adaptor proteins harbor NOS to the cell membrane	• eNOS and iNOS should undergo dual fatty acylation to be capable of membrane anchoring • nNOS binds adaptor proteins to associate with the cell membrane • Adaptor proteins also bind to acylated NOSs to specify their membrane destination • Membrane binding determines NOS catalytic activity by establishing NOS-associated signalosomes
NOS molecules are enriched in specific cell membrane regions	• Sarcolemma of muscle cells, caveolae of endothelial cells, postsynaptic membranes and intercellular adhesions and junctional complexes are rich in NOSs
Locally produced NO is a player in membrane physiology	• NO may be released to the extracellular environment: sarcolemmal NO emission acts as a local vasodilator; while postsynaptic NO is a retrograde neurotransmitter • The membrane NOS pool affects membrane conductance, gap junctional coupling and cell adhesion
The cell membrane and the endoplasmic membrane systems bind distinct NOS pools	• Although NOS binds the cell membrane and the endoplasmic membrane systems by the same principal mechanisms, the locally produced NO plays specific roles in the distinct membrane compartments. For instance, sarcolemmal eNOS and sarcoplasmic reticulum nNOS act antagonistically in the heart muscle

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Chapter 7
The Golgi-System Contributes to NO Homeostasis

7.1 NOS is Anchored to the Golgi-Complex in Certain Cell Types

The association of NOS with the Golgi-complex has been shown in various mammalian cell types such as neurons (Calka et al. 1994; Morin and Stanboli 1994; Wang et al. 1995; Xu et al. 2000), astrocytes (Calka et al. 1994), microglia (Calka et al. 1996), retinal cells (Darius et al. 1995), pancreatic islet cells (Bouwens and Kloppel 1994), cardiomyocytes (Buchwalow et al. 2001), endothelial cells (Morin and Stanboli 1993; O'Brien et al. 1995; Sessa et al. 1995; Fulton et al. 2002; Atochin and Huang 2010), muscle fibers (Percival et al. 2010), lymphocytes (Ibiza et al. 2006) and macrophages (Calka et al. 1996). Acrosome, a specialized form of the Golgi-complex in vertebrate spermatocytes, also contains NOS (Meiser and Schulz 2003). Comparative studies show that NOS is anchored to the Golgi-complex in the neurons of invertebrates (Ott and Elphick 2002; Ott et al. 2007) and NO-induced morphological changes of dictyosomes, the functional equivalents of the Golgi-system, from alga cells have also been reported (Lehner et al. 2009).

7.2 Acylation of NOS May Take Place at the Golgi-Complex

As we discussed in the previous chapter, eNOS and iNOS bear fatty acylated sequences that allow their association with cell membranes (Chap. 6) (Fleming 2010). Fatty acylation of NOS molecules possibly occurs in the cytoplasm or at the cytoplasmic region of the Golgi-system (Fig. 7.1). For instance, members of the DHHC acyltransferase family in the Golgi-complex catalyze the palmitoylation of eNOS (Fernandez-Hernando et al. 2006) (Chap. 6). The association of NOS with the Golgi-complex may therefore be temporal and serve the dynamic posttranslational modification of the newly synthesized NOS proteins. In this scenario, the Golgi-complex ensures fatty acylation thus determining the intracellular destination of NOS.

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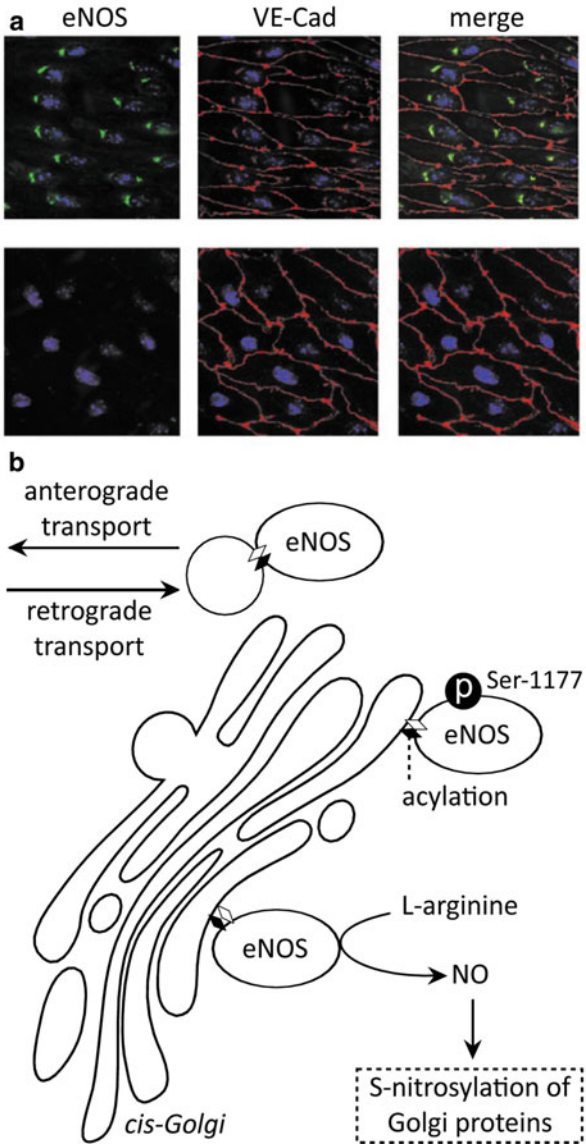

Fig. 7.1 Association of NOS with the Golgi-system in endothelial cells. En face immunostaining of mouse carotid arteries shows eNOS (*green*) localization in the perinuclear Golgi-system (**a**). VE-cadherin (*VE-cad, red*) staining shows the outlines of the endothelial membranes. *Top:* wild-type mouse, *Bottom:* eNOS-knockout mouse; note the lack of eNOS labeling. Original magnification × 600. Reprinted with permission (Atochin and Huang 2010). eNOS is present mainly in the *cis*-Golgi, where its acylation possibly takes place (**b**). By vesicular transport, eNOS is translocated to the plasma membrane (*anterograde transport*) or redistributed to the Golgi-system (*retrograde transport*); possibly, these events are affected by eNOS phosphorylation (e.g. Ser-1177). The main function of eNOS is the S-nitrosylation of several Golgi proteins

However, dissociation of eNOS from the Golgi-complex leads to reduced NO synthesis in endothelial cells (Nuszkowski et al. 2001) which suggests that the binding to the Golgi-system maintains a steady-state of eNOS catalytic activity. It is also possible, that activated eNOS is redirected from the plasma membrane caveolae to the Golgi-complex (Icking et al. 2005). The activation of eNOS by Akt-mediated phosphorylation (Ser-1177 in human, Ser-1176 in mouse and Ser-1179 in bovine), which increases the targeting of eNOS to the Golgi-system, supports this idea (Fulton

et al. 2002; Atochin and Huang 2010). The binding of eNOS to the Golgi-system depends on the acylation pattern of its first 35 amino acids, including N-myristoylation and palmitoylation sites (Sessa et al. 1995; Liu et al. 1997; Sowa et al. 1999; Fulton et al. 2002) (Fig. 7.1). Non-acylated eNOS fails to harbor membranes and is unable to bind the Golgi-complex, and consequently displays reduced NO forming activity (Sessa et al. 1995). The Golgi-system also contains caveolin-1, but it is not associated with eNOS (Govers et al. 2002; Fleming 2010). Importantly, the concentration of caveolin-1 in the cell membrane caveolae (Sowa et al. 1999; Fulton et al. 2002) may be the distinctive signal, which attracts the acylated eNOS to the plasma membrane (Garcia-Cardena et al. 1996; Liu et al. 1996) (Chap. 6). In endothelial cells and muscle cells, both the Golgi-complex and the cell membranes are pools of active NOS, although NO exerts distinct local effects in the two compartments (Qian et al. 2010) (Chap. 6).

During mitosis, the Golgi-system disappears and reassembles in the daughter cells (Morin and Stanboli 1994). NOS becomes redistributed parallel with this disintegration and is rebuilt along with the Golgi-complex in dividing endothelial cells: in early prophase and metaphase, NOS is concentrated near the microtubule spindle, while in anaphase and telophase, NOS is spread in the cytoplasm and finally redistributes in the cytoplasm of the daughter cells (Morin and Stanboli 1994). The mechanism behind the reorganization of the Golgi-system in the daughter cells and the targeting of NOS to the newly formed Golgi-complex is still undefined.

7.3 NO Maintains Golgi-System Architecture and Vesicular Traffic

Early studies on the association of eNOS with the Golgi-complex hypothesized that eNOS is released from the endothelial cells through Golgi-derived vesicles (O'Brien et al. 1995). However, recent works point out that Golgi-linked eNOS is critical for the functioning of the Golgi-system. Scavenging of NO or the knockdown of eNOS evoke morphological alterations of the perinuclear Golgi-complex in endothelial and smooth muscle cells (Lee et al. 2009, 2011) (Figs. 7.2, 7.3) and ablation of nNOS leads to the mislocalization of the Golgi-system in skeletal muscle fibers (Percival et al. 2010). In endothelial and smooth muscle cells the administration of NO-donor compound diminishes these effects (Lee et al. 2009). Mislocalization of the Golgi-system in nNOS-deficient muscle fibers may be a consequence of aberrant microtubule organization (Percival et al. 2010), while in endothelial and smooth muscle cells the changes in the Golgi-system morphology are unrelated to the NO-induced alterations in the cytoskeletal structure (Lee et al. 2011). The Golgi-system morphology is unaffected by the cGMP/PKG pathway (Lee et al. 2011). However, the Golgi-system associated eNOS enhances the overall Golgi protein S-nitrosylation, a candidate mechanism determining Golgi-system morphology (Lee et al. 2011). The fragmentation of the Golgi-system in response to mislocalization of eNOS is

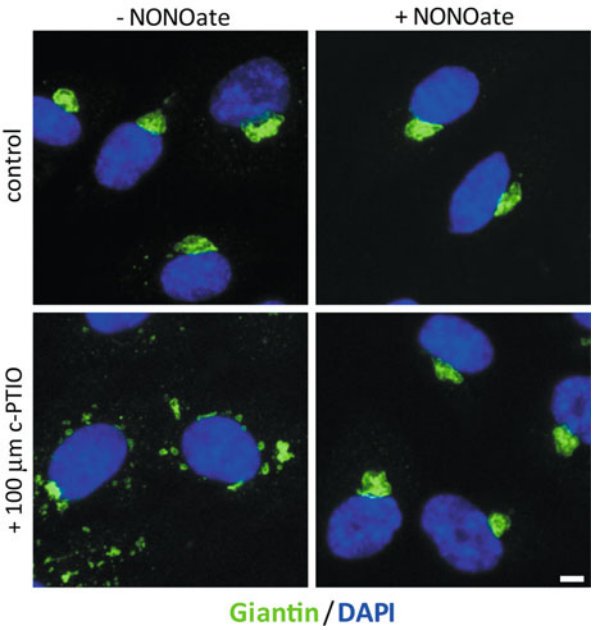

Fig. 7.2 Scavenging of NO causes Golgi-system fragmentation. NO scavenging-induced Golgi fragmentation is inhibited in the presence of a NO donor. HPAEC cultures were treated with the NO-scavenger molecule c-PTIO (100 µM) with or without an NO-donor (400 µM NONOate) for 2 days with daily replenishment. The cultures were fixed and immunostained using an antibody to Golgi-system marker (*giantin*) and with DAPI to stain nuclei. Images of representative cells are illustrated; scale bar 5 µm. Original images with the courtesy of Dr. Jason E. Lee and Dr. Pravin B. Sehgal

a possible consequence of reduced S-nitrosylation of certain Golgi membrane proteins (Iwakiri et al. 2006; Lee et al. 2009; Qian et al. 2010). Specific targets of S-nitrosylation are N-ethylmaleimide-sensitive factor, caveolin-1, and clathrin heavy chain (Iwakiri et al. 2006; Mukhopadhyay et al. 2007, 2008). These proteins are all involved in vesicular transport and vesicle fusion. Lacking NO synthesis, hypo-S-nitrosylation of these proteins may reduce the speed of Golgi-system-mediated protein transport from the endoplasmic reticulum to the plasma membrane (Iwakiri et al. 2006). Other proteins required for vesicular protein transport (e.g. α-SNAP [soluble N-ethylmaleimide sensitive fusion protein attachment protein-a], Vti-1a [vesicle transport through interaction with t-SNAP-receptors homolog 1a], giantin) are also depleted from the Golgi-system in the lack of NO synthesis (Lee et al. 2011).

Some comparative studies suggest that involvement of NO in secretory vesicular transport may also occur in other cell types. For example, in the plant cell NO also S-nitrosylates proteins involved in secretory pathways and affects dictyosome (Golgi-analogue plant organelle) morphology (Lehner et al. 2009). Exogenous NO administration also helps the secretion of pectin and hemicellulose, major products of the dictyosomes in plants (Xiong et al. 2009).

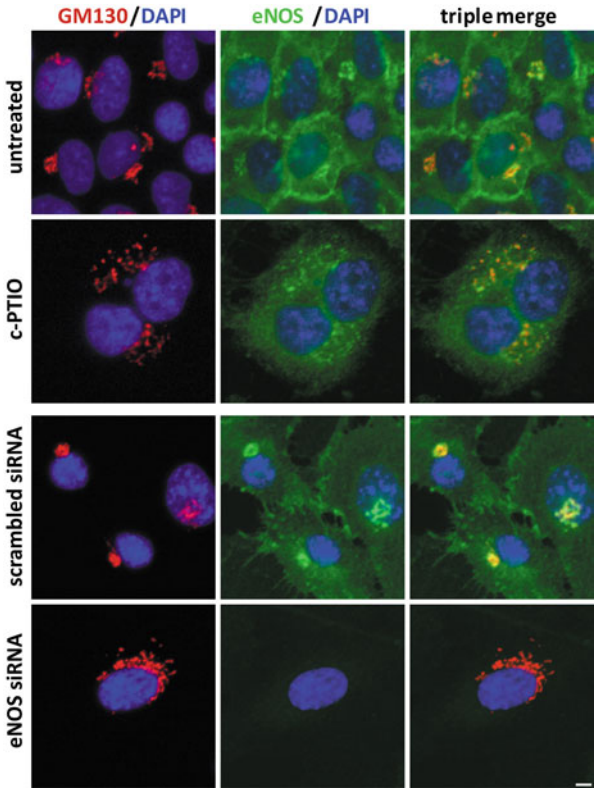

Fig. 7.3 Effects of NO scavenging and eNOS knockdown on the Golgi-system morphology. *Top:* NO-scavenging induced fragmentation of the structure of the Golgi apparatus and eNOS localization away from the plasma membrane in HUVEC-derived immortalized cells. EA.hy926 cells were exposed to 100 μM c-PTIO (NO-scavenger) for two days with daily replenishment. The cultures were fixed and immunostained using an antibody against the Golgi marker/tether giantin (*red*) and against eNOS (*green*) with DAPI for nuclear staining. Images of representative cells are illustrated. *Bottom:* siRNA-mediated knockdown of eNOS in HPAECs leads to Golgi-system fragmentation. The Golgi structure was assayed in HPAEC cultures transfected with eNOS siRNA transfections or scrambled siRNA (negative control). Ninety-six hours after the initial siRNA transfection, cells were fixed for immunofluorescence. Knockdown of eNOS protein levels was verified via immunolabeling against eNOS (*green*) using identical primary and secondary antibody concentrations in all groups with identical exposure times during immunofluorescence data capture. The Golgi structure was assayed by immunolabeling cells with anti-giantin. DAPI was used to stain nuclei. Images of representative cells are illustrated; scale bar 5 μm. Original images with the courtesy of Dr. Jason E. Lee and Dr. Pravin B. Sehgal

7.4 Golgi-Specific NO Signaling Microdomain in the Muscle Fibers

A recent study has revealed the function of Golgi-system associated nNOS in the mammalian striated muscle fibers (Fig. 7.4). The Golgi-system contains an isoform of nNOS (nNOSβ) (Percival et al. 2010). In muscle fibers, nNOS is associated

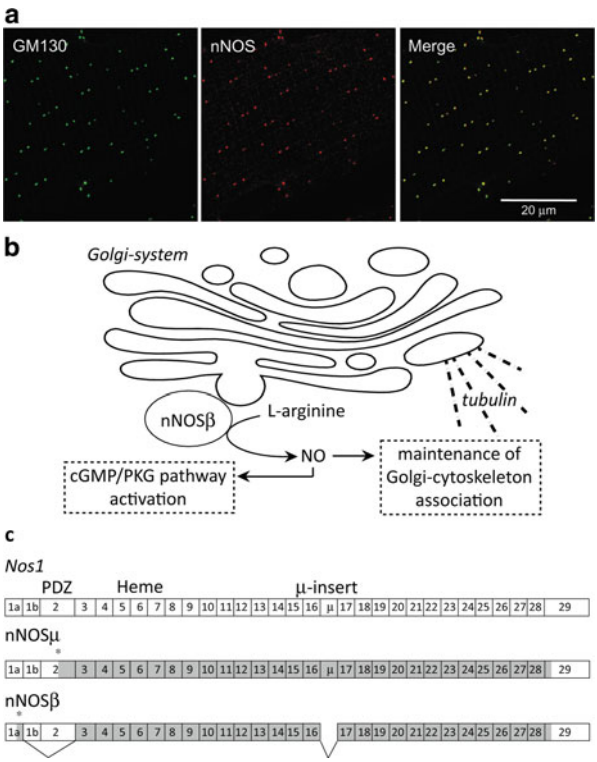

Fig. 7.4 Localization of nNOS in the skeletal muscle Golgi-system. nNOS localizes to the Golgi complex in skeletal muscle (**a**) The gastrocnemius skeletal muscle *cis*-Golgi compartment (*green, left panel*) is labeled with the GM130 (a marker of *cis*-Golgi) antibody. An nNOS (*red, middle panel*) splice variant, thought to be nNOSβ, localizes to the *cis*-Golgi compartment. The extensive colocalization of nNOS and GM130 is evident in the merge image (*yellow, right panel*). Original images, courtesy of Dr. Justin Percival. The Golgi-system anchored NOSβ activates the cGMP/PKG pathway and affects microtubule organization and Golgi-system localization in the muscle fibers (**b**). Exon structure of the mouse *Nos1* gene and two splice variants of nNOS (**c**). Nos1 encodes 31 exons. PDZ—PDZ domain, heme—heme domain, µ-insert—34-amino-acid insert; in skeletal muscle the sarcolemma contains nNOSµ, while the Golgi-system anchors the nNOSβ splice variant. The coding sequences are shown in *gray*; *asterisks* label translation initiation sites. (Percival et al. 2010)

with three subcellular compartments: the nNOSµ isoform is anchored to the sarcolemma and distributed in the cytosol (Lai et al. 2009), and nNOSβ is associated with the Golgi-complex (Percival et al. 2010). Increased intracellular Ca^{2+} levels activate all of these isoforms; however, their NO synthesis evokes spatially different downstream effects (Chap. 6). Activation of the nNOSµ accounts for the attenuation of local vasoconstriction in contracting muscles thus allowing sufficient blood and oxygen perfusion of the active muscles and also diminishing fatigue after exercise (Heydemann and McNally 2009; Lai et al. 2009) (Chap. 6). The physiological function of the cytosolic nNOSµ is still unknown (Percival et al. 2010), although its effect

has been addressed under pathological conditions (Rando 2001; Wehling-Henricks et al. 2009; Suzuki et al. 2010) (Chap. 12). The lack of a Golgi-associated nNOS pool leads to myopathic changes with reduced muscle mass (Percival et al. 2010). Skeletal muscles lacking Golgi-nNOS contain increased numbers of fatigue-sensitive IIb-type fibers, show Golgi-system mislocalization, altered cytoskeletal structure, sarcomere and mitochondrial misorganization (Percival et al. 2010). Possibly, the Golgi-system associated nNOS activates the cGMP/PKG pathway and affects muscle contractility (Percival et al. 2010). These findings on site-specific NO actions further underscore the importance of subcellular NOS distribution in distinct physiological effects of NO.

7.5 The Acrosome Contains NOS

The acrosome is a specialized form of the Golgi-complex, forming a cap-like structure in the head region of the mammalian spermatocyte. This organelle functions as storage for multiple enzymes required to penetrate the oocyte during the fertilization process (Toshimori and Ito 2003). Its content releases during the so-called acrosome reaction[1], when the spermatocyte penetrates the zona pellucida and attaches to the oocyte membrane (Fig. 7.5).

Mammalian spermatocytes are capable of synthesizing NO by oxidizing L-arginine to L-citrulline (Herrero et al. 1997a; Revelli et al. 1999) and the presence of nNOS (Lewis et al. 1996; Meiser and Schulz 2003) and eNOS (Lewis et al. 1996; Revelli et al. 1999; Meiser and Schulz 2003) has been shown by western blotting and immunocytochemical studies in these cells. Spermatocyte NOS isoforms occur in the head region (Lewis et al. 1996; Meiser and Schulz 2003) and their association with the acrosome has also been documented (Meiser and Schulz 2003; Anderson et al. 2009). The administration of L-arginine (Funahashi 2002; O'Flaherty et al. 2004) or NO donor compounds induces acrosome reaction and increases the capacity of spermatocytes for successful fertilization (Sengoku et al. 1998; Revelli et al. 1999; Anderson et al. 2009). In turn, inhibition of NOS (Viggiano et al. 1996; Herrero et al. 1997b; Kameshwari et al. 2003) or scavenging of NO (Revelli et al. 1999; O'Flaherty et al. 2004) reduce acrosome exocytosis and inhibit fertilization. Importantly, factors inducing acrosome reaction, such as follicular fluid proteins (Revelli et al. 1999) or progesterone (Herrero et al. 1997b) also increase NO synthesis and a consequent acrosomal reaction. An influx of Ca^{2+} and certain biomodulators such as leptin and insulin (Lampiao and du Plessis 2008; Anderson et al. 2009) also increase spermatocyte NO synthesis and acrosome reaction, and may help *in vitro* fertilization (Lampiao and du Plessis 2008). However, a premature release of the acrosome content (Anderson et al. 2009), which leads to infertility (Herrero and Gagnon 2001) may be evoked by excessive eNOS activation or NO administration.

[1] The acrosome reaction is a final event in the so-called capacitation, a process ensuring maturation and fertility of spermatocytes within the female genital tract.

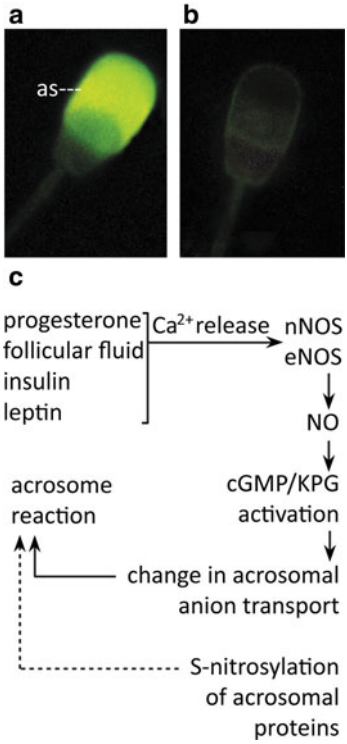

Fig. 7.5 Acrosome, a special form of the Golgi-system, also contains NOS. The acrosome forms a cap-like structure around the spermatocyte head (a–b). Fluorescence pattern of pig spermatozoa stained with FITC-peanut-agglutinin (PNA) for the assessment of acrosome status and sperm viability: (**a**) acrosome-reacted cells with uniform green FITC-PNA fluorescence of acrosome cap (ac); (**b**) acrosome-unreacted cells with no staining of the acrosomal cap; original magnification × 1000. Reprinted with permission (Siciliano et al. 2008). An increase of the Ca^{2+} level activates the acrosome associated NOS isoforms; the released NO activates the cGMP/PKG pathway and S-nitrosylates acrosomal proteins, leading to acrosome reaction

Spermatocyte-derived NO also induces oocyte activation (Kuroda et al. 2000; Petr et al. 2005a, 2005b) helping successful fertilization. The targeting of spermatocyte NOS activity, therefore, may be a tool in assisted fertilization (Revelli et al. 1999) or in anticonceptive intervention (Anderson et al. 2009).

Spermatocyte NO may activate acrosome loss through at least three downstream signaling pathways. NO may activate soluble guanylyl cyclase, leading to increased cGMP levels and the activation of PKG (Revelli et al. 2001b; Anderson et al. 2009). Increased cGMP levels induce acrosome exocytosis and activation of the cGMP/PKG pathway is essential for acrosome reaction (Revelli et al. 2001a, 2002). However, NO-independent activation of particulate guanylyl cyclase (e.g. by atrial natriuretic peptide) also evokes acrosome release (Zamir et al. 1995). That NO affects the anion transport system of the spermatozoa in a cGMP/PKG-independent manner and

contributes to acrosome release (Funahashi 2002) has also been suggested. The production of reactive nitrogen species (RNS) occurs during spermatocyte capacitation (de Lamirande and Lamothe 2009; de Lamirande et al. 2009), therefore, it is hypothesized that protein nitration may be an additional mechanism affecting acrosome reaction. The administration of NO to human spermatocytes evokes S-nitrosylation of several proteins, including proteins associated with the acrosome region (Lefievre et al. 2007). Recent findings show that light irradiation increases spermatocyte NO synthesis (Ankri et al. 2010) along with reactive oxygen species generation (Zan-Bar et al. 2005), which may give rise to the formation of RNS. However, the biological relevance of light on spermatocyte biology and particularly on protein nitration is uncertain.

7.6 Chapter Summary

NOS is associated with the Golgi-system	• NOS binds to the Golgi-system in various cell types. NOS proteins may undergo fatty acylation in the Golgi-system. In endothelial cells, the acylated sequences harbor eNOS to the Golgi-system; in other cell types, the mechanism of NOS-binding is still unknown
Functions of NO in the Golgi-system	• NO can S-nitrosylate Golgi proteins; a requirement for maintenance of the Golgi-system structure • In skeletal muscle fibers the Golgi-system-associated NO synthesis affects muscle contractility and myofiber structure • In the acrosome (a modification of the Golgi-system), NO induces the release of the acrosome content, and thereby affects successful fertilization

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Chapter 8
Phagosomal and Lysosomal NO Synthesis

8.1 NO in Multivesicular Bodies, Phagosomes and Secondary Lysosomes

Engulfment of particles by endocytosis is one of the most ancient and evolutionarily conserved cellular processes in the eukaryotic cell (Chang 2009). Endocytosis starts with the recognition and binding of particles by cell-surface receptors; followed by budding of the cell membrane and the formation of an endosome which internalizes the bounded particles. Finally, the endosome undergoes fusion with lysosomes containing hydrolytic enzymes to degrade the engulfed cargo (Fang 2004).

Late endosomes often enclose intraluminal vesicles that are formed by the endosomal membrane growing inward (Fig. 8.1). These structures are also called multivesicular bodies (Loesch et al. 1997; O'Neill and Quah 2008). They may fuse with the plasma membrane and release their intraluminal vesicle content to the extracellular environment. The secreted vesicles contain bacterial antigen motifs and may function as signals for immune cells (Record et al. 2011). For instance, exosomes derived from bacterially infected macrophages carry bacterial coat components and stimulate bystander macrophages and neutrophils to secrete proinflammatory mediators and increase NO production (O'Neill and Quah 2008). Interestingly, the circulating exosomes of platelets also generate NO in septic shock, which evokes myocardial nitrosative injury (Azevedo et al. 2007). Platelet exosomes are also capable of inducing endothelial NO and peroxynitrite ($ONOO^-$) generation, thus evoking apoptosis and vascular damage (Gambim et al. 2007).

Phagocytosis is a special type of endocytosis: it is required for the engulfment of solid particles such as pathogens, xenobiotics, protein complexes and cell debris of necrotic or apoptotic cells (Fig. 8.1). The endosome formed in the phagocytosis process is termed a phagosome (Weissmann 1964; Hirsch 1965). In vertebrate-type phagocytosing cells such as macrophages, the association of NOS with phagosomes has been shown (Winberg et al. 2007). These cells employ NO synthesis as a pathogen killing mechanism, to evoke nitrosative damage of the engulfed microbes (Malawista et al. 1992). Accordingly, microbial antigens increase NOS-activity,

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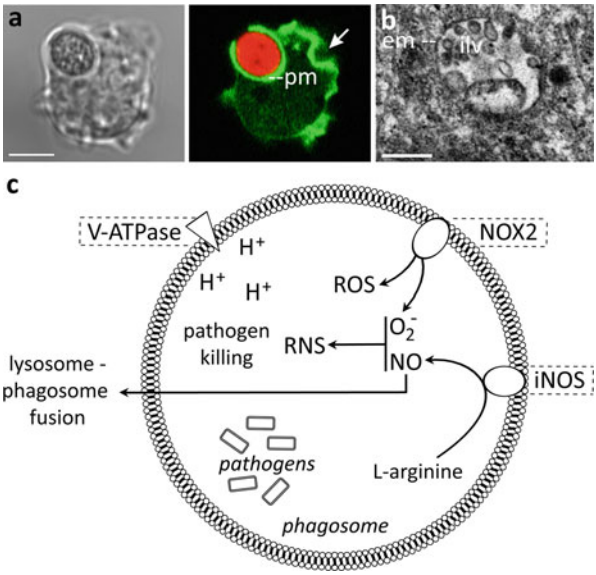

Fig. 8.1 Synthesis of NO in phagosomes. Example of phagocytosis: *Dictyostelium discoideum* amoeba engulfs a rhodamine-labelled yeast cell **a** The arrow shows a newly formed phagocytic cup. The phagosome membrane (*pm*) surrounds the particle. Green fluorescent protein is used to tag actin filaments. Phase contrast (on the left) and confocal image (on the right), scale bar 5 µm. Reprinted with permission (Schleicher and Jockusch 2008). Late endosomes may form multivesicular bodies: the endosome membrane (*em*) surrounds several intraluminal vesicles (*ilv*) which may be released from the cell and activate immune cells **b** Author's TEM image, scale bar 200 nm. The pathogen-containing phagosomes synthesize *NO* which forms *RNS* to kill *pathogens* or facilitate *lysosome-phagosome fusion* by affecting actin organization around the *phagosome*. The vesicular ATPase (*V-ATPase*) which generates an acidic environment in the *phagosome*, and the phagocyte oxidase (*NOX2*) which generates *ROS*, also ensures *pathogen killing* **c**

which is then involved in phagocytosis (Zagryazhskaya et al. 2010), pathogen killing and inflammation (Franchini et al. 1995; Nath and Powledge 1997; Sethi et al. 2001).

The activation of macrophages by bacterial components (e.g. lipopolysaccharide, LPS) or inflammatory cytokines (e.g. interferon-γ, IFNγ) evokes an inflammatory or M1 phenotype acquisition (Benoit et al. 2008). This M1-type polarization of macrophages increases the expression of iNOS, leads to the enrichment of iNOS in the phagosome membrane and evokes a NO-burst (Winston et al. 1999). Vesicular iNOS is derived from a cytosolic iNOS isoform, by a post-translational protein modification, which increases the membrane association of the molecule (Vodovotz et al. 1995). In resting macrophages iNOS is associated with non-lysosomal vesicles, which undergo fusion with phagosomes thus translocate iNOS to the phagosomal membrane upon activation and phagocytosis (Vodovotz et al. 1995).

Phagosomes of M1 macrophages also contain NADPH-dependent phagocyte oxidase which produces superoxide (O_2^-) and hydrogen peroxide (H_2O_2) thus increases ROS generation within the phagosome (Fig. 8.1) (Winberg et al. 2007). With the

activation of phagosomal NO synthesis the phagocyte oxidase activity can also be increased (Brennan et al. 2004) and the generated O_2^- forms $ONOO^-$ with NO. Under the acidotic pH of the phagosome NO also gives nitrous acid (HNO) and other reactive nitrogen species (RNS), which evoke nitrosative damage of the engulfed pathogens (Jordao et al. 2008; Ehrt and Schnappinger 2009). Phagosome NO synthesis also restricts the iron-availability of the engulfed cells thus limiting their survival (von Bargen et al. 2011). Phagosomal NO synthesis not only ensures the elimination of the pathogenic cells, but also helps F-actin assembly around the phagosomes, which facilitates phagosome-lysosome fusion (Winberg et al. 2007) (Fig. 8.1).

Certain intracellular pathogens, such as *Mycobacterium tuberculosis*, *Leishmania donovani* and *Rhodococcus equi*, have evolved defensive mechanisms, by which they arrest the fusion of lysosomes with the phagosome and thus avoid degradation by lysosomal enzymes (Winberg et al. 2007; von Bargen et al. 2011). Various cell surface molecules of the engulfed pathogens mediate the inhibition of the lysosome-phagosome fusion: e.g. lipophosphoglycans or trehalose dimycolate (Jordao et al. 2008; Ehrt and Schnappinger 2009). However, when macrophages are being activated by IFNγ or LPS, their NO burst overshadows these defense mechanisms and lysosomes fuse properly with the phagosomes (Winberg et al. 2007).

Phagocytosing immune cells of invertebrates also synthesize NO, and they may respond with increased NO synthesis to various microbial products (Nieto-Fernandez et al. 1999; Beck et al. 2001). Rhizopoda, the most ancient phagocytosing eukaryotes show NOS-like activity (Rojas-Hernandez et al. 2007) and also display reductive NO synthesis (Risgaard-Petersen et al. 2006). These eukaryotes utilize phagocytosis to engulf unicellular organisms, thus they may be considered the archetypes of phagocytosing immune cells. To date, whether a NO burst occurs during their phagocytosis however, has not been established. Destruction of engulfed pathogens by cytotoxic effects of NO is therefore an attribute of multicellular eukaryotes, and this mechanism is conserved in the evolution of the innate immune system (Tauber 2003; Fang 2004).

8.2 Lysosomes of Granulocytes are Sources of NO

Granulocytes constitutively express iNOS, eNOS, and nNOS and display calmodulin-dependent L-arginine/L-citrulline conversion (Maruo et al. 1999; Cedergren et al. 2003; Heijnen et al. 2006; Saini et al. 2006; Saluja et al. 2010; Saluja et al. 2011). Although NOS is also distributed in the cytoplasm and associated with the nucleus (Heijnen et al. 2006; Saluja et al. 2010; Saluja et al. 2011), electron microscopic analysis has revealed that granulocyte-specific lysosomes, the so-called eosinophil and neutrophil granules are the most important NOS-containing organelles in granulocytes (Fig. 8.2).

A subset of neutrophil granules (the so-called azurophilic granules) and the eosinophil granules also contain heme-peroxidases (EC 1.11.1.7): myeloperoxidase (MPO) and eosinophil peroxidase (EPO), respectively. A product of MPO is

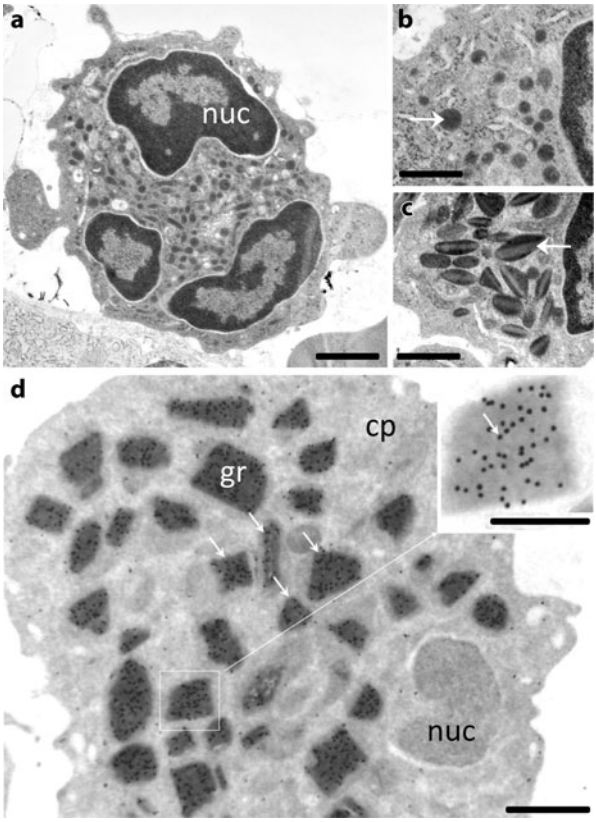

Fig. 8.2 Leukocyte granules contain NOS. Leukocyte granules are lysosome-like vesicles containing various proteins implicated in host defense, hydrolytic enzymes, and plasma membrane components, receptors of complements, chemoattractants and NOS. Some of the granules undergo fusion with the phagosomes and they are involved in the killing and degradation of microorganisms. Granule contents may also be released into the extracellular space, where they play distinct roles in inflammation. TEM images showing a neutrophil granulocyte of mouse **a** neutrophil granules **b** and eosinophil granules **c** form mouse granulocytes. *nuc* – nucleus, *white arrow* points to granules; scale bar 650 nm (a), 300 nm (b, c); Author's images. TEM images showing colloidal gold-labeling of NOS (*white arrows*) in the granules of a human eosinophil granulocyte **d** (Saluja et al. 2010). *cp* – cytoplasm, *gr* – eosinophil granule, *nuc* – nucleus, arrows label NOS signal (colloidal gold); scale bar 200 nm, in insert 500 nm. (Source: With courtesy of Dr. Madhu Dikshit)

hypochlorous acid (HOCl), which is an effective pathogen killing substance, while EPO generates hypobromite, another oxidizing agent which ensures defense against helminths and bacteria (Fang 2004). Both MPO and EPO are responsible for generating RNS from degradation products of NO, such as NO_2^- and peroxynitrous acid (HONOO). In activated granulocytes, NO degrades to NO_2^- or combines with O_2^- to $ONOO^-$, which then forms HOONO (Pryor and Squadrito 1995) (Fig. 8.3). MPO can convert NO_2^- and HOONO to other RNS, such as nitryl chloride (NO_2Cl) and

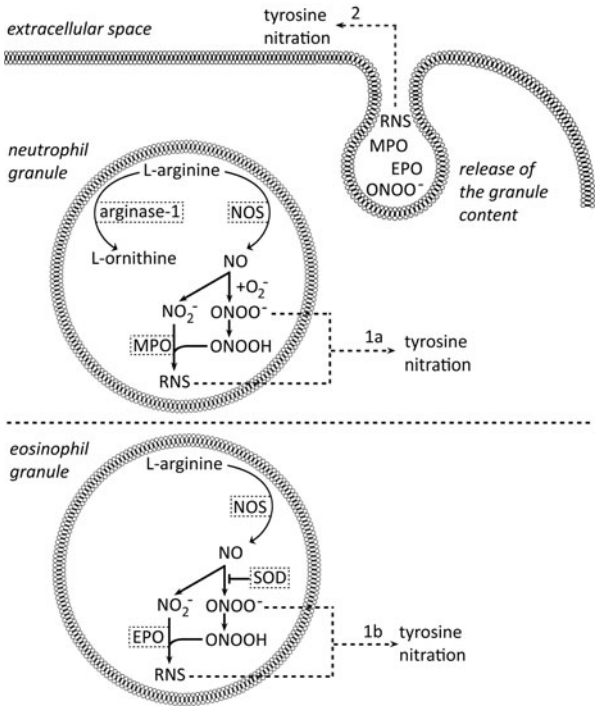

Fig. 8.3 Synthesis of *NO* in leukocyte granules. In the leukocyte granules, *NOS* synthesizes *NO* from *L-arginine*. In *neutrophil granules*, *arginase-1* competes for the substrate with *NOS*, and may thereby limit *NO* levels. Peroxidases (*MPO*, *EPO*) produce reactive nitrogen species (*RNS*) from derivatives of *NO* (NO_2^-, $ONOO^-$, *ONOOH*). Antioxidant enzymes, e.g. *SOD*, may limit the generation of *RNS*. The *RNS* evoke *tyrosine nitration* in the cell (*1a*, *1b*) or in the *extracellular space* (*2*)

nitrogen dioxide (NO_2) (Floris et al. 1993; Eiserich et al. 1998; But et al. 2004). Similarly, EPO also metabolizes NO_2^- to RNS in eosinophil granulocytes (Wu et al. 1999; Takemoto et al. 2007b). Activated human neutrophil granulocytes show increased MPO activity along with their elevated NO production and both NO and NO_2^- are capable of increasing MPO activity (Sethi et al. 2001; But et al. 2004).

Nitrotyrosine is abundant in granules containing both iNOS and peroxidases (Heijnen et al. 2006), suggesting that close vicinity of NO synthesis and peroxidase activity results in tyrosine nitration. In accordance with this scenario, upregulation of iNOS increases the level of 3-nitrotyrosine in eosinophil granulocytes (Duguet et al. 2001) and increased iNOS expression and tyrosine nitration occurs at inflammatory sites infiltrated by neutrophil or eosinophil granulocytes (Wu et al. 1999; Iijima et al. 2001). The lack of iNOS or inhibition of NOS abolishes the generation of intracellular RNS in granulocytes (Numata et al. 1998; Iijima et al. 2001; Koarai et al. 2002). EPO-deficiency also diminishes tyrosine nitration in eosinophil granulocytes in response to allergen challenge in mice, showing that peroxidase activity is required for protein nitration (Duguet et al. 2001). Although $ONOO^-$ evokes tyrosine nitration by itself, MPO and EPO generated RNS play the leading role in nitration of tyrosine residues in granulocytes (Eiserich et al. 1998; But et al. 2004).

8.3 Effects of Protein Nitration Evoked by Granulocytes

The generation of RNS and consequent protein nitration may provide an additional microbial killing mechanism in granulocytes (Malawista et al. 1992; Malawista et al. 1996; Gutierrez-Correa et al. 2000). For instance, tyrosine nitration by resident eosinophil granulocytes of the gastric mucosa (Takemoto et al. 2007b) is involved in defense against pathogens, such as *Helicobacter pylori* (Kuwahara et al. 2000). However, tyrosine nitration by tumor-infiltrating neutrophil granulocytes may also evoke genotoxic damage and contribute to the burden of genetic abnormalities associated with tumor progression (Sandhu et al. 2000). In various inflammatory disorders such as asthma, atopic dermatitis and allergic reactions, granulocyte-evoked tyrosine nitration also accounts for tissue damage and remodeling (Maruo et al. 1999; Kubo et al. 2005; Prado et al. 2006). Production of NO in granulocytes and consequent protein nitration is therefore considered as a cytotoxic, often harmful and inflammation provoking mechanism.

However, tyrosine nitration of chemoattractant molecules, such as interleukin-8 and monocyte chemotactic protein-1 impairs their ability to increase granulocyte chemotactic activity (Sato et al. 2000c; Sato et al. 2000b). Eosinophil granulocytes also display diminished chemotaxis in response to tyrosine nitrated eotaxin, interleukin-5 and RANTES (normal T cell expressed and secreted) (Sato et al. 1999; Sato et al. 2000a). Tyrosine nitration of immunoglobulin-G impairs its ability to induce inflammatory granulocyte activation (Uesugi et al. 2000). Tyrosine nitration of chemotactic factors therefore diminishes granulocyte recruitment to inflammatory sites. Moreover, tyrosine nitration also inhibits granulocyte adherence to endothelial cells, therefore NO may limit the endothelial injury evoked by activated granulocytes (Banick et al. 1997; Su et al. 1998). The activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) also decreases granulocyte rolling and adhesion by a mechanism dependent on NO production (Napimoga et al. 2008). Apart from tyrosine-nitrated proteins, other nitrated organic compounds, such as unsaturated fatty acids also exert an anti-inflammatory profile by attenuation of neutrophil degranulation, O_2^- generation and integrin expression (Coles et al. 2002).

Various immunomodulatory factors are capable of inducing iNOS gene transcription in granulocytes and increased iNOS activity is proportional with tyrosine nitration under certain pathological conditions (Pautz et al. 2010). However, studies with iNOS deficient mice have pointed out that tyrosine nitration is not completely abolished by the lack of iNOS (Kenyon et al. 2002), since other NOS isoforms may also be sources of NO and cell types other than granulocytes also contribute to tyrosine nitration in inflamed tissues (Maarsingh et al. 2009). Collectively, inflammatory activation of granulocytes evokes NO production, and NO is converted to RNS by peroxidases, leading to tyrosine nitration. Protein nitration evokes nitrosative damage in the inflammatory environment; however, nitration of various biomolecules inhibits granulocyte-mediated inflammation.

Apart from the effects of NO-derived RNS, NO also acts through the cGMP/PKG pathway in granulocytes (Wyatt et al. 1993). The NO/cGMP/PKG signaling induces degranulation, the release of inflammation-modulating substances (Wyatt et al.

1993). Moreover, NO is also required for granulocyte chemotaxis and metalloproteinase secretion (Iijima et al. 2001; DiScipio et al. 2006). Under oxidative stress, NO generation also helps the survival of neutrophil granulocytes and contributes to sustained inflammation (Riazantseva et al. 2010). However, it has not been established whether the lysosomal NOS-pool would be the source of NO in these events.

8.4 Arginase-1 Reduces NO Synthesis in Neutrophil Granulocytes

Availability of L-arginine is a key determinant of NO biosynthesis. Neutrophil granulocytes constitutively express arginase-1, which hydrolyzes L-arginine to L-ornithine and urea (Munder et al. 2005; Munder et al. 2006). In neutrophil granulocytes, arginase-1 is confined to gelatinase containing granules, which are also sites of NO synthesis. Within the granules, arginase-1 consumes L-arginine, and thus reduces NO generation by NOS (Jacobsen et al. 2007) (Fig. 8.3). Competition of the two enzymes for the same substrate therefore, determines the level of NO production in the neutrophil granules. It has also been shown that L-ornithine and N_{ω}-hydroxy-L-arginine, an intermediate product of NO biosynthesis inhibit arginase-1, and L-ornithine also reduces uptake of L-arginine in NOS-containing cells (Maarsingh et al. 2009). Interplay between arginase-1 and NOS therefore, may ensure balanced NO production in the neutrophil granules. However, increased consumption of L-arginine by arginase-1 also increases tyrosine nitration (Takemoto et al. 2007a) since reduced availability of L-arginine increases O_2^- generation by the iNOS reductase domain (Xia et al. 1998), leading to production of $ONOO^-$ in the leukocytes (Maarsingh et al. 2009).

Because eosinophil granulocytes do not express arginase-1, the regulation of NOS activity through L-arginine levels is specific to neutrophil granulocytes (Luckner-Minden et al. 2010). Why eosinophil granulocytes are able to evoke higher levels of tyrosine nitration than neutrophil granulocytes (Takemoto et al. 2007b) may be due to the lack of regulation of NOS catalytic activity. In eosinophil granulocytes, catalase and superoxide dismutase (SOD) may counteract the RNS generation, without affecting NO synthesis (Takemoto et al. 2007b).

8.5 Chapter Summary

NO in the endosomes	• Endosome-derived multivesicular bodies may emit exosomes, which contain RNS and cause nitrosative damage in tissues; or activate NO synthesis in immune cells • In phagosomes NO and RNS are pathogen killing agents. Inflammatory stimuli increase iNOS transcription and the iNOS protein is targeted from the cytosol to the phagosome membrane
NO in the lysosomes	• Lysosomes of granulocytes produce NO which leads to protein nitrosylation, affects pathogen killing and inflammation. Substrate restriction may limit NO synthesis within the lysosomes

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Chapter 9
NO Synthesis and Cell Locomotion

9.1 The Association of NO Synthesis with Cilia

Studies on the free-living freshwater ciliate *Paramecium* species provide evidence that NO synthesis affects the ciliary beat and consequent motility of cells (Malvin et al. 2003; Amaroli et al. 2006). In *Paramecium* NO is formed by a calcium dependent nNOS (NOS1)-like protein, which is distributed in the cytoplasm (Malvin et al. 2003; Amaroli et al. 2006). Blocked NO synthesis reduces the ability of cells moving toward zones with optimal temperature (Malvin et al. 2003), supporting the idea that regulation of ciliary activity by cytoplasmic NO synthesis ensures oriented cell movements. In vertebrates, ciliated epithelial cells express eNOS in the airways, oviducts, testes and cerebral ventricles (Xue et al. 1996; Zhan et al. 2003; Konig et al. 2005; Stout et al. 2007). In airway epithelia, eNOS occurs shortly after birth simultaneously with the activation of ciliary movements (Xue et al. 1996); showing that NO synthesis is required for ciliary activity and mucus forwarding over the airway epithelia.

In ciliated epithelial cells, eNOS is localized to the apical zone, in proximity to the cilia (Xue et al. 1996; Konig et al. 2005). The binding of eNOS to the basal microtubule membranes (Xue et al. 1996) and to a cilia-associated tubulovesicular system (Krasteva et al. 2007) has been shown (Fig. 9.1). The activation of NO synthesis by administering L-arginine increases ciliary beat frequency in airway epithelia (Li et al. 2000; Jiao et al. 2010). NOS inhibition leads to the consistent reduction of ciliary beat frequency (Jain et al. 1993; Kim et al. 2001; Alberty et al. 2004) and reduced NO production is associated with higher percentage of immotile cilia in the airways (Pifferi et al. 2011).

The NO/cGMP/PKG pathway may regulate ciliary movement, and control the phosphorylation state of various ciliary proteins (Li et al. 2000; Gertsberg et al. 2004). The presence of PKG in the axoneme (Gertsberg et al. 2004), the anchoring of NO-target proteins to the ciliary basal bodies (Stout et al. 2007) and the stimulatory effect of increased cGMP levels on ciliary beat frequency (Zhang and Sanderson 2003; Sisson et al. 2009) support this possibility. The apical region of the ciliated airway epithelium is abundant in caveolin-3, which ensures the anchoring of eNOS

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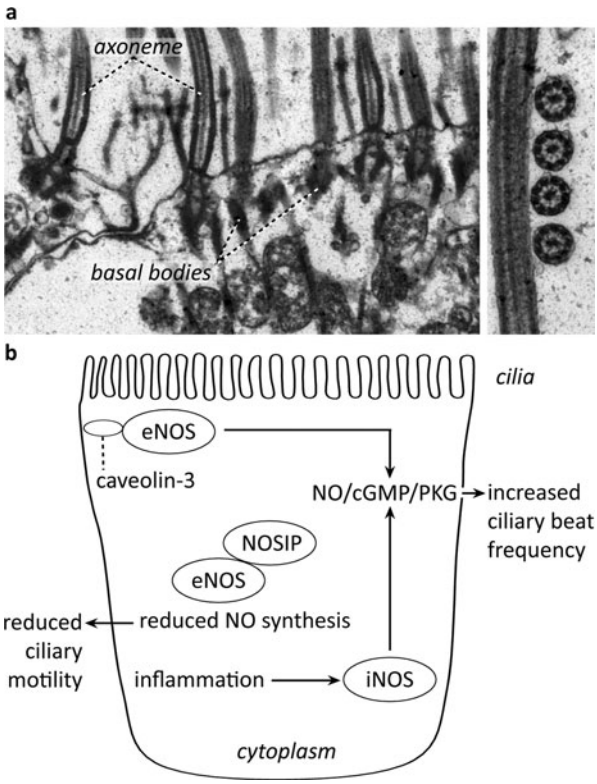

Fig. 9.1 NO synthesis in the ciliated epithelial cells. Structure of the apical region of ciliated epithelia (**a**) on the *left* longitudinal section showing the ciliary axonemes and basal bodies; on the *right* longitudinal and cross section of axonemes. Author's TEM images. The eNOS is located in the apical cell region, anchored by caveolin-3 (**b**). Association with NOSIP leads to eNOS translocation to the cytoplasm and reduced NO synthesis. Under inflammatory conditions, the iNOS level is increased in the cytoplasm, leading to elevated NO synthesis

in proximity of the cilia (Krasteva et al. 2007) (Fig. 9.1). In airway epithelia, eNOS is also associated with the NOS-interacting protein (NOSIP), which affects eNOS compartmentalization and catalytic activity (Konig et al. 2005). NOSIP binds to the carboxyl-terminal region of the eNOS oxygenase domain and promotes uncoupling of eNOS from caveolins (Dedio et al. 2001). This event leads to the translocation of eNOS from the caveolin-3 rich apical membrane regions to intracellular sites, thereby inhibiting NO synthesis (Dedio et al. 2001) (Fig. 9.1).

Although eNOS is the most relevant NOS isoform in ciliary epithelia, low levels of nNOS and iNOS have also been shown in the cytoplasm of airway epithelial cells (Xue et al. 1996; Kim et al. 2001; Jiao et al. 2010). In response to bacterial or inflammatory stimuli, iNOS expression is upregulated, which increases ciliary beat frequency and promotes the effective removal of pathogens from the airway mucosa (Fig. 9.1) (Jain et al. 1995; Ueda et al. 2001; Alberty et al. 2006).

9.2 Nitric Oxide Synthase in the Flagellum

The association of NOS with flagella has been shown in the unicellular euglenoid *Trypanosoma cruzi* and in vertebrate spermatocytes (Lewis et al. 1996; Goldstein

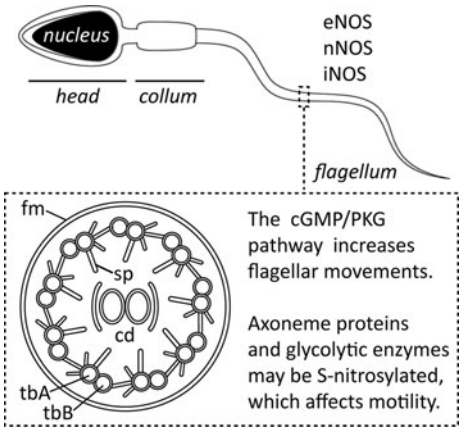

Fig. 9.2 NO synthesis in the spermatocyte axoneme. All of the three mammalian NOSs may be present in the axoneme. NO can activate the cGMP/PKG pathway or S-nitrosylate axoneme proteins. NO may also affect axoneme morphogenesis. *fm* flagellar membrane, *cd* central microtubules, *sp* radial spoke, *tbA*, *tbB* peripheral microtubules

et al. 2000). NO increases sperm motility in fish (Creech et al. 1998; Wilson-Leedy and Ingermann 2011) and mammals (Hellstrom et al. 1994; Lewis et al. 1996; Hassanpour et al. 2007; Lampiao and du Plessis 2008a; Srivastava and Agarwal 2010; Miraglia et al. 2011) and it is required for the hyperactivity reaction[1] of the mammalian spermatocyte (Yeoman et al. 1998). The inhibition of NOS or scavenging of NO reduces sperm motility (Lewis et al. 1996; Donnelly et al. 1997; Kameshwari et al. 2003; Hassanpour et al. 2007; Wilson-Leedy and Ingermann 2011). Similar to its effect on cilia, NO increases flagellar beats through the cGMP/PKG pathway (Fig. 9.2) (Miraglia et al. 2007, 2011). However, the effects of NO may be different on quiescent or activated spermatocytes (Wilson-Leedy and Ingermann 2011). For instance, administering NO inhibits cell respiration and reduces sperm motility in the fish *Oncorhynchus mykiss* (Wilson-Leedy and Ingermann 2011). In the same model, the activation of spermatocytes is associated with increased NO synthesis and this endogenous NO improves spermatocyte motility (Wilson-Leedy and Ingermann 2011).

S-nitrosylation of various proteins involved in flagellar movements (e.g. A-kinase anchoring proteins) may also affect sperm motility (Lefievre et al. 2007). Members of the A-kinase anchoring (AKAP) protein family are scaffold proteins associated with cAMP-dependent protein kinase A (PKA); they facilitate selective phosphorylation of PKA target proteins (Coghlan et al. 1993; Kurokawa et al. 2004; Langeberg and Scott 2005; Dodge-Kafka et al. 2006). AKAPs are present in the axoneme of mammalian sperm flagellum (AKAP4) (Miki et al. 2002), ciliated epithelial cells (AKAP28) (Kultgen et al. 2002; Stout et al. 2007) and in the prokaryote *Chlamydomonas* flagellum (Elam et al. 2009). The lack of AKAPs leads to disregulated cAMP/PKA signaling and impairs flagellar movements (Gaillard et al. 2001, 2006; Miki et al. 2002). AKAPs, however, not only coordinate the PKA activity in the sperm flagellum (Carr and Newell 2007), but are also required for the assembly of the flagellar axoneme. In *Chlamydomonas*, an AKAP is identical with a radial spoke

[1] Hyperactivity of flagellar movements occurs when spermatocytes reach the oocyte.

protein (Gaillard et al. 2001; Wirschell et al. 2008) which is essential for axoneme assembly (Gaillard et al. 2006). Lacking AKAP4, the progressive motility of mouse sperm is impaired due to the shortened flagellum and the imperfect organization of the fibrous sheath (a cytoskeletal structure present in the principal piece of the sperm axoneme) and impaired association of flagellar proteins (Miki et al. 2002; Carr and Newell 2007). S-nitrosylation of axonemal AKAPs therefore, has a potential impact on spermatocyte kinesis: it may affect cAMP/PKA signaling, a key regulator of sperm motility or even the morphogenesis of the flagellar axoneme (Carr and Newell 2007; Wirschell et al. 2008). Of note, proper association of glycolytic enzymes with the axoneme also depends on AKAP4, and glycolytic enzymes are subjects of S-nitrosylation in the human spermatocyte (Miki et al. 2002; Lefievre et al. 2007). This raises the possibility that NO has the potential to affect glucose utilization, the main energy supply of flagellar movements (Fig. 9.2) (Lefievre et al. 2007). Interestingly, the production of NO by oocytes also increases flagellar motility of spermatocytes (Creech et al. 1998), which may facilitate chemotaxis and fertilization (Miraglia et al. 2007).

The flagellar axoneme contains eNOS, nNOS and under inflammatory conditions, iNOS (Fig. 9.2) (Donnelly et al. 1997). A recent study shows that male infertility with asthenozoospermia[2] and reduced percentage of progressive motile sperm may be associated with a missense Glu298Asp polymorphism of the eNOS-encoding gene (Buldreghini et al. 2010). The impaired eNOS activity may explain the reduced NO synthesis and poor motility (Buldreghini et al. 2010) of asthenozoospermic spermatocytes (Lewis et al. 1996). In this scenario, the axonemal eNOS is a positive regulator of spermatocyte movements. Challenging this possibility, another study indicates that in most high motile sperm samples, eNOS and nNOS transcripts are undetectable, whereas, they are expressed in the low motile samples (Lambard et al. 2004). Similarly, the semen of normozoospermic fertile men exhibit lower NO concentrations than those of asthenozoospermic infertile men. Lower NO production is associated with improved spermatocyte motility (Balercia et al. 2004). The effects of NO on spermatocyte motility may be concentration dependent: while endogenous NO synthesis is required for flagellar movements, the overproduction of NO reduces kinesis, possibly due to its cytotoxic effects (Balercia et al. 2004). Concordant with this conclusion, a NO burst evoked by high doses of NO-donor compounds or inflammatory mediators (TNFα and IL-6) leads to spermatocyte damage (Balercia et al. 2004) and reduced motility (Hassanpour et al. 2007; Lampiao and du Plessis 2008b).

It is likely that eNOS is the physiologically important NO source, while iNOS may account for the cytotoxic overproduction of NO. This is supported by the evidences that TNFα and IL-6 increase iNOS gene expression in various cells (Winston et al. 1999) and inhibit Ser-1177 phosphorylation of eNOS thereby reducing its activity (Atochin and Huang 2010). The overproduction of NO may be a result of reduced eNOS activity overshadowed by an increased iNOS expression. Studies with NOS deficient mice also show that ablation of iNOS improves the *in vitro* fertilization rate

[2] Medical condition caused by the high percentage of spermatocytes with reduced motility in the semen.

of spermatocytes (Yang et al. 2005), suggesting that iNOS activity has a negative impact on sperm physiology. However, testicular macrophages (Winnall et al. 2011), Leydig cells, Sertoli cells and the epithelium of the epididymis and vas deferens also produce NO (Zini et al. 1996), therefore changes of NO levels in the semen do not accurately reflect the actual NO synthesis of spermatocytes. Increased eNOS activity along with reduced NOSTRIN expression has been found in spermatogonia, Sertoli cells, stromal cells and vascular endothelia in the testes of azoospermic patients (Xiang et al. 2011). Increased NO synthesis may thereby arise either from the spermatocytes or other NOS-containing testicular cells. This fact makes it difficult to interpret the correlation between sperm motility and the concentration of NO-derived decomposition products in the semen.

9.3 Amoeboid Movements and Interaction of NO with the Cytoskeleton

The presence of NOS has been shown in cells displaying amoeboid movements, such as aflagellate spermatocytes of helminthes (Pfarr and Fuhrman 2000) and the effect of NO on outgrowths of cellular protrusions has been established in differentiating or regenerating neurons (Trimm and Rehder 2004), endothelial cells (Feron and Balligand 2006; Park et al. 2010), migrating leukocytes (Thibeault et al. 2010), fibroblasts and pericytes (Lee et al. 2005).

In mammalian neurons, NO administration evokes a rapid and transient elongation of filopodia, along with a reduction of filopodial numbers (Van Wagenen and Rehder 1999). Increased NOS activity induces filopodial outgrowth (Cheung et al. 2000), the synthesized NO stabilizes growth cone morphology, while the NO-derived nitrosonium ion (NO^+), and peroxynitrite ($ONOO^-$) promotes further filopodial growth (Cheung et al. 2000). Synthesis of NO is therefore required for neuronal growth cone dynamics and path finding, and NO may function as a slow-down and searching signal for growing neuronal processes (Trimm and Rehder 2004). Consistently, in mice with deletion of nNOS, the peripheral nerve regeneration is impaired along with the altered filopodial morphology of the growth cone (Keilhoff et al. 2002). In neuronal cell precursors of the snail *Helisoma trivolvis,* endogenous NO synthesis facilitates the development of growth cones and increases filopodial length (Tornieri and Rehder 2007), showing the evolutionarily conserved role of NO in growth cone dynamics.

The activation of the cGMP/PKG pathway mediates the effects of NO on filopodial development (Fig. 9.3). Stimulation of guanylyl cyclase and increased cGMP levels stabilize filopodial development (Cheung et al. 2000), while inhibition of soluble guanylyl cyclase blocks filopodial elongation (Tornieri and Rehder 2007). Moreover, S-nitrosylation of growth cone proteins may also affect filopodial morphology (Cheung et al. 2000). In *Helisoma* growth cones, a NO-induced, cGMP-dependent ADP-ribosylation of monomeric actin (Welshhans and Rehder 2005) and activation of ryanodine receptor-mediated Ca^{2+} release (Welshhans and

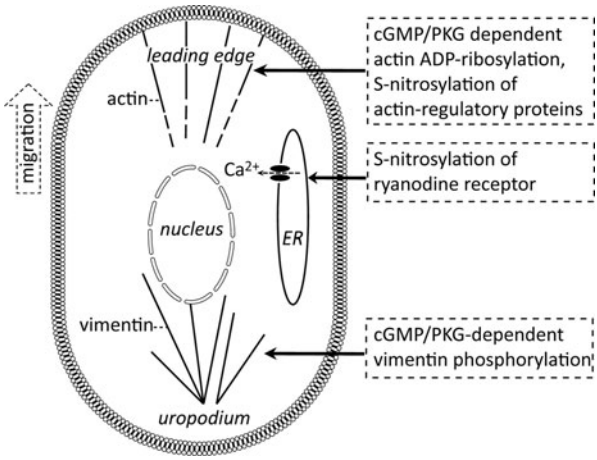

Fig. 9.3 Targets of NO, which can affect cytoskeletal structure. In migrating cells NO increases actin assembly at the leading edge (or filopodium), facilitating migration. The release of Ca^{2+} from intracellular stores and vimentin phosphorylation also affect cytoskeletal reorganization. *ER* endoplasmic reticulum

Rehder 2007) also account for changes in filopodial morphology (Fig. 9.3). Actin is a target of NO-mediated ADP-ribosylation (Clancy et al. 1995) which stimulates F-actin depolymerization (Gorodeski 2000). Ryanodine receptors undergo S-nitrosylation (Lefievre et al. 2007; Donoso et al. 2011) which is required for their channel activity (Wang et al. 2010). It is likely that S-nitrosylation of ryanodine receptors evokes Ca^{2+} release from the endoplasmic reticulum, thus contributing to filopodial morphological changes. Moreover, NO/cGMP/PKG-dependent phosphorylation of regulatory proteins required for actin polymerization, such as VASP (vasodilator-stimulated phosphoprotein) also affects cytoskeletal assembly and evokes the retraction of filopodia (Lindsay et al. 2007).

In migrating endothelial cells, NO enhances pseudopodium protrusion at the leading edge of the cell and helps the formation of the so-called uropodium at the opposite pole (Fig. 9.3) (Kevil et al. 2004). Leading edge of proliferating cells contain caveolin-1, which supports the enrichment of eNOS in the developing membrane protrusions (Garcia-Cardena et al. 1996). Endothelial cells exposed to microgravity also show increased migration along with NO release (Siamwala et al. 2010). However, in macrophages (Jun et al. 1996), neutrophil granulocytes, fibroblasts and pericytes (Lee et al. 2005), NO negatively regulates cytoskeleton assembly and consequent changes in filopodial morphology. The inhibitory effect of NO on actin polymerization reduces leukocyte adhesion and migration activity (Clancy et al. 1995; Jun et al. 1996; Ke et al. 2001). The candidate underlying mechanisms may be the NO-dependent ADP-ribosylation of actin (Clancy et al. 1995; Jun et al. 1996; Ke et al. 2001). Interestingly, disruption of actin polymerization reduces NO synthesis by the inhibition of its catalytic activity and gene expression in activated macrophages (Fernandes et al. 1996).

In activated adherent neutrophil granulocytes, the cGMP target PKG is transiently associated with vimentin intermediate filaments in the perinuclear and uropodial region (Wyatt et al. 1991). Increased NO synthesis through activation

of the cGMP/PKG pathway evokes vimentin phosphorylation (Wyatt et al. 1991) and is involved in cytoskeletal reorganization (Pryzwansky and Merricks 1998). Recent studies have revealed several key functions for dynamic and complex vimentin phosphorylation in cell attachment and migration (Ivaska et al. 2007).

9.4 Chapter Summary

NO synthesis in ciliated cells and in the flagellum	• Cytoplasmic NO synthesis provides autocrine control of ciliary beat frequency in the unicellular eukaryote *Paramecium* species • In vertebrates, ciliated epithelia contain eNOS anchored to the apical cell region. Caveolin-3 and NOSIP determine eNOS compartmentalization and activity in ciliated epithelia. NO increases ciliary beat frequency. Upregulation of cytoplasmic iNOS in airway infections facilitates the mucociliary transport • The flagellar axoneme contains NOSs and NO-target proteins; endogenous NO synthesis helps flagellar movements, although NO overproduction may be cytotoxic
Cytoskeletal rearrangements and NO	• NO affects filopodial growth, cytoskeletal actin polymerization and vimentin phosphorylation

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Chapter 10
Nitric Oxide Synthesis in the Mitochondria of Animal Cells

10.1 Effects of NO on the Mitochondria

Several studies have pointed out that NO affects distinct functions of the mitochondria, such as oxidative phosphorylation, free radical generation, membrane potential and the mitochondrial pathway of apoptosis (Brookes 2004; Taylor and Moncada 2010). The inhibitory effect of NO on mitochondrial respiration has been documented in various cell types (Cleeter et al. 1994; Bates et al. 1996; Koivisto et al. 1997; Brookes et al. 1999). The underlying mechanism is the competitive and reversible inhibition of cytochrome-c oxidase (CcO, Complex IV) and the S-nitrosylation of electron transport chain proteins (Cleeter et al. 1994; Poderoso et al. 1996; Clementi et al. 1998). Although NO competes with O_2 at CcO and thus inhibits respiration, CcO eliminates NO by oxidizing it to NO_2^- under normoxia (Cooper 2002; Cooper and Brown 2008) (Fig. 10.1). Moreover, in the presence of O_2^-, NO forms $ONOO^-$, thus the previously inhibited CcO is being reactivated (Poderoso et al. 1996). NO also mitigates CcO release and administering L-arginine and NOS-cofactors to isolated rat mitochondria increases mitochondrial respiration (McCormack and Denton 1993; Brookes et al. 2000). The effects of NO on the mitochondrial respiration depend on the local NO, O_2^- and $ONOO^-$ concentrations (Fig. 10.2). For instance, in normoxic cells NO binds to guanylyl cyclase with much higher affinity than to CcO (Rodriguez-Juarez et al. 2007) and the inhibitory effect of NO on CcO becomes prominent under O_2 limitations, when the reductive NO synthesis increases (Taylor and Moncada 2010). A sustained mitochondrial NO level may initiate hypoxic signaling and adaptation of the respiratory electron chain to hypoxia (Brookes et al. 2002; Finocchietto et al. 2009) (Fig. 10.1). A possible NO release from the hypoxic cells can evoke local vasodilation and reoxygenation of the affected tissue (Palacios-Callender et al. 2007; Taylor and Moncada 2010).

In the mitochondria, NO also increases the O_2^- and H_2O_2 production due to the reaction of NO with membrane ubiquinol (Poderoso et al. 1996; Finocchietto et al. 2009). The NO-derived reactive nitrogen species along with the NO-mediated O_2^- and H_2O_2 generation can evoke cell death (Brookes et al. 2000; Dedkova et al. 2004; Valdez et al. 2004; Parihar et al. 2008b). Protein nitration, lipid peroxidation, and

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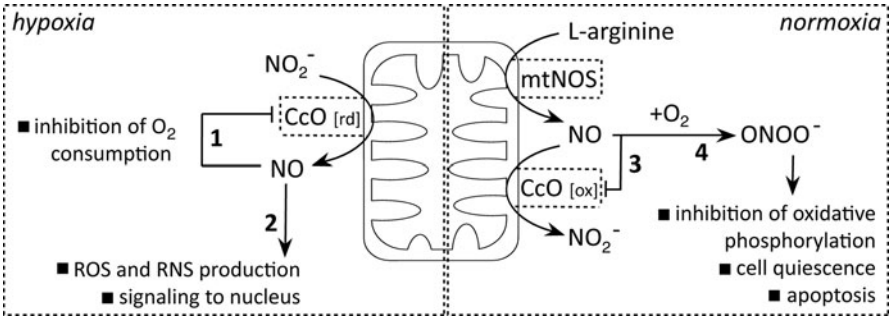

Fig. 10.1 Production and main functions of NO in the hypoxic and the normoxic mitochondrion. Under hypoxia the reduced CcO ($Cco_{[rd]}$) generates NO from NO_2^- and the produced NO inhibits CcO and O_2 consumption (**1**), forms RNS and increases ROS levels (**2**). RNS and ROS function as hypoxia signals to the nucleus and activate NF-κB and stabilizes hypoxia inducible factor signaling (Taylor and Moncada 2010). In normoxic mitochondria mtNOS generates NO from L-arginine and the NO is converted to NO_2^- by the oxidized CcO ($CcO_{[ox]}$). NO competes with O_2 and inhibits CcO (**3**) and forms $ONOO^-$ (**4**). Both NO and $ONOO^-$ inhibit ATP production, counteract cell proliferation and can induce apoptosis (Finocchietto et al. 2009; Poyton et al. 2009). Mitochondrial functions can be affected by external NO derived from other cell compartments

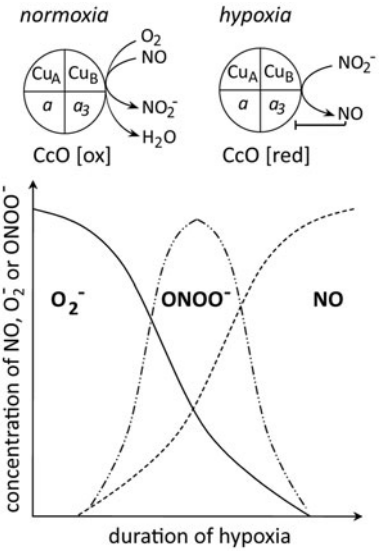

Fig. 10.2 The role of cytochrome-c oxidase (CcO) in determining mitochondrial NO levels. *Top:* CcO contains two heme (*a, a_3*) and two copper (Cu_A, Cu_B) centers. In normoxic mitochondria NO competes with O_2 for the Cu_B-a_3 binding site. The oxidized CcO (CcO [ox]) reduces O_2 to H_2O and converts NO to NO_2^-. In hypoxic mitochondria the reduced CcO (CcO [red]) generates NO from NO_2^- and NO inhibits O_2 binding at Cu_B-a_3 (Taylor and Moncada 2010). *Bottom:* Free radicals produced by mitochondria when cells experience hypoxia. Hypoxia favors NO generation. (Poyton et al. 2009)

cytochrome-c release are the main consequences of high NO levels in mitochondria, triggering the mitochondrial way of apoptosis (Finocchietto et al. 2009). NO also affects mitochondrial membrane potential, the dynamics of mitochondrial Ca^{2+} signaling and it is also implicated in mitochondrial biogenesis (Takehara et al. 1995; Nisoli et al. 2005; Davidson and Duchen 2007; Lores-Arnaiz et al. 2010). Studies using NOS deficient mice show that NO also affects the cellular localization of mitochondria (Percival et al. 2010).

Various techniques have demonstrated the presence of NO within the mitochondria (Lopez-Figueroa et al. 2000; Nohl et al. 2000; Dedkova et al. 2004; Lores-Arnaiz et al. 2010). However, mitochondria may also function as a cellular sink for NO, thus the presence of NO or NO-derivative compounds within the mitochondria does not necessarily reflect a local NO synthesis (Brookes 2004). Production of L-citrulline from L-arginine by the urea cycle is also associated with the mitochondria, which makes it difficult to identify NOS-like activity based on the measurement of L-arginine/L-citrulline conversion (Brookes 2004). Importantly, cellular NO levels are affected secondarily by mitochondrial function (Tirosh et al. 2001), thereby a mitochondrion-dependent and a mitochondrion-derived NO production can exist in the animal cell (Brookes 2004).

10.2 Oxidative NO Synthesis in the Mitochondria

Early studies show the colocalization of eNOS and the mitochondrial enzyme succinate dehydrogenase in mammalian mitochondria (Kobzik et al. 1995; Frandsen et al. 1996) and eNOS has been localized to the inner mitochondrial membrane by immuno-gold labeling (Bates et al. 1995). The possible colocalization of NOS and the respiratory chain member CcO has also been reported (Elfering et al. 2002), although the accuracy of this finding has been challenged (Brookes 2004). Other studies suggest that the mitochondrial NOS (mtNOS) may be identical with nNOS or iNOS, depending on the cell type (Bates et al. 1996; Koivisto et al. 1997; Carreras et al. 2002; Riobo et al. 2002; Valdez et al. 2004). Biochemical analysis shows that the mitochondrial inner membrane contains a Ca^{2+}-dependent mtNOS, which produces NO from L-arginine in a constitutive manner (Bates et al. 1995, 1996). This ability of oxidative NO synthesis is a distinctive characteristic of animal cell mitochondria: in plants and fungi the presence of a mitochondrial NOS has not yet been confirmed (Chaps. 4, 5).

Mitochondria isolated from mice lacking nNOS fail to release NO, confirming that nNOS is responsible for the mitochondrial oxidative NO synthesis (Kanai et al. 2001). Recent studies point out that mtNOS derives from a cytosolic nNOSα, a splice variant of nNOS (Finocchietto et al. 2009). The mitochondrial genome lacks NOS-coding sequences and thus, is unable to synthesize NOS independently from the nuclear genome (Finocchietto et al. 2009). The cytoplasmic nNOSα undergoes processing which results in the excision of its N-terminal PZD domain and a dual fatty acylation (myristoylation and palmitoylation) (Finocchietto et al. 2009). The mitochondrial

nNOSα has lower molecular weight than the cytoplasmic enzyme—e.g. in the rat brain 157 kDa in the cytoplasm and 144 kDa in the mitochondria (Riobo et al. 2002)—and its fatty acylation allows binding to the inner mitochondrial membrane. There is an additional variation in the apparent molecular weight of mitochondrial nNOS in various tissues, e.g. 144 kDa in the rat brain, 159 kDa in the rat gastrocnemius muscle (Carreras et al. 2002; Riobo et al. 2002), which reflects a cell type specific post-translational modification of nNOSα.

Mitochondrial translocation of nNOSα is low in embryos and increases with postnatal development (Carreras et al. 2001; Riobo et al. 2002; Valdez et al. 2004; Finocchietto et al. 2008, 2009). Some adaptive conditions, such as hypothermia or hypoxia also increase the mitochondrial nNOSα pool (Finocchietto et al. 2009). Under inflammatory conditions, iNOS or a lipopolysaccharide-induced NOS isoform can also be present in the mitochondria (Finocchietto et al. 2009; Aguirre et al. 2011). In ischemic cardiomyocytes eNOS can translocate from caveolae to mitochondria (Sun et al. 2012). However, the mitochondrial entry of NOSs is still undefined. The candidate mechanisms may involve Akt-mediated phosphorylation or association with caveolin-1, Hsp90, Hsp70 or dystrophin (Finocchietto et al. 2009).

10.3 Reductive NO Generation

Animal cell mitochondria show reductive NO synthesis from NO_2^-, similar to the NO generation mechanism of certain prokaryote cells (Chap. 2) and mitochondria in plants and fungi (Chaps. 4, 5). The NO_2^-/NO converting ability of mitochondria is associated with the respiratory electron transport chain, which may utilize NO_2^- instead of O_2.

In respiring mitochondria, the ubiquinone/cytochrome be_1 reduces the added NO_2^- to NO, as shown in a study of isolated rat liver mitochondria using the deoxyhemoglobin nitrosylation technique and electron spin resonance-signals to detect the NO released (Kozlov et al. 1999). The reduction of NO_2^- to NO requires NADH, NADPH, flavoproteins, and a functional cytochrome-c complex (Reutov and Sorokina 1998). Since NO_2^- is one of the main decomposition products of NO, this mechanism allows the recycling of NO_2^- to the biologically active NO in the mitochondria (Kozlov et al. 1999; Nohl et al. 2000). Under O_2 limitation, similar to hypoxic yeasts, rat hepatocyte mitochondria produce NO from NO_2^- by CcO (Castello et al. 2006). Under normoxia, CcO oxidizes NO to NO_2^- and uses O_2 as a terminal electron acceptor. When O_2 is limited, CcO is being reduced, and its NO oxidizing ability falls, which leads to local NO and reactive oxygen species (ROS) accumulation (Finocchietto et al. 2009). The possible NO_2^-/NO reducing ability of the reduced CcO may further increase the mitochondrial NO levels. Generation of NO and ROS in the hypoxic mitochondria can increase hypoxic signaling (e.g. stabilization of hypoxia-inducible factor, activation of NF-κB pathway) thereby constitute an important element in the cellular adaptation to O_2 limitation (Taylor and Moncada 2010).

The nitrite reductase activity of deoxygenated hemoglobins such as myoglobin, hemoglobin and neuroglobin are also known in animal cells (Shiva et al. 2011; Tiso et al. 2011). For instance, the addition of deoxymyoglobin and NO_2^- to isolated rat heart and liver mitochondrial homogenates results in NO generation(Shiva et al. 2007, 2011; Tiso et al. 2011). Although accumulating evidence shows that neuroglobin is involved in the regulation of mitochondrial respiration and the cellular redox state (Yu et al. 2009; Brittain et al. 2010), the possible physiological relevance of deoxygenated hemoglobins in mitochondrial NO synthesis should be determined (Smagghe et al. 2008).

As in prokaryotes, plants and fungi, the reductive NO production favors hypoxic conditions (Fig. 10.2). An interesting vertebrate model to study the effects of hypoxia is the goldfish, *Carassius auratus*, since this species may tolerate long-lasting O_2 limitation (Hansen and Jensen 2010). A study on the systemic level of NO metabolites in goldfish shows that exposure to hypoxia for two days causes robust decrease in plasma NO_2^-, suggesting a reduced NOS activity and increased NO_2^- utilization. Since NOS requires O_2 for NO synthesis and hypoxic mitochondria increase their NO_2^-/NO reduction, it is tempting to assume that these changes may explain the observed fall in systemic NO_2^- levels (Hansen and Jensen 2010).

10.4 Mammalian AtNOS1 Ortholog is Present in the Mitochondria

A mammalian ortholog of *Arabidopsis thaliana* NOS-1 (mAtNOS1 or NOA1) has been identified and its association with the inner mitochondrial membrane has been shown in the mouse cell (Zemojtel et al. 2006b). Studies with mAtNOS1 deletion mutants and green fluorescent protein fused mAtNOS1 protein show that the N-terminal 60 amino acid-sequence ensures mAtNOS1 mitochondrial targeting (Zemojtel et al. 2006b). As we have discussed in previous chapters, *Arabidopsis thaliana* NOS-1 (AtNOS1) was first described as a plant-type NOS enzyme associated with the mitochondria (Guo et al. 2003) (Chap. 4). However, its lack of NO forming ability has been demonstrated (Moreau et al. 2008) and that AtNOS1 belongs to the family of small GTP-binding proteins has been clarified (Moreau et al. 2010). It is postulated that AtNOS1 may be a NOS-associated protein, which secondarily affects NO synthesis, thus it is often termed as *Arabidopsis thaliana* NOS-associated protein-1 (AtNOA1) (Sudhamsu et al. 2008; Sun et al. 2010; Majlath et al. 2011). However, there is no definitive evidence to support that AtNOA1 binds to a mitochondrial NOS. It has also been proposed that AtNOA1 affects mitochondrial protein translation and mitochondrial ribosome assembly, which may indirectly alter NO production (Zemojtel et al. 2006a; Kolanczyk et al. 2011).

Although AtNOA1 (AtNOS1) and mAtNOS1 are not functional NO forming enzymes, their close vicinity to the respiratory electron transport chain may allow interaction with the reductive NO synthesis (Zemojtel et al. 2006b). Although it has not yet been validated, this possibility would explain the NO-modulating effects

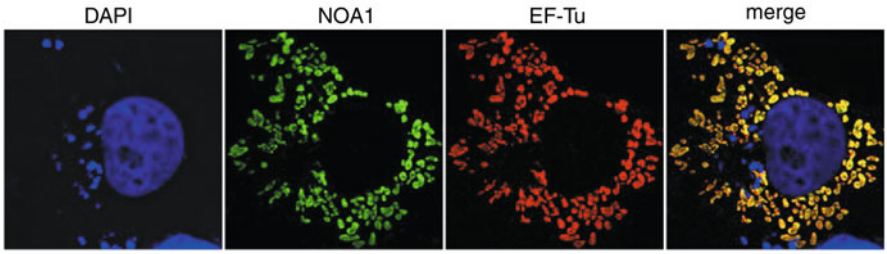

Fig. 10.3 Mitochondrial localization of mouse NOA1, the mammalian ortholog of AtNOS1. COS1 cells were transiently transfected with V5-His-tagged mouse NOA1 (*green*) and FLAG-tagged mitochondrial Tu translation elongation factor EF-Tu (*red*). EF-Tu is a mitochondrial ribosome protein. The nucleus was counterstained with DAPI. Overlapping signals for NOA1 and EF-Tu suggest the colocalization of the two proteins within the mitochondria. The involvement of both EF-Tu and NOA1 in the biogenesis of the mitochondrial ribosome has been confirmed (Kolanczyk et al. 2011). Original images with the courtesy of Dr. Mateusz Kolanczyk

of these proteins. Studies using over- and underexpressing mAtNOS1 in human neuroblastoma and mammary adenocarcinoma cells show that mAtNOS1 is involved in certain mitochondrial functions; increases mitochondrial protein tyrosine nitration, affects mitochondrial transmembrane potential, evokes cytochrome-c release and apoptosis (Parihar et al. 2008a, 2008c). These *in vitro* studies support that mAtNOS1 is involved in the control of the mitochondrial way of apoptosis. Mitochondrial mAtNOS1 also affects NO levels, which might be involved in the initiation of the apoptosis program (Parihar et al. 2008b).

The expression of mAtNOS1 mRNA in the mouse embryonic liver, the developing central nervous system and in the ossification centers of bones validates the *in vivo* relevance of mAtNOS1 (Zemojtel et al. 2006b). The expression pattern suggests that this mitochondrial protein may be involved in hepatic hematopoiesis and bone formation (Zemojtel et al. 2006b). A most recent work has demonstrated that mAtNOS1 is required for normal development of the mouse embryo and extraembryonic tissues in the placenta and the trophoblast (Kolanczyk et al. 2011). The ablation of mAtNOS1 causes embryonic lethality due to severe growth retardation (Kolanczyk et al. 2011). Inactivation of *Noa1* (encoding NOA1, the equivalent of mAtNOS1) impairs mitochondrial protein synthesis and causes global defect of oxidative phosphorylation; consequently, NOA1 deficient cells suffer ATP deficit under nutrient starvation and show reduced viability (Kolanczyk et al. 2011). These studies have pointed out that the mammalian AtNOS1 ortholog is a mitochondrial ribosome protein, required for mitochondrial protein synthesis, ATP production, cell survival and control of apoptosis (Kolanczyk et al. 2011) (Fig. 10.3). This protein may be an interface between the mitochondrial and the caspase-mediated apoptotic pathways, since the lack of mAtNOS1 activates the mitochondrial way of apoptosis and compromises caspase-mediated cell death (Parihar et al. 2008a, 2008c; Kolanczyk et al. 2011).

10.5 Chapter Summary

NO and mitochondrial functions	• Mitochondrial respiration, O_2 consumption, adaptation to hypoxia, mitochondrial biogenesis and cellular distribution of mitochondria are targets of NO • Mitochondrial NO burst is involved in apoptosis
Mitochondrial NO synthesis	• Endogenous NO generation is associated with a NOS-like activity under normoxia and with CcO under O_2 limitation. Targeting mechanism of NOSs to the mitochondria is still undefined

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Chapter 11
Peroxisomes: Where NOS Rests in Peace?

11.1 NOS is Associated with Peroxisomes in Animal Cells

Peroxisomes or microbodies are 0.2–1 μm sized vesicular or tubular organelles surrounded with a single membrane bilayer (Singh 1996; Schrader and Fahimi 2008). They are present in almost all types of animal cells and particularly abundant in vertebrate hepatocytes where they comprise ~1% of the total cell volume (Fig. 11.1) (Schrader and Fahimi 2008). Peroxisomes are involved in lipid metabolism (oxidation of pipecolic, phytanic and very-long chain fatty acids; synthesis of plasmalogens and bile acids) and they are the key organelles of free radical detoxification (Parsons 2004; Schrader and Fahimi 2008). Although an endosymbiont origin of peroxisomes has been hypothesized, recent findings show that peroxisomes are specific to eukaryotes and are possibly derived from the endoplasmic reticulum (Gabaldon et al. 2006).

The peroxisome matrix contains more than 50 enzymes (e.g. the complete human peroxisomal proteome is encoded by 85 genes; for more details see Peroxisome Database, www.peroxisomedb.org), including H_2O_2-producing oxidases, H_2O_2-decomposing catalase (CAT), enzymes of the fatty acid oxidation and amine synthesis (Schrader and Fahimi 2004, 2008; Schluter et al. 2010). The association of NOS with the peroxisome matrix has been detected in rat and human hepatocytes (Stolz et al. 2002; Collins et al. 2003; Loughran et al. 2005) and lipopolysaccharide-stimulated mouse dendritic cells (Heijnen et al. 2006). The presence of 3-nitrotyrosine has also been shown in mouse peroxisomes, which is indicative of ongoing NO generation and protein nitration within these organelles (Heijnen et al. 2006).

11.2 Debated Function of Peroxisomal NOS in Animal Cells

Compared to the NOS-associated peroxisomal functions in plant cells (Del Rio 2011) (Chap. 4), the biological relevance of peroxisomal NO synthesis in animal cells is still debated (Fig. 11.2). An early study suggests that the activity of peroxisomal enzymes is affected by NO: increased NO synthesis inhibits CAT activity and stim-

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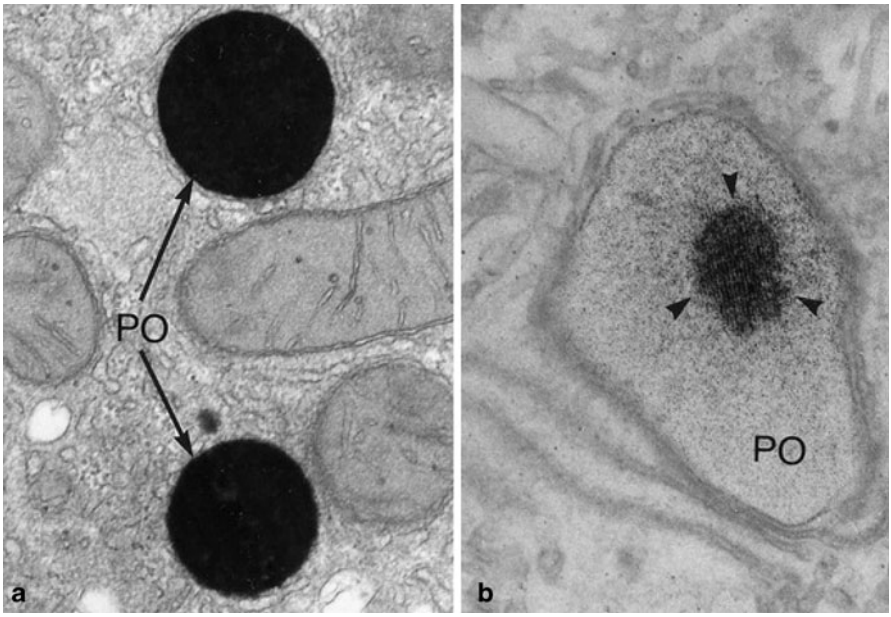

Fig. 11.1 Peroxisome ultrastructure in rat liver. Cytochemical localization of characteristic peroxisomal proteins catalase (**a**) and urate oxidase (**b**) in the peroxisome (*PO*) matrix. Catalase is stained with the alkaline diamino-benzidine technique. Mitochondria in vicinity of peroxisomes are also visible. Magnification × 28,600. Urate oxidase is visualized using the cerium method. Note the dark staining of the crystalline core of the peroxisome (*arrowheads*). Magnification × 50,400. Images reprinted with permission. (Schrader and Fahimi 2008)

ulates peroxisomal β-oxidation in human fibroblast cultures (Kremser et al. 1995). Since cGMP inhibits the activity of peroxisomal enzymes (CAT, acyl-CoA-oxidase and dihydroxyacetone-phosphate acyltransferase), it has been postulated that NO produced in response to cytokine stimuli affects peroxisomal functions by cGMP synthesis (Dhaunsi et al. 2004). Interestingly, L-carnitine, which displays antioxidant benefits and acts against lipid peroxidation, also prevents this cGMP-mediated impairment of peroxisomal enzyme activities (Dhaunsi et al. 2004). Moreover, L-carnitine inhibits NOS activity in cells with functional peroxisomes (Koeck and Kremser 2003), suggesting that changes in peroxisomal free radical decomposition might influence NOS. The possible role of NO in peroxisomal metabolism and the putative interplay between peroxisome function and NO synthesis, however, remains unknown.

A more recent study challenges the impact of NO on peroxisome activity and shows that peroxisomes are intracellular deposits of inactivated iNOS (Loughran et al. 2005) (Fig. 11.2). This study shows that two subcellular reserves of iNOS exist in cytokine-stimulated hepatocytes: a cytoplasmic and a peroxisome-specific iNOS pool (Loughran et al. 2005). The peroxisomal iNOS pool shows reduced catalytic activity, which might be explained by monomerization of iNOS and the low tetrahydrobiopterin (BH_4) availability within the peroxisome matrix (Loughran et al. 2005). Momomeric iNOS generates O_2^- which may be harmful in the cytoplasm

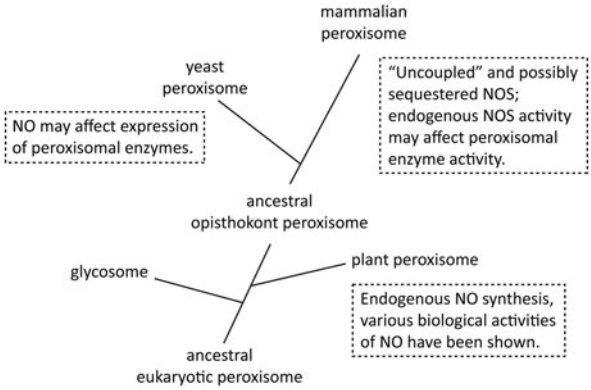

Fig. 11.2 The possible phylogenic tree of peroxisomes and functions of the peroxisomal NO synthesis. Proteomic analyses show the high level of evolutionary plasticity of peroxisomes (Gabaldon et al. 2006). NOS may be associated with the peroxisomes of evolutionarily distinct eukaryotes, although there is a considerable difference between plant, fungi and animal cells regarding the impact of their peroxisomal NO synthesis. Glycosomes contain glycolytic enzymes and occur in the unicellular parasite *Trypanosoma* species (Parsons 2004); *opisthokonts* represent a common ancestral group of fungi and metazoa. (Wainright et al. 1993)

(Xia et al. 1998). Consequently the deposition of monomeric iNOS within the peroxisomes might be a protective mechanism against NOS-induced oxidative damage. The low BH_4 environment within the peroxisome creates "uncoupling" conditions for the iNOS, possibly further increasing its O_2^- production. Peroxisomal iNOS activity thus results in $ONOO^-$ generation which may account for the protein nitration observed in the peroxisome matrix (Heijnen et al. 2006). The oxygenase domain of iNOS is capable of degrading $ONOO^-$ and the produced reactive nitrogen intermediate leads to nitration and inhibition of iNOS (Marechal et al. 2007) (Fig. 11.3). Similar activity of nNOS and *Bacillus subtilis* NOS has also been detected (Marechal et al. 2010), suggesting that an autocatalytic inhibition of NOSs by $ONOO^-$ cleavage may be an evolutionarily conserved mechanism. Its relevance in peroxisomal $ONOO^-$ turnover, however, should be addressed by future studies.

Collectively, capturing of iNOS within the peroxisomal matrix keeps NOS inactivated (Fig. 11.3). Multiple factors ensure the inactivation of peroxisomal NOS: monomerization, low cofactor and substrate availability and a possible autocatalytic nitration event (due to the increased O_2^- and $ONOO^-$ generation of the monomeric NOS). However, this model is feasible only in long-lived cells such as hepatocytes, where the transient upregulation of iNOS expression is followed by a "recovery" state, in which iNOS expression declines. When iNOS is no longer necessary, peroxisomes may sequester cytoplasmic iNOS molecules in these cells. However, in other cells expressing iNOS, such as granulocytes or macrophages, increased iNOS expression evokes a cytotoxic NO-burst and leads to the death of NO-producing cells (Winston et al. 1999). These cells, therefore, do not require safe removal of iNOS, which might explain the lack of association of peroxisomes and iNOS in e.g. granulocytes and macrophages (Vodovotz et al. 1995).

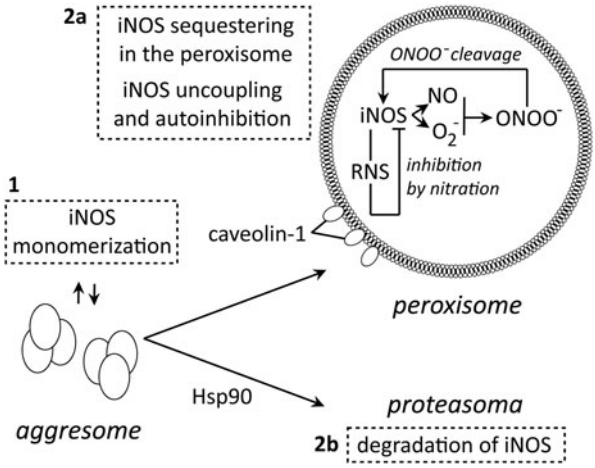

Fig. 11.3 Hypothetical trafficking pathway of momomeric iNOS to the peroxisome. iNOS monomers produce free radicals in the cytoplasm, consequently the cells eliminate iNOS by proteolytic degradation in the proteasoma, or by sequestering it into the peroxisomes. *Aggresomes* may represent a decision point: Hsp90 may guide iNOS to the proteasome or iNOS may be activated again by dimerization in the cytoplasm. Association of iNOS with the peroxisomal caveolin-1 may help peroxisomal targeting and ultimate inactivation. Within the peroxisomes iNOS produces NO and O_2^- (due to the low BH_4 levels), which form $ONOO^-$. Cleavage of $ONOO^-$ by the monomeric iNOS generates reactive nitrogen species (*RNS*), which nitrate the iNOS molecule further diminishing its activity

This scenario implies that liver peroxisomes act as intracellular burial places of iNOS, although the mechanism of iNOS import to the peroxisomes is still an open debate (Fig. 11.3). The peroxisome matrix contains monomeric iNOS molecules, however, iNOS monomerization alone is not sufficient to serve as a peroxisome-targeting code (Loughran et al. 2005). The palmitoylation of iNOS at the cysteine-3 residue allows its binding to intracellular membranes (Vodovotz et al. 1995; Navarro-Lerida et al. 2006; Villanueva and Giulivi 2010). iNOS is also capable of establishing a microtubule association for intracellular trafficking (Navarro-Lerida et al. 2006; Villanueva and Giulivi 2010). Hepatocyte peroxisome membranes also contain microdomains enriched in caveolin-1 (Woudenberg et al. 2010), and caveolin-1-dependent sequestration of iNOS has been shown in detergent-insoluble compartments (possibly identical with peroxisomes) in human carcinoma cells (Felley-Bosco et al. 2002). These interactions may allow iNOS to target the peroxisome membrane, however, the mechanism of entry is still unknown.

Peroxisome membranes contain transport proteins (belonging to the peroxins family), which facilitate the import of folded or oligomerized proteins into the peroxisome matrix (Wolf et al. 2010; Rucktaschel et al. 2011). This import mechanism depends on peroxisomal targeting signals (PTS) which are recognized by distinct peroxins (Wolf et al. 2010). The sequence of iNOS lacks canonical PTSs, however, a variation of two peroxisomal targeting sequences (PTS1 and PTS2) is present in the iNOS molecule, which might allow iNOS to enter the peroxiomes (Loughran et al. 2005). Of note, the lack of PTSs does not exclude peroxisomal import, since some

peroxisomal proteins are targeted to the peroxisome matrix through interactions with PTS-containing protein partners (Wolf et al. 2010). Peroxin 5p (Pex5p) also allows peroxisomal entry of proteins lacking PTS (van der Klei and Veenhuis 2006) and it is likely that Pex5p may help peroxisomal traffick of iNOS (Del Rio 2011).

11.3 Aggresome: Another Sink for Unwanted NOS Proteins?

Aggresomes are unique subcellular compartments anchored to microtubules and formed by the aggregation of misfolded proteins (Johnston et al. 1998). These structures sequestrate proteins ultimately destined for degradation. It has been shown that iNOS may occur in aggresomes and catalytic activity of aggresomal iNOS is reduced (Kolodziejska et al. 2005) (Fig. 11.3). For instance, in cytokine stimulated human bronchial epithelial cells, iNOS is progressively sequestered in aggresomes and this process correlates with marked reduction of NO synthesis (Kolodziejska et al. 2005; Pandit et al. 2009).

More recently, other NOS isoforms have also been described in aggresomes: in cortical neurons nNOSα and nNOSβ occur in aggresomes (Corso-Diaz and Krukoff 2010). Evidence supports that Hsp90 inhibits the aggresomal storage of nNOS and facilitates its proteasomal degradation (Corso-Diaz and Krukoff 2010). It is tempting to speculate that aggresomal deposition of NOS may be followed by its translocation to the peroxisomes or to the proteasomal system. It has also been shown that aggresomes function as reservoirs for latent iNOS and they delay its proteolysis (Kolodziejska et al. 2005) (Fig. 11.3). Although the role of aggresomes in NOS protein turnover is still largely unknown, aggresomal inclusion of NOS may be a decision point: NOSs may be temporarily stored in the aggresomes and than reused or degraded. Peroxisomal deposition may be an ultimate and irreversible inactivation mechanism of iNOS.

11.4 Chapter Summary

NOS in the peroxisome	• iNOS may be present in the peroxisomes of certain animal cell types, in a monomeric and uncoupled form, which produces free radicals
Inactivation of NOS in the peroxisomes	• Low BH_4 levels and lack of L-arginine supply limit NO production • iNOS undergoes self-nitration, which further inhibits its activity
How can NOS enter the peroxisomes?	• Peroxisome membrane contains caveolin-1, which may facilitate peroxisomal NOS targeting • iNOS contains a variant of PTS sequences, which may help peroxisome entry
Biological function of peroxisomal NOS	• Peroxisomal enzyme activities may be affected by NO, although it is debated that NOS has a specific function in the peroxisomes of animal cells

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Chapter 12
Subcellular Redistribution of NOS

12.1 Membrane Targeting and Release of eNOS from the Caveolae

In endothelial cells, eNOS is present at the plasma membrane caveolae and at the Golgi-system (Oess et al. 2006) (Chaps. 6 and 7). eNOS has a dynamic relocation ability, possibly mediated by vesicular transport or cytoskeletal components (Oess et al. 2006; Schilling et al. 2006), although the details of this anterograde and retrograde transport are still unknown (Fig. 12.1). For instance, insulin triggers anterograde eNOS translocation from the Golgi-system to the cell membrane caveolae (Wang et al. 2009). This mechanism requires eNOS/caveolin-1 palmytoylation and the phosphatidylinositol 3-kinase (PI3K) pathway (Wang et al. 2009). As a result, insulin increases the caveolar eNOS pool and phosphorylates eNOS, increasing its catalytic activity (Wang et al. 2009; Fleming 2010). Lacking caveolin-1, eNOS does not translocate to the caveolae and due to the lack of the inhibitory caveolin-1 effect (Chap. 6), a cell destructive NO production can occur (Wang et al. 2009). Similarly, the phosphorylation of caveolin-1 also leads to the release of eNOS and consequent nitrosative injury (Mastronardi et al. 2010). In cardiomyocytes, the lack of the caveolin-1 also leads to eNOS release and nitrosative cell damage (Wunderlich et al. 2006). Dissociation of eNOS from the caveolae and its redistribution to the mitochondria, along with increased S-nitrosylation of mitochondrial proteins has been reported in hypoxic cardiomyocytes (Sun et al. 2012). Under pathological conditions eNOS may lose its ability to anchor the cell membranes (Mukhopadhyay et al. 2007). For example, cholesterol depletion from the cell membrane impairs the caveolar structure and eNOS releases into the cytosol (Nuszkowski et al. 2001). Hypochlorite-modified low-density lipoproteins, which accumulate in atherosclerotic plaques, also evoke eNOS release from the cell membrane and from the Golgi-system (Nuszkowski et al. 2001). A cytosolic eNOS mislocalization in pulmonary hypertension is associated with the impairment of the Golgi-system structure and reduced NO-bioavailability in vascular cells (Mukhopadhyay et al. 2007, 2008; Lee et al. 2009, 2011).

The retraction of eNOS from the cell membrane caveolae occurs physiologically in endothelial cells, mediated by NOSTRIN, an eNOS-associated protein (Schilling

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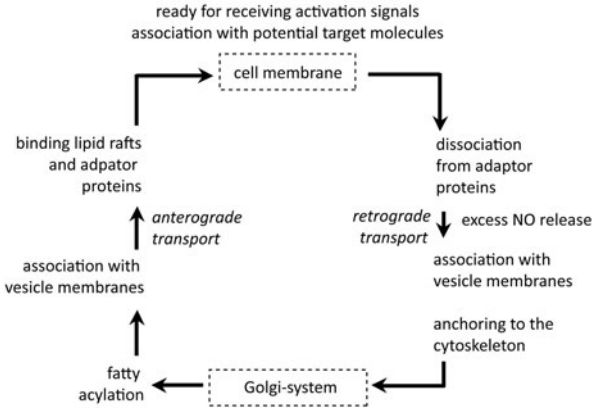

Fig. 12.1 Redistribution of eNOS between the Golgi-system and the plasma membrane caveolae. Fatty acylation possibly takes place at the Golgi-system and vesicles transport eNOS to the plasma membrane. The main binding partner of eNOS is caveolin-1, which anchors it to the plasma membrane caveolae. Regulatory proteins and NO-targets (e.g. guanylyl cyclase) are also associated with the membrane-bound eNOS, ensuring a properly balanced NO synthesis. Dissociation of eNOS from the cell membrane increases NO synthesis without a negative feedback. The retrograde eNOS transport may also be part of its redistribution to the Golgi-system

et al. 2006) (Chap. 6). A synthetic peptide (a fragment of the NOS reductase domain) also evokes retrograde eNOS transport from the cell membrane to the perinuclear Golgi-system (Hutchinson et al. 2009). In this process, the eNOS activity increases because of its lack of binding to caveolin-1 (Hutchinson et al. 2009). In snail neurons a similar redistribution of a NOS-associated protein has been found from cytosol to membranes, leading to reduced NO synthesis (Rőszer et al. 2010).

12.2 Mislocalization of Sarcolemmal nNOS in Muscle Dystrophies

In skeletal muscle nNOSμ is associated with the sarcolemmal membrane as a member of the dystrophin glycoprotein complex (DGC) (Oess et al. 2006; Lai et al. 2009), therefore DGC integrity is essential for the recruitment of nNOSμ to the sarcolemma (Fanin et al. 2009). Two proteins of the DGC, α1-syntrophin and dystrophin are responsible for the stabilization of nNOSμ: α1-syntrophin binds the N-terminal PDZ domain of nNOSμ, while dystrophin anchors the complex to other members of the DGC (Brenman et al. 1995; Kameya et al. 1999; Oess et al. 2006; Lai et al. 2009; Li et al. 2011a). Dystrophin deficiency (e.g. in Duchenne muscular dystrophy or in *mdx* muscle dystrophic mouse) (Brenman et al. 1995), lack of α1-syntrophin (Kameya et al. 1999), instability or disintegration of the DGC, reduce the sarcolemmal nNOSμ pool and lead to nNOSμ mislocalization to the sarcoplasm (Fanin et al. 2009). Clinical studies show that in patients with inherited or acquired

myopathic conditions, neurogenic conditions and hypotonia, the sarcolemmal nNOS staining is reduced or absent along with DGC disintegration (Finanger Hedderick et al. 2011). Similarly, in mouse models of unloading or denervation-induced muscle atrophy and amyotrophic lateral sclerosis, nNOS dissociates from α1-syntrophin and redistributes to the cytoplasm (Suzuki et al. 2010). In these pathologies, the expression pattern of DGC components is not affected and the mechanism of nNOS release from the intact DGC is still undefined (Suzuki et al. 2010).

The absence of sarcolemmal nNOS and its relocation to the cytoplasm is accompanied with muscle weakness, increased fatigability and progressive decline of muscle mass (Brenman et al. 1995; Suzuki et al. 2010), Under physiological conditions, sarcolemmal NO synthesis evokes vasodilation in the arteries supplying the skeletal muscles (Percival et al. 2010) (Chap. 6). The locally produced NO increases the blood and oxygen supply of the contracting fibers since it antagonizes the α-adrenergic vasoconstriction of the small muscle arteries (Heydemann and McNally 2009). In muscle dystrophies, the lack of sarcolemmal NO synthesis and the consequent impairment of blood flow aggravates muscle disease (Grange et al. 2001). However, mice lacking nNOS do not show muscle dystrophy, although their skeletal muscle bulk decreases along with the reduction of maximum tetanic force and increased susceptibility to contraction-induced fatigue (Stamler and Meissner 2001). Another study shows that simultaneous ablation of nNOSμ (sarcolemmal) and nNOSβ (Golgi-system associated) is required to impair muscle performance (Percival et al. 2010). The lack of sarcolemmal NOS and the consequently impaired blood flow therefore can explain only the functional ischemia, but this effect alone is not sufficient to induce muscle dystrophy.

Accordingly, in nNOS knockout or selective nNOS inhibitor-treated mice, denervation-induced muscle atrophy is slightly blunted (Suzuki et al. 2010). Similarly, the elimination or inhibition of nNOSμ in dystrophin-deficient mice increases muscle force (Li et al. 2011b). These findings suggest that failure to anchor nNOS to the sarcolemma impairs muscle function more severely than the lack of nNOS. Since dystrophic *mdx* mice exhibit nNOS mislocalization in the muscle fibers, high levels of lipid peroxidation and protein nitration along with the reduced muscle force, the underlying mechanism may be a nitrosative damage evoked by the mislocated nNOSμ (Li et al. 2011b).

It has been shown that sarcolemmal nNOSμ binds to caveolin-3, another protein of the DGC, which inhibits NO synthesis (Chap. 6). This inhibitory effect is lost with the dissociation of nNOSμ from the DGC. The lack of caveolin-3 also leads to muscle dystrophy (e.g. rippling muscle disease), although nNOSμ is not dislocated from the sarcolemma (Kubisch et al. 2003; Gazzerro et al. 2011). In muscle fibers of dystrophic patients with caveolin-3 deficiency, an increased nNOS activity has been shown, and a consequent nitrosative injury may provide explanation for the impaired muscle physiology (Gossrau 1998). In this scenario, the loss of nNOSμ-caveolin-3 interaction—either by the dislocation of nNOSμ or the lack of caveolin-3—leads to an increased NO synthesis which evokes nitrosative damage of the muscle fibers (Li et al. 2011b).

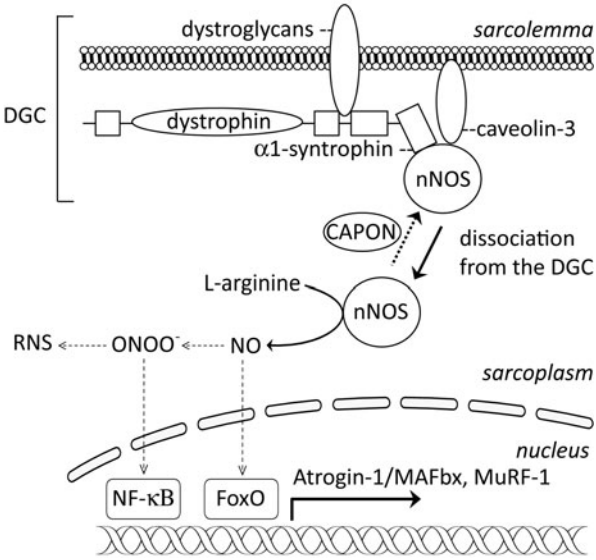

Fig. 12.2 Effects of nNOSμ mislocalization in the skeletal muscle fibers. The sarcolemmal nNOSμ anchors to the dystrophin-glycoprotein complex (*DGC*) through α1-syntrophin and dystrophin. In various forms of muscle dystrophies, the instability of DGC or the lack of α1-syntrophin or dystrophin leads to the release of nNOSμ. In muscle atrophy, nNOSμ also dissociates from the DGC and relocates to the sarcoplasm. CAPON counteracts the dissociation. Lack of nNOSμ-caveolin-3 interaction increases NO production of the mislocated enzyme. Increased production of reactive nitrogen species (*RNS*) may lead to the nitrosative damage of muscle proteins. Sarcoplasmic NO synthesis by the mislocated nNOS also activates FoxO transcription factors and upregulates genes involved in the catabolism of muscle proteins. Similarly, the NO-derived $ONOO^-$ increases DNA binding of NF-*κB*

Another model, explaining the association between atrophic muscle pathology and nNOS mislocation postulates, that sarcoplasmic NO synthesis activates the transcription of genes involved in muscle protein degradation. The mislocated nNOS synthesizes NO in the sarcoplasm and consequently activates FoxO (Forkhead Box O) transcription factors. FoxO transcription factors upregulate gene expression of muscle-specific E3 ubiquitin ligases: MuRF-1 (Muscle RING-Finger-1) and atrogin-1/MAFbx (Muscle Atrophy-F-box), both are involved in ubiquitination and proteasomal degradation of muscle proteins (Biedasek et al. 2011) (Fig. 12.2).

It has also been shown that $ONOO^-$ but not NO, causes NF-*κB* activation and increased degradation of muscle-specific proteins in differentiated myotubes *in vitro* (Bar-Shai and Reznick 2006) (Fig. 12.2). Antioxidant treatment and inhibition of tyrosine nitration downregulate NF-*κB* activation and slow down the proteasomal degradation of muscle-specific proteins. In *mdx* mice, NF-*κB* inhibition also increases sarcolemmal integrity and muscle strength (Rando 2001).

Other findings suggest that mislocation of nNOS may impair muscle NO homeostasis and physiology by other possible mechanisms. Sarcolemmal nNOS interacts

through its PDZ domain with the Ca^{2+}-efflux pump PMCA4b (plasma membrane Ca^{2+}-ATPase 4b), therefore, nNOS mislocation possibly affects this channel function (Stamler and Meissner 2001). Moreover, sarcolemmal NO synthesis is involved in the control of muscle contractions, glucose uptake and the nitrosylation of ryanodine receptors of the sarcoplasmic reticulum (Stamler and Meissner 2001; Percival et al. 2010) (Chap. 6). Alterations of NO synthesis therefore, can affect the contraction force by alterations in glucose metabolism and changes in the intracellular Ca^{2+} levels. Moreover, increased expression of sarcoplasmic iNOS also accounts for nitrosative stress of the dystrophic muscle fibers. It causes nitrosative damage of the sarcolemma, leading to its increased fragility and impaired mechanical stress resistance (Louboutin et al. 2001). Moreover, it increases proteasomal degradation of insulin receptor substrate-1, provoking insulin resistance (Sugita et al. 2005). Impaired NO homeostasis of the muscle fibers also alters mitochondrial respiratory chain activity (Bates et al. 1996; Soraru et al. 2007; Finocchietto et al. 2008), which may also cause fatigue and atrophic changes.

12.3 CAPON/nNOS Redistribution in Cardiomyocytes and Skeletal Muscle Fibers

Both nNOSα and nNOSμ are associated with NOS1AP (NOS1-associated protein), also known as CAPON (carboxy-terminal PDZ ligand of NOS1) (Jaffrey et al. 1998) (Chap. 6). Under physiological conditions, nNOS is enriched in the sarcoplasmic reticulum of cardiomyocytes (Oess et al. 2006) and colocalizes with CAPON (Beigi et al. 2009). In the sarcoplasmic reticulum, nNOS/CAPON complexes are structurally associated with ryanodine receptor 2 (RyR) and cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), and the locally synthesized NO affects Ca^{2+}-release (Chap. 6) (Danson et al. 2005). Changes in this subcellular distribution of CAPON/nNOS complexes negatively affect ion currents in cardiac cells (Fig. 12.3).

A genome-wide association study has identified a correlation between human electrocardiography alterations and allele variations of CAPON, suggesting that CAPON may affect cardiac electrophysiology, possibly through the modulation of nNOS activity (Arking et al. 2006; Post et al. 2007). Supporting this possibility, the overexpression of CAPON diminishes the L-type Ca^{2+}-channel current and enhances the outward rectifier potassium current; and consequently reduces the action potential duration in guinea pig cardiomyocytes (Beigi et al. 2009). Cardiac nNOS overexpression similarly suppresses the L-type Ca^{2+}-currents, due to the association of nNOS with this sarcolemmal channel (Burkard et al. 2007).

Myocardial infarction translocates CAPON and nNOS to caveolin-3 rich cell membrane caveolae in mouse cardiomyocytes (Beigi et al. 2009) (Fig. 12.3). This transition of nNOS to the sarcolemma affects plasma membrane calcium ATPase activities (Burkard et al. 2007; Oceandy et al. 2007). Sarcolemmal nNOS interacts with Na^{+}-K^{+}-ATPase and with the plasma membrane Ca^{2+}-calmodulin-dependent Ca^{2+}-ATPase 4b (PMCA4b) through the interaction of the C terminus of PMCA4b

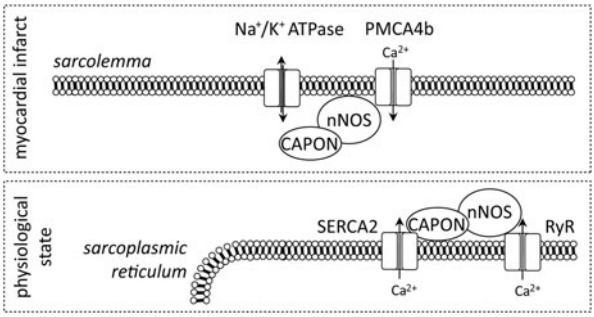

Fig. 12.3 Pathological CAPON/nNOS relocation in the cardiomyocytes. Under physiological conditions, nNOS and CAPON are associated with ion channels of the sarcoplasmic reticulum and affect the dynamics of intracellular Ca^{2+} signaling. In myocardial infarct, CAPON and nNOS are translocated to the sarcolemma, where the locally produced NO disturbs ATPases and the Ca^{2+} entry into the cell

and the nNOS PDZ domain (Danson et al. 2005; Oess et al. 2006). It is possible that the transition of nNOS/CAPON to the plasma membrane in myocardial infarction may allow a similar interaction between nNOS and sarcolemmal ion channel proteins (Beigi et al. 2009). PMCA4b affects nNOS signaling in the heart (Oceandy et al. 2007), however, the impact of a PMCA4b-nNOS interaction through redistribution of CAPON is yet unexplored.

In the skeletal muscle, CAPON may counteract the dissociation of sarcolemmal nNOS, thus redistribution of nNOS/CAPON to the DGC may mitigate muscle dystrophies (Fig. 12.2). For instance, a CAPON-like gene in *Caenorhabditis elegans* compensates a muscle dystrophic phenotype (Segalat et al. 2005). Similarly, in dystrophic muscles of *dxm* mouse models, prominent CAPON transcription has been shown (Segalat et al. 2005). Regenerating muscles also express CAPON, and NO synthesis may increase its transcript level (Segalat et al. 2005). Treatments mitigating the dystrophic phenotype in *dxm* mice also increase CAPON expression, which suggests that CAPON may restore the compromised association of nNOS with the sarcolemma (Segalat et al. 2005).

12.4 Uncoupling of the PSD95/nNOS Interface: Potential Medical Benefits

The association of nNOS with PSD95 establishes interaction between NMDA receptors and nNOS at postsynaptic densities and affects nNOS phosphorylation (Chap. 6). A recent study suggests that disruption of this interaction may be a novel target in the reduction of hyperalgesia. Synthetic inhibitors of the nNOS/PSD95 interaction (IC87201 and tat-nNOS, the cell permeable fusion protein containing the PSD95 binding domain of nNOS residues 1-299), block NMDA-induced cGMP production

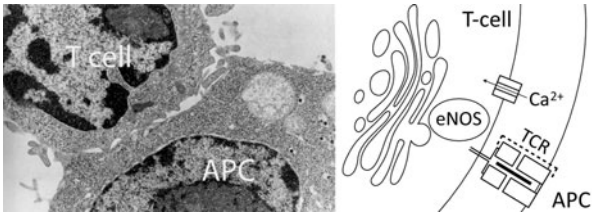

Fig. 12.4 The association of eNOS with the immunological synapse. *On the left:* Association of a T-cell and an antigen presenting cell (*APC*) shown by TEM. The interface of the two cells forms the immunological synapse. Reprinted with permission (Krummel and Cahalan 2010). *On the right:* The Golgi-system translocates to the immunological synapse, ensuring the local NO production by eNOS in the vicinity of the T-cell receptor complex (*TCR*). The Ca^{2+} influx associated with the establishment of the immunological synapse possibly contributes to eNOS activation in the Golgi-system

in primary hippocampal cultures without affecting nNOS catalytic activity (Florio et al. 2009). Intrathecal administration of these inhibitors reverses NMDA-induced thermal hyperalgesia in mice and mechanical allodynia induced by chronic constriction of the sciatic nerve (Florio et al. 2009). Disassembly of nNOS/PSD95 thereby reduces hypersensitivity in acute and chronic pain. Similarly, selective uncoupling of nNOS from PSD95 might be neuroprotective (Cao et al. 2005). Experiments using a panel of decoy constructs targeting the PSD95/nNOS interaction suggest that this interaction and subsequent NO production are critical for glutamate-induced p38 stress-activated protein kinase activation and the consequent neuronal cell death (Cao et al. 2005). These studies suggest that the uncoupling of interaction at specific PDZ domains can generate potential therapeutic applications. Of note, nNOSβ, the nNOS splice variant lacking PDZ domain mislocates into the cytoplasm and sustains its catalytic activity, causing neuronal cell damage (Brenman et al. 1995, 1997).

12.5 Redistribution of the Golgi-System and the Associated NOS Pool

Cytoplasm and the Golgi-system of T-lymphocytes contain eNOS. In activated T-cells, the Golgi-system, along with its eNOS pool, translocates to the proximity of the so-called immunological synapse (Ibiza et al. 2006; Nagy et al. 2010) (Fig. 12.4). This is a special interface at the plasma membranes of T-cells and antigen presenting cells; it is rich in cell surface receptors required for T-cell activation, such as the major histocompatibility complex and T-cell receptor. The association of the T-cell and the APC, triggers the establishment of the immunological synapse, evokes increase in intracellular Ca^{2+} and enrichment of cell organelles at the immune synapse, such as mitochondria (Quintana et al. 2007) and the Golgi-complex (Fernandez et al. 2009; Nagy et al. 2010). Because of the Golgi-system translocation, eNOS is being associated with the immunological synapse and T-cells start to release NO (Fernandez et al.

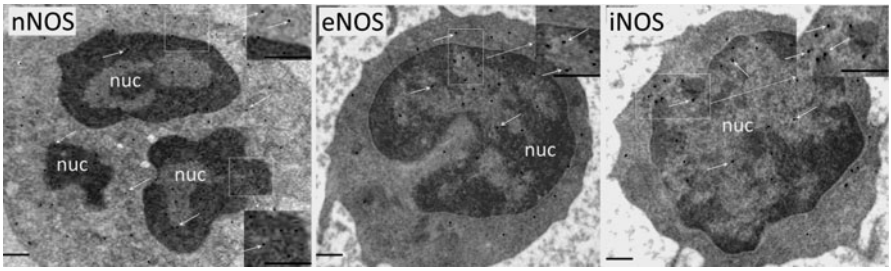

Fig. 12.5 Nuclear localization of various NOS isoforms in leukocytes. TEM images of a human neutrophil granulocyte, a monocyte and a lymphocyte, showing immunogold labeling (*black spots*, indicated with *arrows*) of nNOS, eNOS and iNOS, respectively (Saluja et al. 2011). nuc—cell nucleus; With the courtesy of Dr. Madhu Dikshit

2009; Nagy et al. 2010). The increased eNOS activity is perhaps associated with the increased intracellular Ca^{2+} level (Fernandez et al. 2009; Nagy et al. 2010). Synthesis of NO at the immunological synapse activates several pathways involved in T-cell activation: increases phosphorylation of CD3ζ chain, ZAP-70, TCR-dependent extracellular signal-regulated kinases (ERKs), increases IFN-γ synthesis and reduces production of interleukin-2 (Ibiza et al. 2006). The locally produced NO increases S-nitrosylation at the Golgi-system and activates the N-Ras pathway (Ibiza et al. 2008). Therefore, the Golgi-system eNOS-derived NO potentiates T cell receptor signaling at the immunological synapse (Ibiza et al. 2006). Synthesis of NO is pivotal in the process of T-cell activation and alterations in the lymphocyte NOS activity are associated with inflammatory disorders, such as rheumatoid arthritis and systemic lupus erythematosus (Fernandez et al. 2009; Nagy et al. 2010).

12.6 NOS in the Nucleus: A Transient or Permanent NOS Pool?

Several studies show that NOS may be associated with the cell nucleus (Fig. 12.5). The nuclear envelope anchors nNOS in neurons (Xu et al. 2000), iNOS in cardiomyocytes (Buchwalow et al. 2001) and an unspecified NOS isoform in reactive microglia and macrophages (Calka et al. 1996). Both eNOS and iNOS are present in the nuclei of brown adipose tissue cells (Giordano et al. 2002). Perinuclear Golgi-system of endothelial cells (Lee et al. 2011) and juxtanuclear endoplasmic reticulum of snail neurons (Rőszer et al. 2010) may also be sites of NO synthesis. Nuclei of granulocytes and monocytes also contain iNOS and nNOS (Saini et al. 2006; Kumar et al. 2010; Saluja et al. 2010, 2011) (Fig. 12.5).

Some studies suggest that NOS and its associated proteins occur in the nucleus transiently, due to their translocation from the cytoplasm. For example, in activated neutrophil granulocytes the NO-dependent PKG translocates into the nucleus and occurs in the euchromatic regions (Wyatt et al. 1991). Similarly, CAPON colocalizes with Ser-847 phosphorylated nNOS in neurons of the mouse hippocampus and the

cerebral cortex and inflammatory stimulus (peritoneal injection of LPS) relocates CAPON to the nuclei of the neurons (Shao et al. 2011). NOSIP is also present in the nuclei (Dreyer et al. 2004) and during cell division, NOSIP translocates from the nucleus to the cytoplasm and anchors eNOS to the actin cytoskeleton (Schleicher et al. 2005). This mechanism leads to a cell-cycle dependent transient eNOS inactivation (Schleicher et al. 2005). The nuclear translocation of eNOS has been described as a response to lysophosphatidic acid in hepatocytes and endothelial cells *in vitro* (Gobeil et al. 2006).

Recent studies show that nuclear compartmentalization of eNOS regulates gene expression, and affects Ca^{2+} homeostasis of the nuclei (Provost et al. 2010). For example, genes encoding iNOS and microsomal prostaglandin E synthase-1 are up-regulated by NO and this effect is associated with the translocation of eNOS to the nucleus (Gobeil et al. 2006). Moreover, nuclear localization of guanylyl cyclase has also been found in rat hepatocytes and stimulation of the nuclear NO synthesis activates the mitogen-activated kinase pathway and NF-κB binding to DNA (Gobeil et al. 2006). While it is known, that NO affects gene expression by various mechanisms (Kroncke 2001; Fish et al. 2007), it is still uncertain whether nuclear association of NOS is required for these effects. Many examples show that the cytoplasmic or the mitochondrial NO synthesis affects transcription (Lushchak et al. 2010; Taylor and Moncada 2010; Biedasek et al. 2011), therefore a physical association of NOS with the nucleus is not required for modulating genes. The functional relevance of the redistribution of NOS and NOS-associated proteins between the cell nucleus and extranuclear cell compartments is therefore still largely unknown.

12.7 Dynamic NOS-Pools of the Cell

Studies discussed in this monograph support the idea that NO synthesis has been engaged with specific subcellular compartments during the evolution of the cell. For example, the reductive NO synthesis is associated with the prokaryote cell membrane and its derivatives in the eukaryote cell, while the oxidative NO synthesis by NOSs takes place mainly in the cytoplasm and the intracellular membrane systems. The recent findings summarized in this chapter show that NOS can be redistributed between certain membrane compartments (e.g. sarcolemma, caveolae, endoplasmic reticulum, Golgi-system) and the cytoplasm (Fig. 12.6). This dynamic redistribution ability of NOS determines NO synthesis and the local effects of NO. Thereby subcellular redistribution of NOS between cytoplasm and membrane compartments is a key mechanism in the cellular NO homeostasis. Targeting NOS to given subcellular destinations is therefore a potential therapeutic approach to control local NO synthesis and effects of NO. Future studies in this precise area of NO biology may allow the better treatment of pathologies associated with dysregulated NOS activity. This research line can also branch out to new horizons, to define the evolution of NOS compartmentalization and the better understanding of the signals orchestrating intracellular sorting of NOS.

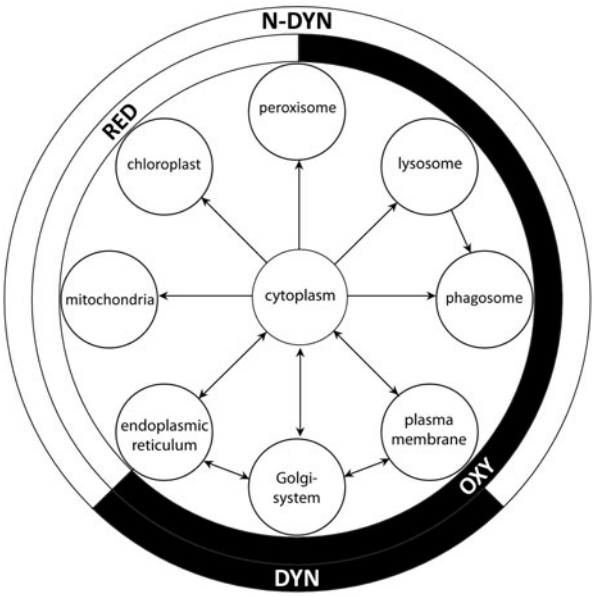

Fig. 12.6 Distribution of NO-synthesizing enzymes between subcellular units. There is a redistribution ability of NOS between the cytoplasm, the endoplasmic reticulum, the Golgi-system and the plasma membrane, therefore these compartments form a dynamic cellular NOS-pool (*DYN*). In these compartments the NOS redistribution affects NO forming activity and the local effects of NO. NOS can be allocated from the cytoplasm to other organelles, forming a non-dynamic NOS pool (*N-DYN*) with limited redistribution ability. All of these units show oxidative NO generation (*OXY*) by NOSs, although some subcellular units also display reductive NO generation (*RED*). Arrows indicate the possible movements of NOS

12.8 Chapter Summary

Physiological changes in subcellular NOS distribution	• In endothelial cells, there is an intracellular traffic of eNOS to the cell membrane caveolae. Redistribution to the Golgi-system is also possible • In activated T-cells the Golgi-system and its NOS pool translocates to the immune synapse • NOS and NOS-associated proteins are able to translocate to the cell nucleus or from the cell membrane to intracellular membranes or cytosol
Pathologies and NOS mislocalization	• Lack of NOS-anchoring proteins or cholesterol ablation from the cell membrane can evoke NOS mislocalization • The release of NOS from cell membranes leads to the impairment of the regulation of NOS activity. Nitrosative damage, reduced NO availability, reduced S-nitrosylation at the Golgi-system are all consequences of NOS mislocalization • Release of nNOS/PSD95 association has impact in reducing neurotoxic NO synthesis

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Appendix

Image Information

This monograph contains original illustrations with the exception of Figs. 2.3, 2.9 (left panel), 3.1 (lower panels), 3.6, 4.3, 4.4, 6.7a (© Dreamstime.com LLC, Brentwood, US stock photo gallery contributors Jacub Pavlinec, Constantin Sava, Vasiliy Koval, Mauro Rodrigues, Mikeexpert, Konuchilo, Kuhar, Grafoo, Antikainen, Marpalusz and Duncan Noakes); 2.8a, 7.2, 7.3, 7.4a, 8.2c, 10.3, 12.5 (published with courtesy), 7.1a, 7.5a, 8.1a, 11.1, 12.4 (reprinted with permission). Three dimensional molecule models were reconstructed with Chimera 1.5 software using secondary structure prediction generated by a local-meta-threading-server (LOMETS), University of Michigan, US.

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Glossary

Abscisic acid (ABA), also known as abscisin II or dormin, involved in seed and leaf primordia dormancy and also in the autumn abscission (fall of leaves).

Alternative oxidase is a bypass enzyme in the electron transport chain of plants and some fungi; it provides an alternative route for electrons to reduce oxygen; responsible for the "cyanide resistant" respiration.

Anaerobiosis is a form of life in the lack of O_2.

Auxin is a plant hormone, with major roles in morphogenesis and growth.

Cytokinins are plant hormones which promote cell proliferation, plant growth, the development of apical dominance and senescence.

Fenton-chemistry is a reaction of iron (Fe^{2+}) and hydrogen peroxide (H_2O_2), giving hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$$

Filopodia are fine cell extrusions of the migrating cells formed on the surface of the leading edge of the lamellipodia.

Fruiting body or sporocarp is a multicellular, spore-forming structure of fungi, which represents the sexual reproduction phase.

Growth cone is the growing end of an axonal process, which is searching for synaptic contacts, thus its acrin cytoskeleton is in dynamic reorganization.

Hypoxia is a deprivation of O_2 supply; in aerobe cells, it develops under 1.5–3% [O_2], although this value might vary greatly among cell types and cell compartments.

Lamellipodium (plural, lamellipodia) is the leading edge of migrating cells, formed by an actin cytoskeletal network.

Lipopolysaccharides (LPS) are covalently bound lipid and polysaccharide molecules; they are constituents of the outer cell membrane of Gram negative bacteria; they are used to induce inflammatory response (e.g. macrophage activation) under experimental conditions.

Normoxia is a sufficient O_2 supply; for aerobe cells ~3–10% [O_2], depending on cell type; ambient 21% [O_2] concentration represents a hyperoxic state.

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Programmed cell death is commonly used terminology instead of "apoptosis" in plant cell biology.

Rubisco is the most commonly known shorter name of *r*ib*u*lose-1,5-*bis*phosphate *c*arboxylase *o*xygenase; a chloroplastic enzyme of the Calvin cycle, required for carbon fixation.

Transcription factors are proteins that bind specific DNA sequences thus control transcription.

Vascular plants or Cormophyta is an old classification term in botany, which refers to all plants with axis (stem or cormus) and root (radix); these plants have a developed vascular system, which transports water and dissolved assimilates.

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