Chapter 2

The Hidden Face of Nitrogen Oxides Species: From Toxic Effects to Potential Cure?

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Abstract

Nitrogen oxide (NOx) species represent ones of the most threatening air pollutants due to their prevalence and harmful impact on the environment and human health. The term NOx gathers mainly nitric oxide (NO) and nitrogen dioxide (NO_2), mostly produced by anthropogenic activities such as transport and industries. Several cellular constituents were already described as NOx targets. These include membranes, proteins, respiratory chain enzymes, lipids, and DNA. Such damages lead to pathologies of lungs, cardiovascular system, and skin because these organs represent the first barrier toward the environment. On the other hand, NOx is also naturally synthetized by several organisms, playing a mediator role in essential cellular functions. However, few data are yet available on NOx activity toward microorganisms. Here, we review data concerning the double face of NOx, including their use in the medical field against pathogens' infections that highlight the versatility of these compounds.

Keywords: NOx, pollutants, signalization, physiopathology, treatment

1. NOx species: the genesis of a major air pollutant

"Yet, pollution is the largest environmental cause of disease and death in the world today, responsible for an estimated 9 million premature deaths" stated The Lancet Commission in 2017. This phenomenon represents an alarming threat for human health, as a major cause of respiratory and cardiovascular pathologies as well as infertility. Moreover, those atmospheric pollutants have severe impacts on the environment and participate in climatic change,

acidification, eutrophication, and ecosystem disturbances. Several international and national organizations (e.g. WHO, EEA, and INERIS) aim at reducing the global emission of pollutants, thanks to environmental policies as Kyoto and Gothenburg protocols signed in 1997 and 1999, respectively. After that, an encouraging decrease in air pollutants' levels was measured between 2000 and 2015 [1].

Atmospheric pollutants can be classified into four families: classical, indoor, and organic or inorganic air pollutants. Among the classical ones, which are the principle in amount, sulfur dioxide (SO_2), particle matter, ozone (O_3), and nitrogen oxide (NOx) species are found [2]. The term NOx refers to a wide range of nitrogen-derived compounds, where nitric oxide (NO) and nitrogen dioxide (NO_2) are predominant [3]. Those compounds can be naturally produced at low level by lightnings [4] and volcanic eruptions [5]. However, NOx is mainly generated by anthropogenic activity (e.g., road transport, energy production, industry, and agriculture) (**Figure 1**) [6].

Even if NO and NO_2 represent the main species of NOx, nitrogen exists in several oxidation states in the environment, from N (−III) to N (+V). NO takes a central place in the series of reactive nitrogen species (RNS) [7]. Oxidation and reduction of NO result in the formation of several RNS, including nitrate or ammonium. NO_2 is formed by the reaction of NO with O_2 and can be dimerized to give N_2O_4. The formed reactive species can, in turn, be involved in a wide range of reactions (**Figure 2**).

Nitrogen is an essential constituent of vital macromolecules (nucleic acids and proteins). This atom also constitutes the dinitrogen (N_2) gas, usually abbreviated nitrogen. This gas represents 78% of atmospheric air and is continuously recycled in our environment. First, the atmospheric nitrogen is fixed in the soil by prokaryotes to form ammonia (NH_3), which could be taken up by plants. NH_3 is then converted into ammonium (NH_4^+). Ammonium could be also

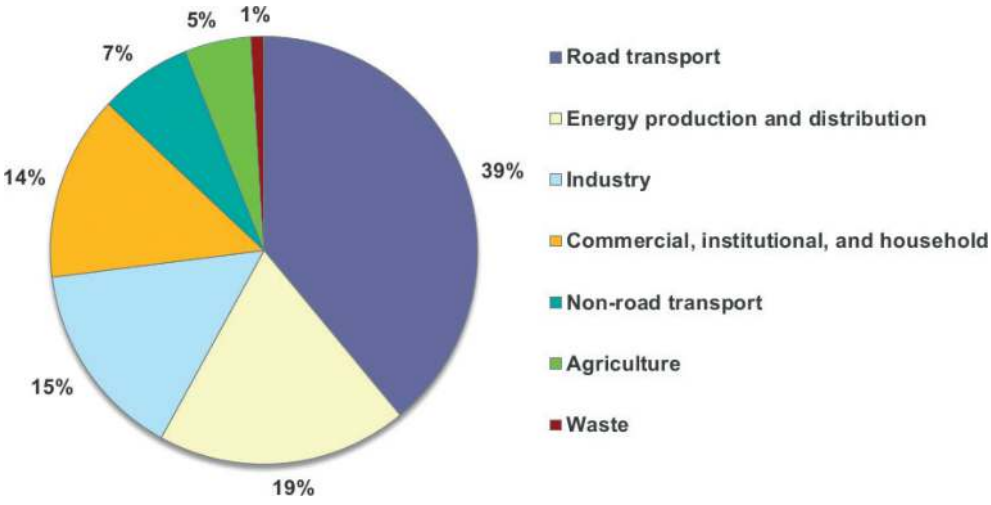

Figure 1. NOx sources linked to anthropogenic activities (according to [1]).

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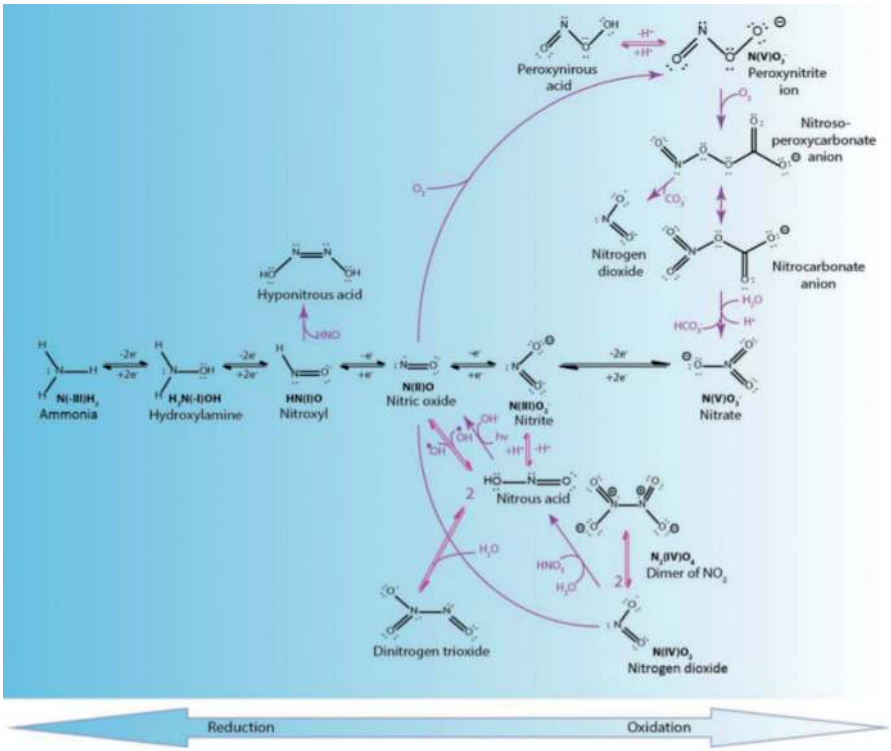

Figure 2. Redox relationships of NOx with other RNS. Black arrow: oxidation and reduction of NO; purple arrow: a wide range of reactions; roman numbers: oxidation state of the nitrogen atom; color gradient: reduction/oxidation state from (–III) to (V).

formed by mineralization of organic matter by decomposers (fungi, worms, and prokaryotes). The second part of the nitrogen cycle is the nitrification of ammonium by prokaryotes leading to nitrite (NO_2^-) formation, which is then further oxidized into nitrate (NO_3^-). Finally, nitrate could be assimilated by plants for their growth or converted into N_2 by denitrifying bacteria (**Figure 3**).

Moreover, nitrogen is also the precursor of NOx. Indeed, NOx formation can be separated into four steps. First, NO is formed in the atmosphere in combination with nitrogen, resulting from the global biogeochemical nitrogen cycle, and oxygen, following combustion (natural or anthropic). Then, this highly reactive compound can be oxidized by oxygen (O_2) to form NO_2. Alternatively, nitrogen dioxide can also be directly formed through catalytic ammonia combustion or nitrosyl chloride oxidation. Then, a temperature-dependent equilibrium is establishing between NO_2 and its dimeric form, nitrogen tetroxide ($N_2O_4^-$). Finally, a photochemical reaction between NO_2 and hydroxyl radicals leads to the generation of ozone (O_3^-) (**Figure 4**).

Even if NO and NO_2 are classified as RNS, their characteristics are very different suggesting their diverse behavior and reactivity. For example, the solubility of NO is very low

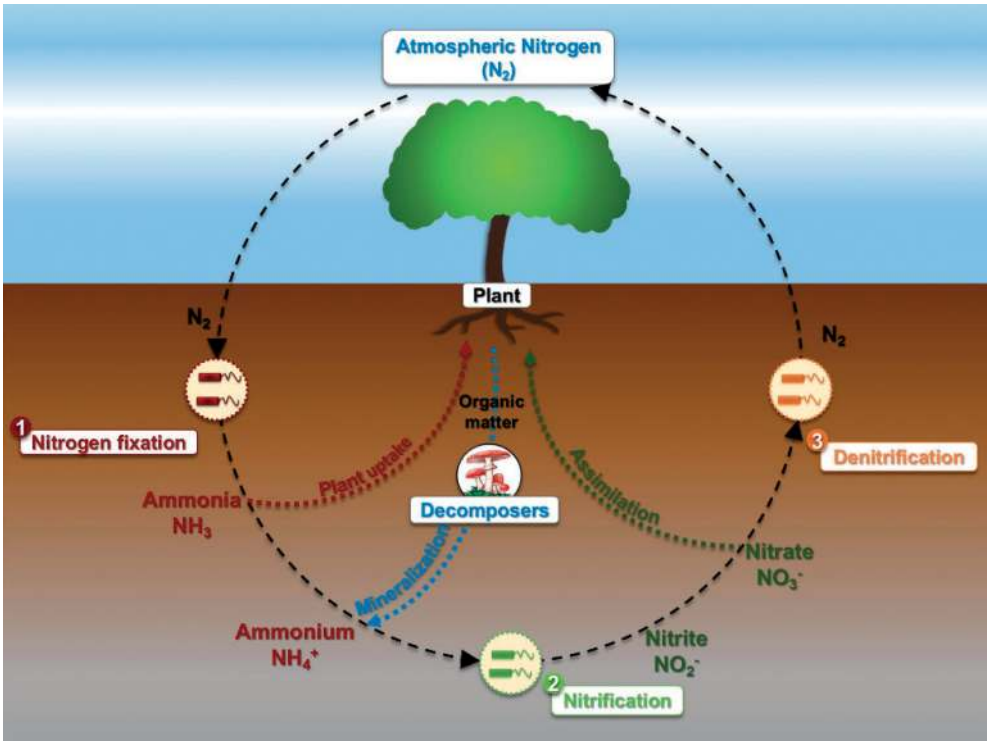

Figure 3. Nitrogen cycle at the interfaces among air, soil, and ecosystem. (1) Fixation of gaseous nitrogen by prokaryotes leading to ammonia (NH_3) and ammonium (NH_4^+); (2) nitrification of NH_4^+ by prokaryotes resulting in nitrite formation (NO_2^-) subsequently oxidized into nitrate (NO_3^-); (3) denitrifying prokaryotes catalyzing the conversion of nitrate into gaseous N_2.

(5.7×10^2 g/L), and NO_2 is highly reactive in water resulting in the formation of HNO_2 and HNO_3 [8]. Moreover, the half-life time of those compounds highly differs because NO_2 half-life is around 35 h, whereas that of NO is almost impossible to determine because of its high reactivity [9]. The permeability of both compounds is also totally unrelated: NO has a lipophilic behavior with consequently a high-membrane permeability. This can be illustrated by the high-diffusion ability of NO toward the membrane. Conversely, the permeability coefficient of NO_2 is estimated about 5 cm s^{-1} suggesting a lower diffusion power toward biological membranes [10]. In spite of these differences, the penetration of NO and NO_2 inside cells and their high reactivity are at the origin of their pathogenic potential on human health, particularly when physiological elimination thresholds are exceeded. Since skin, respiratory tract, and lungs are the first barrier toward those gases, these organs are evidently the principle targets of these compounds and their derived species [11–13]. However, their diffusion deeper in the organism can lead afterward to other severe effects, for instance, on the cardiovascular and immune systems [12, 14]. Human health effects range from reversible (nausea, breathing difficulties, and asthma symptoms) to irreversible (cardiovascular defects, emphysema, and immunopathologies), including cancer induction in the worst cases (**Figure 5**) [15].

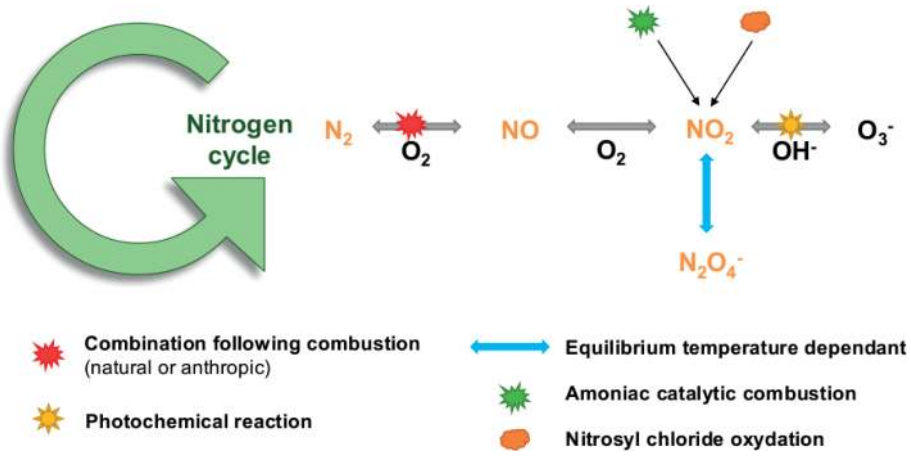

Figure 4. Atmospheric genesis of NOx (adapted from [3]).

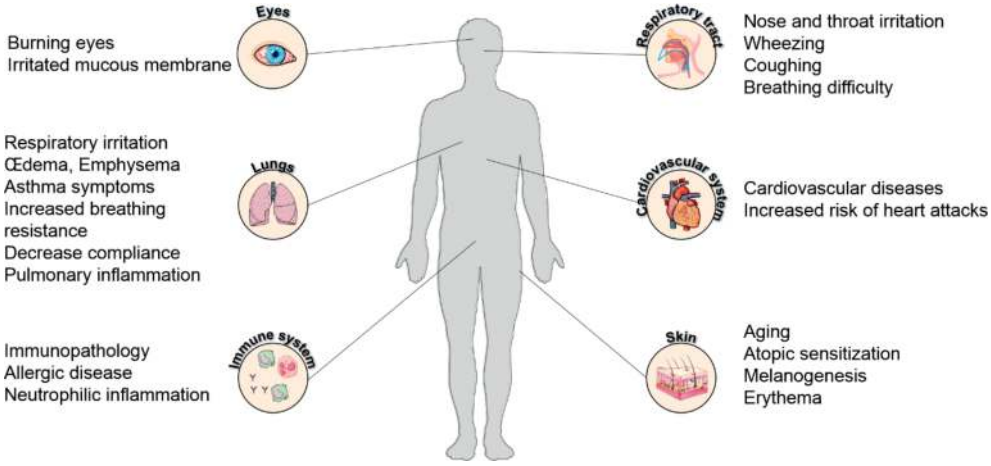

Figure 5. Representation of NOx targets and associated pathologies [11–15].

Thanks to epidemiological and clinical studies, thresholds of NO and NO_2, whose chemical structure and conversion factors are represented **Figure 6A**, have been determined for different human health effects (**Figure 6B** and **C**, respectively). However, no guideline values are available for NO (**Figure 6D**) due to its complete and rapid reaction [16]. Thus, in order to limit health issues, international organizations, such as WHO, set up guidelines for atmospheric NO_2 limit value (**Figure 6E**).

NOx is then highly toxic compounds and has a myriad of deleterious impacts on the human physiology when the bearable thresholds are exceeded. However, NOx is also naturally produced by cellular processes in a wide range of living organisms. In this case, NOx can have totally different effects as discussed later.

A

1ppm = 1.23µg/m³ 1ppm = 1.88 µg/m³

B

Time (min)	10	20	30	60
Lethal effects	1000	850	750	600
Irreversible effects	150	120	100	80
Reversible effects	ND	ND	ND	ND

C

Time (min)	10	20	30	60
Lethal effects	118	98	88	73
Irreversible effects	60	55	50	40
Reversible effects	5	5	5	5

D

NO guidelines		µg/m³	ppm
1 hour mean	Limit value		
	Alert threshold	ND	
Annual mean			

E

NO_2 guidelines		µg/m³	ppm
1 hour mean	Limit value	200	0.1
	Alert threshold	400	0.2
Annual mean		40	0.02

Figure 6. Key features concerning NOx. (A) Lewis structure of NOx and conversion factors; (B) NO threshold (ppm) according to [9]; (C) NO_2 threshold (ppm) according to [9]; (D) and (E) NO and NO_2 guideline values, respectively, according to [1]. ppm, parts per million; ND, not determined.

2. The double face of NOx in the eukaryotic world

To better understand why NOx impact on so many organs, it is essential to focus on the units forming each part of our body: the eukaryotic cells themselves.

2.1. Exogenous NOx: a hostile intruder inside the eukaryotic cells

Much work was already performed on NOx targets at the cell scale. NO and NO_2, thanks to their chemistry properties mentioned earlier, are highly diffusible through living membranes and exhibit a strong reactivity. It is easy to imagine the potential of such compounds. Since NO is highly reactive in lipophilic media, it can react inside the lipid bilayer, first protection of cells against the environment. Indeed, polyunsaturated fatty acids (PUFAs) are susceptible to NO in the presence of O_2 and $ONOO^-$ [17]. The interaction of these toxic compounds with PUFA double bounds leads to the formation of nitrated FA. This process is considered as a protective strategy because it redirects O_2^- and $ONOO^-$-mediated cytotoxic reactions to other oxidative pathways. Post-translational modification of several proteins is also observed in the presence of NOx and RNS. For example, tyrosine nitration on phenol residue mediated by peroxynitrite derived from OH and NO_2 leads to 3-nitrotyrosine formation [18]. This molecule represents a useful biomarker of nitrosative stress in various pathologies such as atherosclerosis [19]. Protein thiol residues are highly susceptible to NO and $ONOO^-$ leading to the formation of nitrosothiol (RS-NO) and nitrothiol (RS-ONO), respectively [20]. This reversible modification modulates the activity of several proteins similar to phosphorylation. For example, S-nitrosylated cysteine thiol residue can be denitrosylated by S-nitrosoglutathione reductase or thioredoxin systems [21]. NO and other RNS can also nitrosylate [Fe—S] cluster of transition metal centers, which are essential for protein function. This modification can activate a wide range of enzymes such as the soluble guanylate cyclase (sGC) mentioned below

[22]. On the contrary, this reaction can also alter the protein function, for example, in the case of hemoglobin [23]. NO and other higher RNS products ($ONOO^-$ or NO_2) are able to generate nitration, nitrosation, or deamination reactions on DNA bases leading to mutagenesis [24]. This phenomenon is enhanced by the inhibition of DNA repair triggered by NO [25]. More recently, a role of NO in epigenetic modification was suggested. To be more precise, this simple molecule seems to modulate histone acetylation and methylation through direct and indirect modulations of histone acetyltransferases and deacetylases, lysine demethylases, histone methyltransferases activity, thus modifying the expression of several genes [26]. These pleiotropic activities of NO and derived compounds highlight here the necessity of fine regulation pathways to prevent the development of several diseases.

2.2. Endogenous NO: an essential mediator of cellular signalization

Interestingly, NO can also be produced by several living organisms: plants, animals, and bacteria, thanks to a specific enzyme called nitric oxide synthase (NOS) [27, 28]. The large distribution of this enzyme through the different reigns emphasizes the importance of NO synthesis. Evolutionary studies highlight the necessity for the first living organisms during primitive era to eradicate toxic O_3 present in the paleoatmosphere as a survival strategy. Indeed, the liberation of gaseous NO in extracellular environment could have subsequently neutralized O_3, thus limiting harmful oxidative reactions [29]. In eukaryotic cells, three isoforms of NOS have been described [30]. The neuronal nNOS (NOS-1) and the endothelial eNOS (NOS-3) are constitutively expressed but are only activated through calcium-dependent mechanisms. The third one is the inducible iNOS (NOS-2) expressed in macrophages following infection by pathogens, virus, or tumors. Contrary to NOS-1 and 3, NOS-2 is constitutively functional. Interestingly, these NOSs have high structural similarities with an oxygenase and a reductase domain (**Figure 7**) [27].

To be fully functional, NOS requires to associate with homodimers, and this form of NOS is crucial for the generation of NO [32]. When conditions are favorable (high level of L-arginine

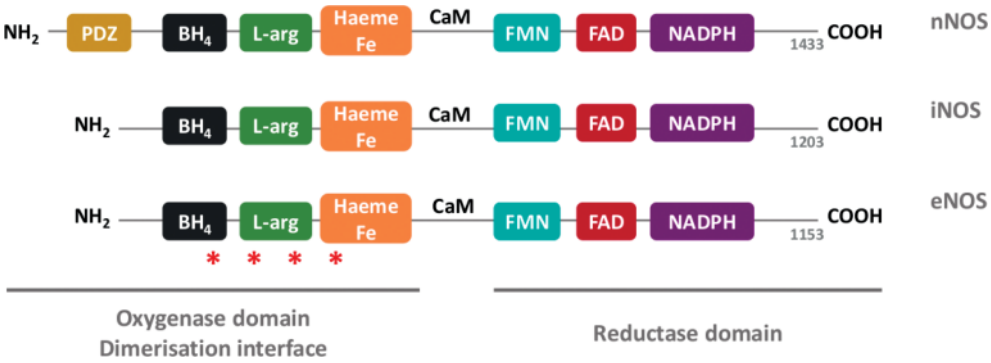

Figure 7. The structural similarities of NOS (adapted from [31]). BH_4, tetrahydrobiopterin; L-arg, L-arginine-binding site; Heme Fe, iron protoporphyrin IX; CaM, calmodulin-binding site; FMN, flavin mononucleotide-binding site; FAD, flavin adenine dinucleotide-binding site; NADPH, nicotinamide adenine dinucleotide phosphate-binding site; *, palmitoylation and myristoylation of eNOS oxygenase domain.

and cofactors like BH_4), this enzyme catalyzes the conversion of L-arginine into L-citrulline using O_2 and leading to NO formation with a ratio of 1:1 [33]. However, if these parameters are not available or if NOS remains monomeric, the enzyme only produces superoxide (O_2^-) [32]. Even if NOS isoforms catalyze the same reaction, their distribution is largely related to their respective functions.

2.2.1. The endothelial eNOS and vasodilatation

Endothelial nitric oxide synthase (eNOS) can be activated through calcium-dependent and independent pathways. On the one hand, through activation of specific receptors, such as acetylcholine muscarinic receptors, bradykinin receptors, and H1 histamine receptors, distributed in the endothelial cell membrane, agonists can trigger an increase in intracellular concentration of calcium (Ca^{2+}) through the well-known polyphophoinosides pathway. Indeed, activation of those receptors stimulates membrane-associated PLCϒ and PI3K activation. This results into inositol trisphosphate (IP3) formation, which induces the release of intracellular calcium stock from endoplasmic reticulum [34]. Then, Ca^{2+} binds to calmodulin (CaM), which could later fix on the calmodulin-binding domain of eNOS controlling its enzymatic activity. On the other hand, in response to hormones or growth factors acting on their corresponding receptors, phosphate kinase A (PKA) or B (AKT) pathway can be induced mediating a phosphorylation cascade. The post-translational phosphorylation of eNOS on three specific sites (Ser617 and Ser1179 for AKt, Ser635 and Ser1177 for PKA) enhances the activity of the enzyme [35]. Moreover, the lipidation of eNOS (palmitoylation and myristoylation) could also enhance its activity [36]. This other post-translational modification promotes eNOS association with cell membrane and is stabilized by interaction with membrane chaperone proteins (caveolin-1 and HSP70/90). Both mechanisms are essential for linking upstream signal transduction pathway to eNOS activity in cells [37]. eNOS could then produce a large amount of NO, which latter diffuses freely inside smooth muscles. Herein, NO activates soluble guanylate cyclase (sGC) by reaction with the heme of the enzyme leading to the increase of cyclic guanosine monophosphate level (cGMP). cGMP subsequently activates phosphate kinase G (PKG) favoring a cytosolic Ca^{2+} reuptake into sarcoplasmic reticulum [38]. The decrease of intracellular level of Ca^{2+} leads to the relaxation of smooth muscle. Moreover, NO can inhibit caspases 3 and 8 and thus apoptosis through protein nitrosylation. NO released by endothelium is also reducing platelet aggregation and platelet or leukocyte adhesion (**Figure 8**) [39, 40].

Another source of NO inside endothelial cells was also described. Indeed, nitrite and nitrate reservoirs could be converted into NO by several enzymes such as cytochrome P450, hemoglobin, myoglobin, and others, under specific conditions [42]. However, several pathways could abolish NO production. Indeed, NO can be eliminated in combination with reactive oxygen species (ROS). eNOS could be inactivated by several phosphatases. The removal of eNOS membrane sequestration by interaction between NOS-interacting protein (NOSIP) and NOS-traffic inducer (NOSTRIN) complex favors eNOS recycling [36]. Since NO plays a central role in this phenomenon, any abnormality altering its production leads to pathogenesis and vascular disorders such as atherosclerosis and hypertension [43].

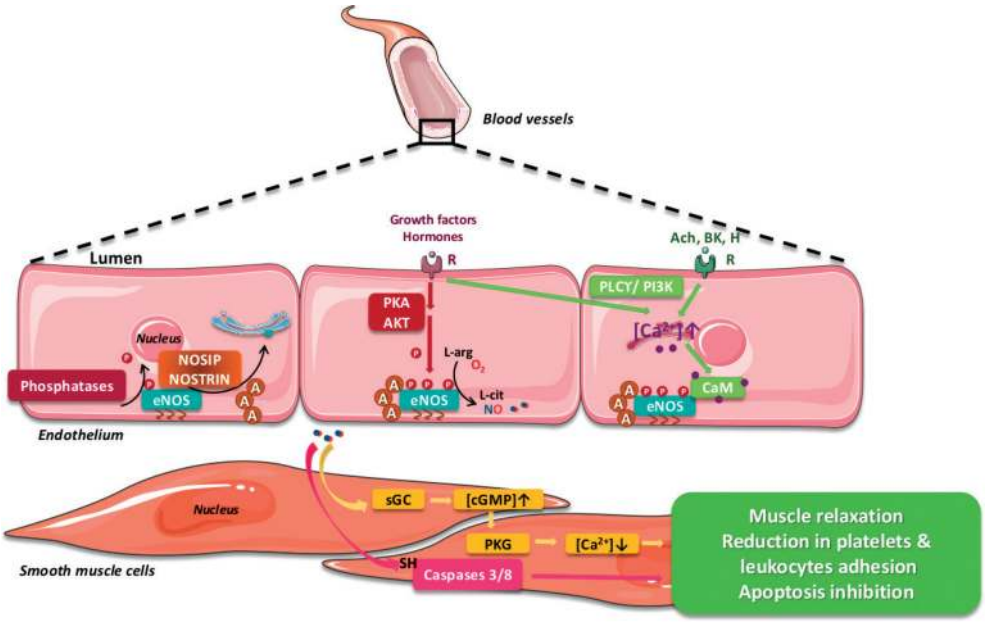

Figure 8. Physiology of vasodilatation: implication of eNOS and NO (adapted from [41]). eNOS, endothelial nitric oxide synthase; ach, acetylcholine; BK, bradykinin; H, histamine; R, receptor; CaM, calmodulin; PLCϒ, phospholipase C ϒ; PI3K, phosphoinositide 3-kinase; PKA, phosphate kinase A; AKT, phosphate kinase B (PKB); sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate; PKG, phosphate kinase G; A, protein complex of HSP70, HSP90, and caveolin-1; NOSIP, NOS-interacting protein; NOSTRIN, NOS traffic inducer.

2.2.2. The neuronal nNOS: a crucial element in neurotransmission

nNOS, also called NOS-1, was the first isoform discovered in the neuronal tissue. It should be distinguished between two subfamilies: nNOSα and nNOSμ, thanks to their locations and functions. Indeed, nNOSα is found in central nervous system and plays a major role in the neurotransmission at neuronal synapses, whereas nNOSμ is found in skeletal and cardiac muscle where NO controls muscle contractility and local blood flow.

2.2.2.1. nNOSα in the central nervous system

Glutamate is the major excitatory neurotransmitter in the brain. When deliver into the synapse, this molecule is activating a specific receptor (NMDAR) located inside the postsynaptic membrane. Activated NMDAR thus allows the entry of calcium. Ca^{2+} are able to activate calmodulin by physical interaction [44]. Afterward calmodulin binds to nNOSα, which, in turn, is activated. Interestingly, nNOSα can indirectly interact with the NMDAR through its fixation on an adapter protein PSD95 by their PDZ interaction domain [45]. This allows the high speed of NO synthesis in response to NMDAR activation. Thereafter, NO can diffuse back to the presynaptic area leading to an increase glutamate release in the synaptic cleft and thus neurotransmission. CAPON, a chaperon protein of DexRas, was found altering the level

of nNOSα present at the membrane [46]. Indeed, CAPON competes with the PDZ domain of nNOSα and thus promotes its detachment from the NMDAR-PSD95 complex. nNOSα interaction with CAPON-DexRas complex allows the activation of DexRas, a small protein G, through s-nitrosylation on its thiol residue [47]. Then, DexRas activates the MAPK cascade and the transcription of several genes [47]. Several shutdown systems are available to limit this process, which could dramatically affect the brain physiology by synapses hyperactivation. Indeed, kinases, such as PKC or PKII, can phosphorylate nNOSα leading to its loss of activity [48]. nNOSα can also be sequestrated by two membrane proteins (caveolins 1 and 3) preventing its interaction with calmodulin (**Figure 9**).

As mentioned above, any disorder affecting NO synthesis can lead to dramatic consequences in the brain. Indeed, a lack of NO could impair the neurotransmission and hence the neuronal plasticity, such as long-term potentiation (LTP) in the hippocampus or long-term depression (LTD) in the cerebellum [50]. NO ensures also the correct irrigation of brain by its vasodilator effect on peripheral blood vessel. The synthesis of NO can be altered by a deficiency or a loss of activity of nNOS. Other regulatory elements, such as the decrease of L-arginine precursor availability or cofactors crucial for the catalytic activity of NOS, contribute also to nNOS inactivation. Conversely, in some several brain pathologies (ischemia, strokes, neurodegenerative disorders including Parkinson's and Huntington's diseases, and amyotrophic lateral sclerosis), a large amount of NO is produced [51]. It is hypothesized that NO or derived nitrogen species interact with various proteins inducing several toxic effects. Thus, pharmacological regulation of NO synthesis offers important strategies for the treatment of neurodegenerative and muscle diseases.

2.2.2.2. nNOSμ in peripheral neurotransmission

On the other hand, nNOSμ is located in skeletal and cardiac muscle where NO controls muscle contractility and local blood flow essential to support muscular effort. When the excitatory neurotransmitter acetylcholine (Ach) is released at the neuromuscular junction, it is activating specific acetylcholine nicotinic receptors present on muscle cells [52]. Activation of these receptors allows the release in the cell sarcoplasm of calcium from exogenous and/or endogenous

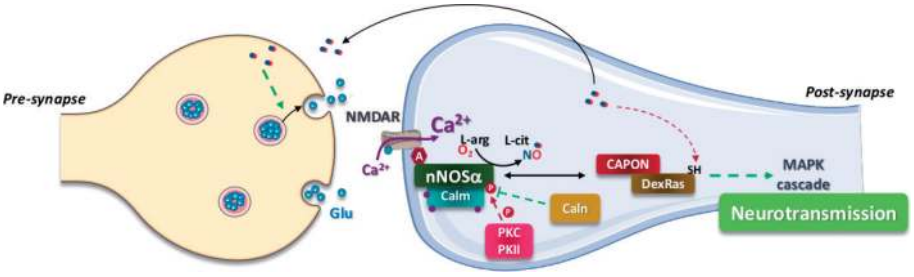

Figure 9. The neurotransmission mediated by NOSα in central nervous system (CNS) (adapted from [49]). Glu, glutamate neurotransmitter; NMDAR, N-methyl D-aspartate type-glutamate receptor; A, PSD95 protein adapter between nNOSα and NMDAR; CaM, calmodulin; PKC, phosphate kinase C; PKII, Ca2+/CaM-dependent protein kinase II; CaN, calcineurin; CAPON, C-terminal PDZ domain ligand of neuronal nitric oxide synthase; MAPK, mitogen-activated protein kinase.

sources. This process is also enhanced by the activation of a voltage-dependent Ca^{2+} channel (CaCn) [53]. The increase of Ca^{2+} from exogenous origin activates ryanodine receptor present on the sarcoplasmic reticulum, which, in turn, delivers a large amount of Ca^{2+}. When the Ca^{2+} threshold is reached, it is binding on the calmodulin protein. Calmodulin can then interact with the nNOSμ to activate the generation of NO. Interestingly, nNOSμ interacts through its PDZ domain with adapter proteins (syntrophins) linked to a membrane protein complex called dystrophin [54]. Produced NO could then diffuse back to the motor neuron to enhance the liberation of neurotransmitters or the vasodilatation of blood vessels, thus increasing the local blood flow to support muscle needs during contraction efforts (**Figure 10**) [55].

NO synthesis is also well regulated by calpain (Calp). Indeed, this protein interacts with the PDZ domain nNOSμ provoking its detachment from the dystrophin/syntrophin membrane complex [56]. Interestingly, in muscular Duchenne dystrophy, the damages caused by dystrophin absence are enhanced in case of simultaneous lack of nNOSμ [57]. Thus, NO donors may offer a potential avenue for therapy.

2.2.3. The inducible iNOS and the innate immunity

NO also takes an important part in innate immunity. Indeed, NO and derived compounds are cytotoxic weapon against tumor cells, microorganisms, and viruses [58]. NO is generated by inducible nitric oxide synthases (iNOS and NOS2) within macrophages. However, more recently, studies have shown that this enzyme is also expressed in other cell line (vascular endothelial cells, hepatocytes, pulmonary, and colonic epithelial cells) [59]. Unlike nNOS or eNOS, its expression is stimulated by several pathways after activation of specific receptors by endotoxins (LPS) or cytokines (TNFα, IFNϒ, and IL1β). Briefly, the LPS endotoxins are carried by an LPS-binding (LPB) protein to its specific Toll-like receptor 4 (TLR4). Then, the transduction

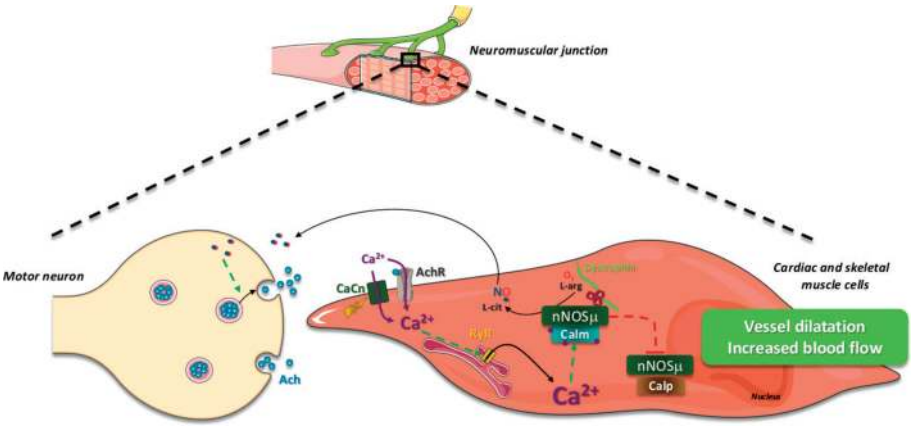

Figure 10. nNOSμ activity at the neuromuscular junction (adapted from [49]). Ach, acetylcholine; CaCn, voltage-dependent Ca^{2+} channel; AchR, acetylcholine receptor; yellow lightning symbols, membrane depolarization; Ca^{2+}, calcium; RyR, ryanodine receptor; L-cit, L-citrulline; L-arg, L-arginine; CaM, calmodulin; A, syntrophin α1, β1 and β2 adapter between dystrophin and nNOSμ; Calp, calpain.

of the signal leads to the transcription of NF-κB [60]. This transcription factor is essential for the expression of *iNOS* gene. On the other hand, cytokines produced by infected cells activate pathways responsible for the expression of several transcription factors (NF-κB, AP1, and IRF1) also involved in the transcription of iNOS [61]. Both pathways are necessary to fully upregulate iNOS expression. Moreover, iNOS is constitutively active and exhibits a high affinity for calcium explaining its Ca^{2+}-independent activity. iNOS produces a large amount of NO until substrate depletion [62]. Then, NO acts directly or through derived compounds (mostly $ONOO^-$ and NO_2) generated through the reaction of NO with ROS formed by NADPH oxidase. These molecules can be generated inside (phagosome) or outside the cells. Altogether, NO and derivatives could act in synergy on several components within the targeted hostile elements such as proteins (thiols, metal centers, and tyrosine residues), nucleic acids, and lipids [63, 64]. Altogether, these modifications lead to irreversible damages impairing cellular metabolism and ultimately inhibit growth and replication mechanisms and even cell death (**Figure 11**).

After infection, NO generation is abolished by neutralization of iNOS activity. Indeed, iNOS undergoes a posttranslational modification called polyubiquitination leading to its proteasomal degradation. Physiological aggresome, an alternative pathway for protein degradation, is also involved in iNOS inactivation [66]. The degradation of NO can also be modulated by its own interaction with ROS. The depletion of L-arginine precursor also shuts down iNOS activity.

Overexpression of iNOS is observed during chronic infection leading to an excessive level of NO and RNS. This phenomenon can impair the host physiology because these high reactive compounds represent mutagenic sources. Thus, the NO balance is a crucial point to

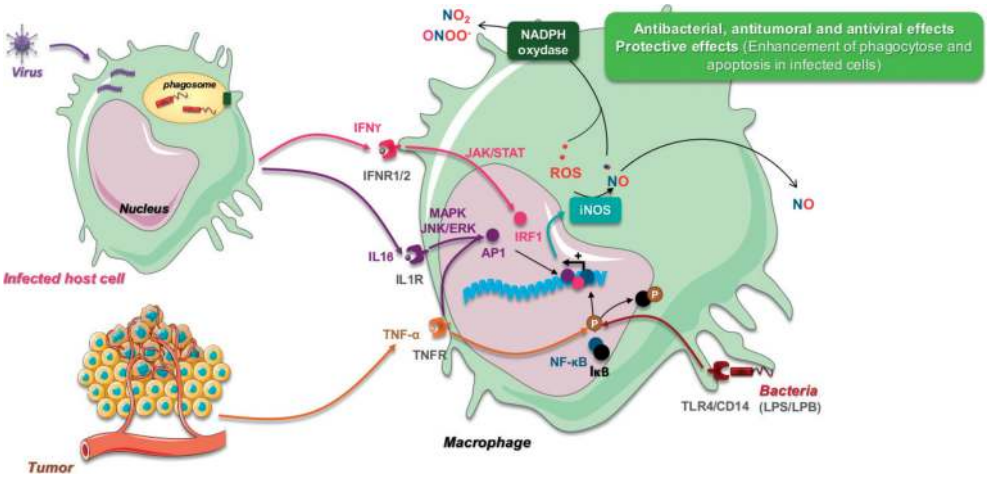

Figure 11. Implication of iNOS in the innate immunity (adapted from [65]). IFNϒ, interferon ϒ; IFNR1/2, IFNϒ receptors 1 and 2; JAK, Janus kinase; STAT, signal transducer and activator of transcription; IRF1, interferon response factor 1; IL1β, interleukin 1β; IL1R, IL1β receptor; MAPK, mitogen-activated protein kinase; JNK, C-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; AP1, activator protein 1; TNF-α, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; NF-κB, nuclear factor κB; TLR4, Toll-like receptor 4; CD14, cluster of differentiation 14; LPS, lipopolysaccharide; LPB, LPS-binding protein; ROS, reactive oxygen species.

control in order to limit its tumorigenic potential. The development of iNOS inhibitors and NO-releasing agents offers interesting therapeutic strategies to struggle against cancer and microbial infections.

3. NOx versus prokaryote: "Catch me if you can"

3.1. NOx at the center of prokaryotic life, virulence, and death

Several environmental, commensal, and pathogenic prokaryotes possess an arsenal of proteins dedicated to the nitrogen metabolism under anaerobic conditions [67]. Indeed, an enzymatic chain is involved in the denitrification pathway responsible for the production of NO through nitrite reductase (NIR) as an intermediate. The outcome of this mechanism is the formation of dinitrogen (N_2). This process is crucial for the growth of bacteria in the absence of oxygen because it is coupled with ATP generation (**Figure 12**).

Interestingly, a shortened NOS-like protein called bNOS was discovered in Gram-positive bacteria [28]. This enzyme is deprived of the classical reductase domain already described in other NOS isoforms. However, bNOS is able to recruit other proteins exhibiting a reductase catalytic activity [69]. Similar to mammalian isoforms, bNOS catalyzes the production of NO using L-arginine precursor. However, NOx is key elements in the innate immunity to avoid infection. Indeed, similar to eukaryotic cells, prokaryotes are also sensitive to NOx activity. Thence, such compounds could alter DNA, proteins, and metal centers leading to severe alterations of bacterial growth and replication.

As previously described, NO could be endogenously produced by bacteria but is also a powerful exogenous antimicrobial agent. This highlights the requirement of systems to manage a bearable threshold of NOx within the cell. First, bacteria have developed NO sensing system

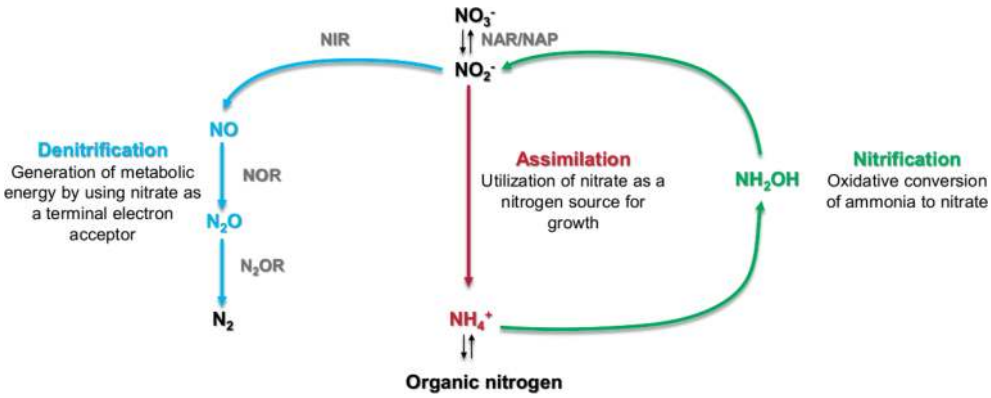

Figure 12. Bacterial nitrogen cycle (adapted from [68]). NO_3^-, nitrate; NO_2^-, nitrite; NO, nitric oxide; N_2O, nitrous oxide; N_2, dinitrogen; NH_2OH, hydroxylamine; NH_4^+, ammonia; NAR/NAP, nitrate reductase; NIR, nitrite reductase; NOR, nitric oxide reductase; N_2OR, nitrous oxide reductase.

through non-heme iron cluster conjugated with a wide range of detoxification mechanisms. Several pathways involving reaction of NO with non-heme iron cluster were described, mainly in *Escherichia coli* [70]. Indeed, NO can lead to a raise of the repression of sulfur assimilation operon (*suf*) exerted by ferric uptake regulator protein (Fur). This reaction is mediated by Fur nitrosylation through NO-derived S-nitrosoglutathione (GSNO). *Suf* products are required for the biogenesis of the [Fe—S] cluster, which, in turn, gives a lure to get rid of NOx collateral damages. The flavohemoglobin (hmpA) is another response against the nitrosative stress. This enzyme catalyzes the conversion of NO into nitrous oxide (N_2O) under anaerobic conditions. Interestingly, in the presence of NO, the S-nitrosylation of cysteine residues on MetR and FNR inactivates both the proteins. Thus, the repression exerted by MetR and the fumarate and nitrate reductase regulatory protein (FNR) is raised by *hmpA* allowing its transcription. Furthermore, NO-responsive transcriptional factor (NorR) is directly activated by NO and enhances the expression of NorVW protein catalyzing the reduction of NO into N_2O. Redox-sensitive cysteines also take part in the NO-sensing systems. Indeed, NO-activated superoxide regulon (SoxR) protein leads to the transcription increase of superoxide dismutase (*sodA*) removing O_2^- that may react with NO to form NO_2 and $ONOO^-$ [71]. Similar to SoxR, peroxide regulon (OxyR) protein is activated by S-nitrosylation. This protein induces gene expression for protective products against nitrosative stress at least by limiting the S-nitrosylation [72]. For example, the expression of catalase KatA, which could buffer free NO, is activated by OxyR [73]. Altogether, these sensing systems lead to the efficient detoxification by generation of less toxic compounds or scavenger molecules.

Moreover, bacteria also develop repairing system of DNA and [Fe—S] cluster damages related to nitrosative stress. Similar to eukaryotic cells, five DNA repair pathways are available in prokaryotes. Among them, the base excision repair (BER), to repair deaminated base, and the nucleotide excision repair (NER), to remove cross-linking in DNA, are predominant [74]. Concerning the [Fe—S] cluster repair, the iron sulfur cluster S protein (IscS), a cysteine desulfurase, can denytrosylate these protein clusters in the presence of L-cysteine [75]. Moreover, Isc regulator (IscR) protein is able to sense nitrosylated [Fe—S] cluster and thus enhances the formation and/or repair of [Fe—S] cluster [76, 77].

NO could also alter tricarboxylic acid (TCA) metabolic pathway and bacterial respiration through reaction with aconitase and cytochromes of the electron transfer chain, respectively [78]. However, these mechanisms are crucial for the generation of ATP and hence the energetic needs of bacteria [79]. To counteract the inefficiency of this enzyme, some bacteria are able to reprogram their metabolism. For example, *Pseudomonas* spp. uses the citrate lyase, phosphoenolpyruvate carboxylase, and pyruvate phosphate dikinase to convert citrate into pyruvate and ATP [80].

In nonlethal concentrations, NO interplays a signaling role in bacteria through several proteins possessing heme-nitric oxide/oxygen-binding domain and is referred under the name H-NOX proteins. H-NOX exhibits a highly conserved domain among bacteria and also shared in mammals (sGC) [81]. All H-NOX are histidine-ligated protoporphyrin IX hemoprotein able to fix NO on its ferrous iron. The H-NOX-encoding genes are found in operons with diverse bacterial signaling genes [82], whose majority is now divided in two classes: containing (i) histidine

kinase (HK) or (ii) diguanylate cyclase (DGCs) and phosphodiesterase (PDE). H-NOX could modify the function of proteins implicated in virulence such as biofilm formation and motility or even *quorum sensing* [83]. For example, H-NOX alters the function of diguanylate cyclase and enhances the phosphodiesterase activity. Altogether, this leads to the biofilm dispersion through the decrease of c-di-GMP, an essential mediator in this process. On the contrary, a more sophisticated system relative to histidine kinase (HnoK) activity leads to the accumulation of c-di-GMP in response to NO favoring the biofilm adhesion and formation. Another important virulence trait could be also modulated by H-NOX as in *Vibrio* species a cross-talk with QS seems to exist. However, a few studies are yet available.

More recently, an antibiotic resistance modulation was promoted by a bacterial NO_2 gaseous exposure. Indeed, an upregulation in expression of MexEF-OprN efflux pump was observed coupled with an increase in related antibiotic resistance [84]. The link between NO_2 and this efflux pump is yet not established, but the authors suggest here a potential role of this protein in the releasing of NOx outside the cells. Previous studies also highlight a correlation between NO endogenously generated by bNOS and increase aminoglycoside resistance [85]. NO and NO_2 could directly interact with antibiotic leading to a less toxic compound and exerted a repression of bacterial respiration preventing drug uptake.

To resume, bacteria are able to produce NO and RNS. Following endogenous burst or exogenous aggression (pollution, inflammation reactions), a physiological threshold is exceeded. However, bacteria developed an ingenious system to counteract the potential effects of NO and derived products. Various interconnected signaling pathways are implicated in this phenomenon: (i) NO detection, (ii) detoxification, (iii) repairing mechanisms, and (iv) metabolic reprogramming. Finally, these fundamental modifications modulate bacterial community behavior (QS) and virulence traits, such as biofilm and antibiotic resistance. This last point is particularly attractive in medicine. Indeed, the emergence of several antibiotic multiresistant pathogens represents a big threat for human that brings us to the last question of this review: "Does NOx therapy represent the new trends in human health care?"

3.2. The novel therapeutic strategies mediated by NOx

A lot of therapeutic strategies exploiting NOx-mediated pathways has recently emerged, particularly to treat cancer. Here we propose to classify them in three families: (i) NO-donor compounds, materials, or nanoparticles; (ii) modulators of NOS activity; and (iii) last but not least, generators of gaseous NO.

The first family NO donors consist of NO-chelating compounds that can release NO under specific conditions within the organism after administration. As shown below, several NO donors have been developed during the last decade. Their global—direct or not—anticancer effects have been exploited in the case of various carcinoma (**Table 1**).

For further information, we strongly recommend to read the review of Huang et al. [86]. Another extension of the use of NO-donor compounds is their graft on material and nanoparticles. Indeed, prosthetic biomaterials used in medical devices were modified through covalent or noncovalent binding of two NO donors (diazeniumdiolates and S-nitrosothiols).

NO-donor classes	Representative molecule	NO-release pathway	Physiological impact
Organic nitrates	Glyceril trinitrate (GTN)	Enzymatic biotransformation needed	Anticancer activity
Diazeniumdiolates	NONOates	Spontaneous and selective	Antiproliferative activity
Metal nitrosyl complexes	Sodium nitroprusside (SNP)	Spontaneous	Invasion suppression Radiosensitization
1,2,5-oxadiazole N-oxides	Furoxans	Thiol-dependant	Neuroprotective
S-nitrosothiols	S-nitroso-N-acetylpenicillamine (SNAP)	Metal ions- or superoxide-dependant	Therapeutic potential in cancers
Sydnonimine	3 morpholinosydnonimine (SIN1)	pH-dependant	Therapeutic potential in cancers

Table 1. The different classes of NO donor (adapted from [86]).

Several clinical trials have shown their usefulness in the context of preventing thrombus formation [87]. NO-donating nanoparticle systems represent a new promising tumoricidal agents, thanks to their unique properties: (i) strengthening NO-donor stability, (ii) loading a large amount of NO, and (iii) possibility to trigger NO releasing [88].

The modulation of NOS activity is another interesting strategy. In some pathologies inducing exacerbating inflammation (sepsis), a localized "nitrosative burst" occurs, and inhibition of NOS function is a crucial element. Thus, several L-arginine precursor analogs (N^{ω}-substituted L-arginine) were developed and tested. However, since L-arginine is the common point between all NOS isoforms, these analogs represent nonselective drugs. Moreover, such therapy did not reach clinical studies on human stage despite its effects on canine vascular tone [89]. Thus, investigation on a more selective inhibition of iNOS, crucial for inflammation, was conducted through transgenic animals. Unfortunately, iNOS knockout mice exhibit an increase in mortality following polymicrobial sepsis [90]. More recently, researcher focused on increasing NO delivery within cancers. Thus, thanks to technical progress and scientific knowledge updating, iNOS-based suicide gene therapy was investigated through viral vector use. This treatment exhibits promising tumoricidal effects and appears interesting for its specific and localized iNOS expression on animal models [91].

The third strategy is the direct gaseous delivery of NO in the treatment of various infection diseases related to antibiotic resistant bacteria. Ghaffari et al. provided a considerable work on this topic. First, they developed an ingenious delivery system usefulness for the monitoring of gNO effects on microorganisms (bacteria and fungi) and eukaryotic cells [92]. Then, they reported an effective concentration of gNO up to 200 ppm in continuous 4 h delivery exhibiting bacteriostatic effects on a representative group of microorganisms (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Candida albicans*, and *Group B Streptococcus*). Moreover, no toxic effects were observed on representative mammalian cell lines (dermal fibroblasts) of wound-healing pathology such as leg ulcer or burn injuries [93]. Thus, these promising results placed gNO as a potential topical antibiotic agent [94]. Later, they also investigated the potential use of inhaled gNO in the treatment of pathogenic infection in the case of cystic fibrosis. Indeed, this pathology is a threatening pulmonary disease where microbial infection could be lethal. However, a continuous exposure of gNO at such concentration can lead to severe

impact on the human health, particularly through methemoglobinemia. This phenomenon occurs when hemoglobin losses its vital function of oxygen carrier following the saturation of its iron heme with NO. Thus, an intermittent high-dose short-duration exposure was tested to determine the dose/duration most effective treatment. An adapted lung mammalian model was also used (macrophages and monocytes and pulmonary epithelial cells) to appreciate the potential effects of this cure on human health [95]. This study reached phase I clinical studies using a promising NO treatment at 160 ppm for 30 min three times daily for two periods of 5 days [96]. However, as reviewed by Petit et al., it is important to keep in mind that "administration of inhaled NO is associated with and unavoidable codelivery of NO_2," thanks to the spontaneous high reactivity of NO with O_2 [97]. They also reported that the higher NO doses are used, the faster is the formation rate of NO_2. In contrast, a direct gaseous NO_2 exposure could also be benefit in the struggle against undesirable microorganism because it seems to impact their metabolism, social behavior, and growth [84]. Moreover, a continuous high-dose NO_2 exposure seems to lead in membrane alteration through an increased permeability [98].

4. Conclusion

In summary, this review of the literature shows that NOx is ubiquitous and essential in our lives. We are exposed to these compounds through environmental contaminations, but they also result from physiological processes, infections, diseases, or even drugs. NO and derivatives are implicated in a large diversity of vital functions including brain functions; motricity; cardiac, vascular, and pulmonary functions; and immunity. For these reasons, NOx is key molecules of major biochemical interest. As all active substances, NOx can exert both beneficial and adverse effects with regard to the exposition dose. The physiological adaptation to NOx results from a fine-tuned equilibrium where detoxification plays crucial roles. Myriad of finely regulated processes has emerged in response to nitrosative stress. Our knowledge still remains incomplete, and future studies are necessary to finely precise the synergy of each NOx implemented in nitrosative stress, especially when they are presented in gaseous phase that remains less explored. However, in spite of all previously described mechanisms to counteract the nitrosative stress, abnormal NOx thresholds could trigger a wide range of pathologies and even death. These antagonists, positive and negative effects, of NOx are actually intriguing. So, many researches are now focusing on NOx producing pathways to find the most effective treatments and drugs. More investigations are still needed to better understand the real potential of NOx as antitumor, antibacterial agents, and their safe clinical use.

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Conflict of interest

We have no conflict of interest to declare.

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