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Comprehensive Invited Review

Reactive Nitrogen Species: Molecular Mechanisms and Potential Significance in Health and Disease

M. Carmen Martínez and Ramaroson Andriantsitohaina

Reviewing Editors: Markus Bachschmid, Srinivas Bharath, Maria Ciriolo, Isabella Dalle-Dorne, Sergey Dikalov, Jeffrey Keller, Pamela Maher, and Hugo Monteiro

INSERM, U771, CNRS UMR, 6214, and Université d'Angers, Angers, France.

Abstract

Reactive nitrogen species (RNS) are various nitric oxide–derived compounds, including nitroxyl anion, nitrosonium cation, higher oxides of nitrogen, *S*-nitrosothiols, and dinitrosyl iron complexes. RNS have been recognized as playing a crucial role in the physiologic regulation of many, if not all, living cells, such as smooth muscle cells, cardiomyocytes, platelets, and nervous and juxtaglomerular cells. They possess pleiotropic properties on cellular targets after both posttranslational modifications and interactions with reactive oxygen species. Elevated levels of RNS have been implicated in cell injury and death by inducing nitrosative stress. The aim of this comprehensive review is to address the mechanisms of formation and removal of RNS, highlighting their potential cellular targets: lipids, DNA, and proteins. The specific importance of RNS and their paradoxic effects, depending on their local concentration under physiologic conditions, is underscored. An increasing number of compounds that modulate RNS processing or targets are being identified. Such compounds are now undergoing preclinical and clinical evaluations in the treatment of pathologies associated with RNS-induced cellular damage. Future research should help to elucidate the involvement of RNS in the therapeutic effect of drugs used to treat neurodegenerative, cardiovascular, metabolic, and inflammatory diseases and cancer. *Antioxid. Redox Signal.* 11, 669–702.

I. Introduction

Nitric oxide ($NO^{\cdot}$) is a ubiquitous intracellular messenger able to regulate physiologic functions such as neural and cardiovascular activities. However, during pathologic conditions, $NO^{\cdot}$ can become deleterious because of its high reactivity with other free radicals, such as the superoxide anion ($O_2^{\cdot -}$). Although both $NO^{\cdot}$ and $O_2^{\cdot -}$ at low concentrations are not toxic in a physiologic environment, an imbalance between the production of these two radicals can be partially responsible for alterations of the molecular mechanisms regulating cell life. This disequilibrium is mainly due to a diminished elimination or an increased production for $O_2^{\cdot -}$ or a reduction in the release or enhanced scavenging of $NO^{\cdot}$. $NO^{\cdot}$ and $O_2^{\cdot -}$ react by an enzyme-independent mechanism to form peroxynitrite ($ONOO^-$), a strong oxidant that reacts with most biologic molecules, causing cell damage. $ONOO^-$ is not the only reactive nitrogen species (RNS); RNS refers to various nitrogenous products, such as $NO^{\cdot}$, nitroxyl (HNO), nitrosonium cation (NO^+), higher oxides of nitrogen, *S*-nitrosothiols (RSNOs), $ONOO^-$, and dinitrosyl iron complexes, excluding NO_3^- (225) (Table 1). Each of these compounds has distinctive properties in terms of reactivity, half-life, lipid solubility, and biologic activity (225). Thus, RNS induce reactions including nitrosylation of sulfhydryls (*S*-nitrosylation) or metals and nitration of tyrosine residues. In addition, cross-talk between reactive oxygen species (ROS) and RNS, through chemical reaction or functional interaction or both, is possible, and it can exacerbate the deleterious effects. It has been suggested that when RNS production becomes excessive, they become deleterious to target cells, and this could play a role for a variety of the degenerative processes of some human diseases (37).

By analogy with ROS and oxidative stress, when the generation of RNS in a system exceeds its ability to neutralize

Table 1. Main Reactive Nitrogen Species in Alphabetic Order

Name	*Formula*	*Formation*
Dinitrogen trioxide	N_2O_3	From NO· and O_2
Nitric oxide or nitrogen monoxide	NO·	From NOS
Nitrite	NO_2^-	From NO·
Nitrogen dioxide	NO_2·	From $ONOO^-$decomposition
Nitronium cation	NO_2^+	From $ONOOCO_2^-$decomposition
Nitrosonium cation	NO^+	From NO·
Nitrosoperoxycarbonate anion	$ONOOCO_2^-$	$ONOO^-$ and CO_2
Nitroxyl	HNO	From one-electron reduction of NO·
Nitryl chloride	Cl-NO_2	From NO_2^- and HOCl
Peroxynitrite	$ONOO^-$	From NO· and $O_2^{\cdot -}$
S-Nitrosothiols	RSNOs	From covalent addition of an NO· group to a cysteine thiol/sulfhydryl

and eliminate them, nitrosative stress occurs (233). This review summarizes the main molecular mechanisms involved in the physiologic effects of RNS as well as the potential pathophysiologic consequences subsequent to the damage induced by RNS on various molecular targets. First, we outline the mechanisms of formation and removal of RNS, highlighting the potential cellular targets and the expected cell effects. Next, we give an overview of the physiologic role of RNS in the living cell, and finally, we look at the consequences of RNS in health, including diseases such as neurodegenerative and cardiovascular pathologies, metabolic and inflammatory diseases, and cancer.

II. RNS Formation and Mechanisms of Action

$NO^{\cdot}$ plays a dual role in the nitrosative stress. On the one hand, $NO^{\cdot}$ is the main RNS produced by cells, and on the other hand, it is the main source for the other RNS. $NO^{\cdot}$ is produced by NO synthases (NOS) from L-arginine and oxygen. It promptly diffuses within cells and through cell membranes, constituting a second messenger leading, for example, to the relaxation of smooth muscle cells or synaptic transmission (see later). The three isoforms of NOS are NOS 1 or nNOS (for neuronal), NOS 2 or iNOS (for inducible), and NOS 3 or eNOS (for endothelial). Although NOS isoforms catalyze the same reactions, they differ in their expression, regulation, and physiologic/pathophysiologic roles (226). Both nNOS and eNOS are constitutively expressed; nNOS is expressed predominantly in neurons, and eNOS, in endothelial cells (183). Moreover, both isoforms are present in other cell types, such as smooth muscle cells (183). In addition, constitutive eNOS is able to produce low levels of $NO^{\cdot}$, and it is accepted that their activation is "beneficial" for cells, as demonstrated by using eNOS knockout mice (215), whereas nNOS produce moderate levels of $NO^{\cdot}$. By contrast, when iNOS expression is induced, predominantly in macrophages, under proinflammatory conditions, large quantities of $NO^{\cdot}$ are produced (183). Regarding the regulation of the three isoforms of NOS, both nNOS and eNOS are regulated by intracellular Ca^{2+}/calmodulin, as well as by the interaction of other proteins such as hsp90 and caveolin. In contrast, iNOS is active even at low concentrations of Ca^{2+}, and it is regulated mainly at the level of transcription.

The direct action of $NO^{\cdot}$ is limited by its biologic half-life *in vivo* (~1 sec) and by the relatively short distance that this molecule can traverse. Under physiological stress conditions, $NO^{\cdot}$ binds to the heme iron of guanylyl cyclase (GC) and activates GC and cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent pathways. Indeed, $NO^{\cdot}$ released from endothelial cells diffuses toward neighboring smooth muscle cells and activates GC. The cGMP produced interacts with protein kinase G (PKG), which phosphorylates contractile proteins, decreases cytosolic Ca^{2+} levels, evokes myosin light-chain dephosphorylation, and induces vasorelaxation (219).

Although NO_2^- has been considered the final metabolite together with NO_3^- in the decomposition of $NO^{\cdot}$, recent data show that this RNS can be considered an intermediate in the complex pathway. NO_2^- is an oxidation product of NOS-derived $NO^{\cdot}$ (212). $NO^{\cdot}$ can be formed by xanthine oxidase–catalyzed reduction of NO_2^- under hypoxic conditions (206, 343) and participates in its protective effects. However, Patel's group (60, 100, 101) describes the cytoprotective effect of NO_2^- independent of the xanthine oxidase pathway. NO_2^- reacts with hemoglobin, which behaves as a nitrite reductase, and decreases the ability of hemoglobin to reduce $NO^{\cdot}$ availability (100). $NO^{\cdot}$ can react with the heme group of hemoglobin and therefore limits its $NO^{\cdot}$ bioactivity (60). Thus, this mechanism may have a potential role in vasodilatation during hypoxic conditions (101).

More reactive than $NO^{\cdot}$, $ONOO^-$ is a RNS resulting from the rapid reaction between $NO^{\cdot}$ and $O_2^{\cdot -}$. $ONOO^-$ is a strong oxidant that mainly nitrates tyrosine residues in nonenzymatic reactions. Moreover, in a biologic system, $ONOO^-$ rapidly reacts with CO_2 (in equilibrium with physiologic levels of HCO_3^-), which leads to the formation of $CO_3^{\cdot -}$ and $NO_2^{\cdot -}$ radicals that, in turn, oxidate and nitrate proteins (312). $ONOO^-$ can react with CO_2, resulting in the formation of an unstable nitrosoperoxycarbonate anion adduct ($ONOOCO_2^-$) that can serve as a source of several reactive intermediates (326). $ONOOCO_2^-$ reacts with guanine in DNA to form a variety of oxidation and nitration products, such as 8-nitro-2′-deoxyguanosine (77). $ONOOCO_2^-$ rapidly decomposes to yield NO_2^+ and CO_3^{2-} (66). Finally, $ONOO^-$ can decompose to NO_2^- and NO_3^-. In particular, the formation of NO_3^- from $ONOO^-$ depends mainly on the iso-

merization of ONOOH and the transient $ONOOCO_2^-$ adduct (312).

A. Posttranslational modifications induced by RNS

When NO· was identified as the molecule accounting for the endothelium-derived relaxing factor, only the modifications on protein-bound transition metals (as described earlier for GC) induced by NO· were studied. However, experiments performed in recent years have shown that RNS can induce posttranslational modifications of a large number of proteins. Like phosphorylation, the most common posttranslational protein modification, the different modifications evoked by RNS on proteins alter protein structure and, consequently, protein function and interactions with downstream signaling targets. Three main posttranslational modifications can be induced by RNS: (a) *S*-nitrosylation, (b) glutathionylation, and (c) tyrosine nitration.

1. *S*-Nitrosylation. NO· can induce *S*-nitrosylation of regulatory protein thiol groups through its autooxidation with O_2 and the formation of dinitrogen trioxide (N_2O_3), a putative nitrosylating species. *S*-nitrosylation is the covalent addition of an NO· group to a crucial cysteine thiol/sulfhydryl to form an *S*-nitrosothiol derivative (RSNO) (Fig. 1). Such posttranslational and temporary protein modifications, which are independent of enzymatic activity, regulate the function of a large number of mammalian, plant, and microbial proteins with a susceptible thiol group. Overall, the susceptibility of protein cysteine sulfhydryl to *S*-nitrosylation is increased by a consensus motif of amino acids comprising nucleophilic residues surrounding "the critical cysteine" (119). This mechanism of *S*-nitrosylation has protective effects in different systems, preventing some critical protein thiols from further irreversible oxidative modifications by ROS. Thus, Stamler's group (112) has suggested that ROS can alter the balance between phosphorylation and *S*-nitrosylation of key signaling proteins. Among these proteins, protein kinases and phosphatases, metabolic enzymes, membrane receptors and channels, cytoskeletal elements, and transcription factors are potential candidates to be *S*-nitrosylated (for a review, see ref. 119). In consequence, alterations in the regulation of protein *S*-nitrosylation or in the levels of *S*-nitrosothiols are often linked to pathophysiologic states including human diabetes (207), cystic fibrosis (105), or mouse sepsis (184).

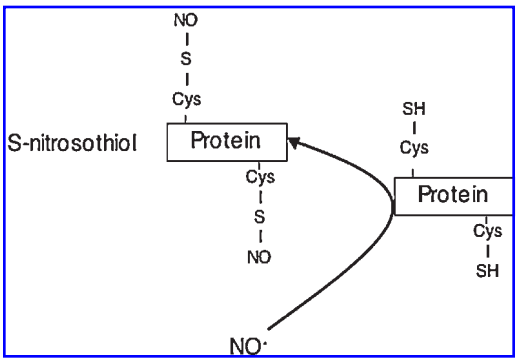

FIG. 1. S-Nitrosylation of proteins. Coupling of an NO· moiety to a reactive thiol group of cysteine residues of peptides and proteins allows the formation of S-nitrosothiols.

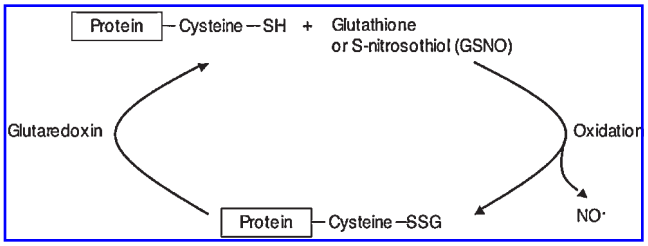

FIG. 2. Mechanism(s) of glutathionylation. Reactions between cysteine residues and glutathione or S-nitrosothiols such as GSNO are able to release NO·.

Because of the lability of the S-NO· complex, detection of *S*-nitrosylated proteins becomes difficult, and, in general, indirect methods of detection are used. New techniques to facilitate quantification of *S*-nitrosylation of proteins should allow a better understanding of the consequence of this posttranslational modification in cell function.

2. Glutathionylation. Glutathionylation, also referred to as glutathiolation, is the addition of glutathione (GSH), or other low-molecular-weight thiols, to the cysteine sulfhydryl residues of proteins and represents the most important thiolation. This reaction is reversible and independent of enzyme activity. After *S*-nitrosylation of GSH and generation of *S*-nitrosoglutathione (GSNO), the formed GSNO molecule can mediate posttranslational modifications (*i.e.*, modification of protein thiols *via* S-nitrosylation and glutathionylation) (32, 112) (Fig. 2). Thus, *S*-nitrosylation can be considered an intermediate signaling event. Then GSNO may account for remote or long-lasting effects of NO·, serving to store, transport, and deliver NO· *in vivo* to its neighboring proteins (217). Thus, GSNO has been suggested as the main physiologic mediator of NO· effects. GSNO decomposes slowly to generate NO· (349), and denitrosylation reactions can be accomplished by GSNO reductase (182). GSNO reductase, also called alcohol dehydrogenase of class III, is the major enzyme that catalyzes GSNO metabolism. GSNO reductase generates glutathione disulfide (GSSG) and ammonia, and allows the regulation of the intracellular levels of *S*-nitrosothiols (115, 182). By using GSNO reductase–deficient mice, Liu *et al*. (184) demonstrated that this enzyme is indispensable for *S*-nitrosothiol metabolism, regulation of blood pressure, and protection from nitrosative stress in response to endotoxin and bacteria. Thus, protein *S*-glutathionylation is considered an adaptive cellular response protecting essential proteins involved in regulatory pathways from permanent loss of function as a consequence of nitrosative stress (152). However, further studies are needed the better to understand the biologic consequences of this posttranslational modification on the regulation of the function of target proteins.

3. Tyrosine nitration. Tyrosine nitration is a two-step process in which the first reaction is the generation of tyrosyl radical by oxidation of tyrosine by reactive species generated from $ONOO^-$. In the second step, tyrosyl radical reacts with NO_2 to form 3-NO_2-Tyr (3-nitrotyrosine) (260) (Fig. 3). Nitration of tyrosine residues is widely used as a biomarker of $ONOO^-$ generated from the reaction between NO· and $O_2^{\cdot -}$. $ONOO^-$ is typically more reactive and toxic than NO· *per se*.

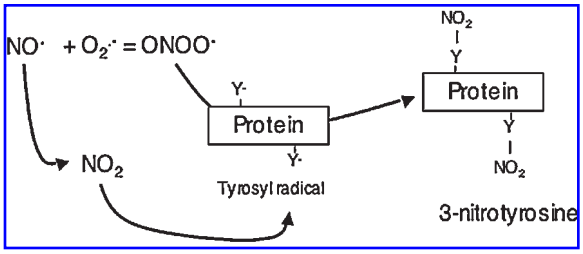

FIG 3. Formation of $ONOO^-$ from the NO· and $O_2^{\cdot -}$ as reaction and the subsequent nitration of tyrosyl radical to form nitrotyrosine. High concentrations of $ONOO^-$ lead to nitration of proteins on tyrosine residues. Tyrosine nitration is a two-step process: first, the generation of tyrosyl radical by oxidation of tyrosine by reactive species generated from $ONOO^-$, and second, the reaction of tyrosyl radical with NO_2 to form 3-NO_2-Tyr(3-nitrotyrosine).

Originally, $ONOO^-$ has been suggested to be a strong biologic oxidant that can react directly with sulfhydryls, iron–sulfur, and zinc–thiolate groups (for review, see ref. 238). In addition to tyrosine nitration, $ONOO^-$ participates in hydroxylation and oxidation reactions (245). In particular, tyrosine nitration may alter several protein activities, and increased circulating levels of 3-nitrotyrosine are correlated with cardiovascular diseases such as coronary artery disease (294). Furthermore, it has been suggested that low amounts of $ONOO^-$ (under physiological stress conditions) can activate pathways leading to the activation of Akt, a kinase that, in human skin primary fibroblasts, is at least in part responsible for antiapoptotic and survival signals (154). Moreover, low physiological levels of $ONOO^-$ can interact with the tripeptide GSH, resulting in reversible *S*-glutathionylation of proteins (3). Another RNS, NO_2, has been described as a nitrating molecule.

Several authors consider that tyrosine nitration is related to nitrative stress rather than to nitrosative stress. In addition, another concept, nitroxidative stress, may replace that of nitrative stress. However, taking into consideration the number of original articles in which RNS-induced nitration is referred to as nitrosative stress, we use this term in this review.

Although data obtained in *in vitro* studies clearly show that nitration of proteins could alter protein structure and function, a large number of questions remain to be answered to explain completely the biologic significance of protein nitration *in vivo*. Comparison of proteomic posttranslational modification analysis of tissues obtained from patients *versus* "healthy" tissues could contribute to the discovery of new elements for a better understanding of disease etiology, and then, to the development of new tools for research against diseases.

Very recently, a novel mechanism was described. Sawa *et al.* (282) showed that the nitrate derivative of cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), generated through iNOS activation, is able to form protein Cys-cGMP adducts by *S*-guanylation. In addition, 8-nitro-cGMP can regulate the redox-sensor signaling of protein Keap1, suggesting that 8-nitro-cGMP can be considered a second messenger of NO·.

B. Relation between oxidative and nitrosative stresses

Oxidative stress is closely related to nitrosative stress. In general, the excessive production of ROS or the failure of cellular removal of these species or both are responsible for increased oxidative stress. Once ROS levels are enhanced, reactions between ROS and RNS take place and lead to nitrosative stress. Although oxidative stress is not the focus of this review, we consider it necessary to comment briefly on the main ROS implicated in the generation of nitrosative stress. Elevated levels of $O_2^{\cdot -}$ and hydrogen peroxide chiefly contribute to the development of oxidative stress, and their subsequent reactions with RNS result in nitrosative stress. The half-lives of ROS vary considerably from a few nanoseconds for the most-reactive compounds, such as ·OH, to seconds and hours for more-stable ROS, such as hypochlorous acid (311). ROS are generated by a wide array of enzymes that include NADPH oxidases, xanthine oxidase, peroxidases, lipoxygenases, and cyclooxygenases, and complex I and III of the respiratory chain in the mitochondrion. NADPH oxidases are enzymes with catalytic and regulatory subunits that catalyze single-electron reductions of molecular oxygen by using NAD(P)H as electron sources (106, 165). Conversely, xanthine oxidase produces $O_2^{\cdot -}$ or hydrogen peroxide as products of the terminal steps of purine metabolism, metabolizing hypoxanthine and xanthine to uric acid with concomitant generation of $O_2^{\cdot -}$ (25). In addition, xanthine oxidase can generate NO· under hypoxic situations, either from organic nitrites in the presence of xanthine as a substrate (76) or when NOS cannot produce NO· (175). However, the physiologic relevance of this pathway *in vivo* remains to be elucidated.

Under certain conditions, NOS themselves can generate $O_2^{\cdot -}$ as a consequence of the electron transfer within the active site becoming "uncoupled" from L-arginine oxidation; instead, molecular oxygen is reduced to form $O_2^{\cdot -}$ (331, 332). Various molecular mechanisms could contribute to NOS uncoupling. Limited availability of the substrate L-arginine may reduce NO· synthesis; under these conditions, the Ca^{2+}/calmodulin-activated NOS resembles the plasma membrane–associated NADPH-dependent oxidase of neutrophils (255). Also, the interaction between heat-shock protein 90 and NOS seems to regulate the balance between NO· and $O_2^{\cdot -}$ productions (257). Tetrahydrobiopterin (BH_4), which acts as an essential cofactor for all three NOS isoforms (6), appears to have the main role in the regulation of this equilibrium. Recent evidence suggests that BH_4 depletion is responsible for NOS uncoupling, because of its oxidation and/or reduced synthesis as described in mice with hypertension (168).

III. RNS Neutralization

The mechanisms that protect mammalian cells from the deleterious effects of nitrosative stress remain to be clarified. Nonenzymatic protection against RNS may be provided by cellular antioxidants, such as GSH, metalloporphyrins, selenium compounds, uric acid, β-carotene, and vitamins E and C, as well as by the elimination of NO· by its rapid reaction with hemoglobin. Proteins such as thioredoxin (243), GSH peroxidases (31), superoxide dismutases (SOD) (143), or γ-glutamyl transpeptidase (131) have been recognized as potential systems able to remove RNS. Thioredoxin proteins

are classically defined by their ability to reduce disulfides to dithiols. Besides GSH, thioredoxin constitutes a major protective mechanism. Thus, both thioredoxin and GSH reduce protein thiols and various ROS, such as $O_2^{\cdot -}$ and hydrogen peroxide (243). Both systems operate independently and, moreover, GSH levels are higher than those of thioredoxin (73, 342). GSH peroxidases are selenoproteins able to reduce hydroperoxides to the corresponding alcohols by means of GSH. In addition to their antioxidant activities (GSH peroxidases are the main intracellular antioxidant), they interact with redox-regulated transcription factors, such as the NF-κB and Nrf2/Keap1 systems (31), suggesting that GSH peroxidases can be involved in the regulation of the inflammatory process. SODs are metalloenzymes that catalyze the conversion of $O_2^{\cdot -}$ to hydrogen peroxide, which immediately becomes a substrate for the enzyme catalase and other hydrogen peroxide–catalyzing enzymes such as GSH peroxidases and peroxiredoxins. Up to now, three different SOD isoforms have been described, and they ensure the major scavenging systems in the cytoplasm and nucleus (SOD1 or CuZn-SOD), mitochondrion (SOD2 or Mn-SOD), and extracellular space (SOD3 or EC-SOD) (143). Transcription of the *sod* genes is increased in response to cell stress, leading to increased activity to protect cells from damage.

All of these elements underscore the variety of RNS and the subsequent reactions that can be observed, and suggest that the reactions associated with RNS are, in general, more complex than those of ROS.

IV. Molecular Targets of RNS: Cellular Effects of RNS

RNS can react with lipids, DNA and RNA bases, metal cofactors, and proteins (Fig. 4).

A. Lipids

Regarding lipids, RNS can react with cellular lipids directly or indirectly, generating a spectrum of products, many of which contain functional groups capable of modifying proteins (361). The reactive lipid products can be divided into three major classes: first, lipid peroxides are products derived from the enzymatic action of 15-lipoxygenase (8); second, reactive lipids with electrophilic properties, such as 15-deoxy-Δ12,14-prostaglandin J2, and 4-hydroxynonenal (50, 166); finally, receptor agonists including nitrolinoleic acid (LNO_2) and lysophosphatidylcholine (50). It is interesting to note that, under certain conditions, lipids reacting with RNS may become donors of $NO^{\cdot}$, and, consequently, no deleterious effects are described. For example, LNO_2, a product of $NO^{\cdot}$-dependent linoleic acid nitration, is the most abundant, biologically active oxide of nitrogen in the human vascular compartment (15). LNO_2 acts as a signaling mediator *via* both receptor-dependent and receptor-independent pathways. Thus, LNO_2 is a specific and a high-affinity endogenous ligand for peroxisome proliferators–activated receptor-γ (PPARγ) (286) (Fig. 5). Besides, LNO_2 activates cAMP-dependent protein kinase pathways in cells involved in inflammation. This reaction confers cell protection against the inflammatory process. In the vascular compartment, LNO_2 elicits endothelium-independent relaxation by stimulating GC and generation of cGMP. Recently, Schopfer and colleagues (287) identified the intermediate product between LNO_2 and GC as $NO^{\cdot}$ and propose LNO_2 as a hydrophobically stabilized $NO^{\cdot}$ donor (Fig. 5). The nitro derivative of oleic acid (OA-NO_2), as well as LNO_2, is able to react with GSH and to form GS-OA-NO_2 and GS-LNO_2 (16) (Fig. 5). Baker and colleagues (16) report that the reversible adduction of thiols by nitro-fatty acids is a mechanism for reversible posttranslational regulation of protein function by nitro-fatty acids. Both OA-NO_2 and LNO_2 have been described recently as endogenous antiinflammatory mediators able to repress NF-κB–dependent gene expression and subsequently to reduce inflammatory response in vascular cells (62).

Another aspect is the ability of nitrated products of unsaturated fatty acids, such as OA-NO_2 and LNO_2, to inhibit vascular smooth muscle cell proliferation by inducing growth arrest of these cells in the phase G_1/S of the cell cycle. This effect of the nitroalkenes (the nitration products of unsaturated fatty acids formed *via* $NO^{\cdot}$-dependent oxidative reactions) is associated with an upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$. In addition, modifications at the level of the translocation of the nuclear factor

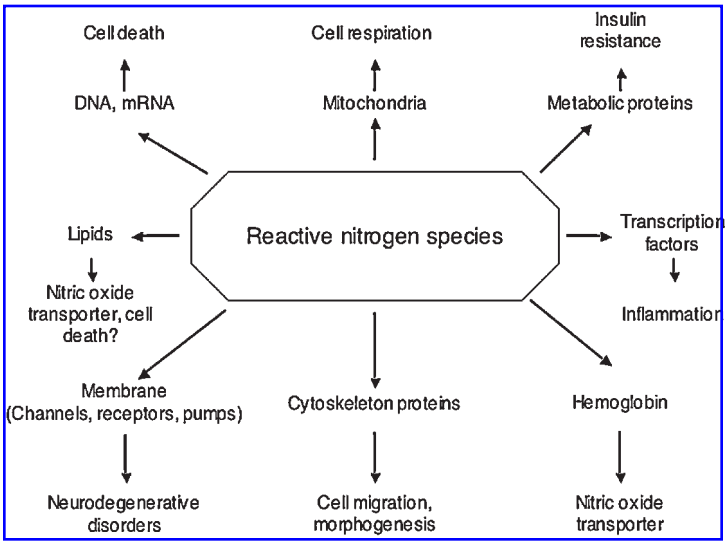

FIG. 4. Molecular targets of reactive nitrogen species (RNS). RNS are able to act on a variety of targets responsible for their pleïotropic effects.

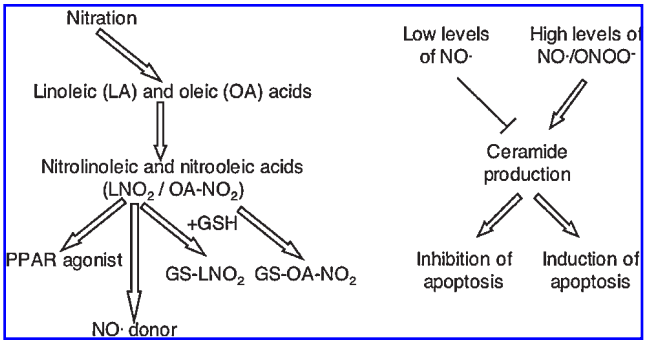

FIG. 5. Interaction of RNS with lipids. Through the nitration of linoleic (LA) and oleic (OA) acids, lipids can become $NO^{\cdot}$ donors. Nitrolinoleic (LNO_2) and nitrooleic ($OA\text{-}NO_2$) acids protect cells from inflammation, through the activation of peroxisome proliferators–activated receptor-γ (PPARγ). Moreover, LNO_2 and $OA\text{-}NO_2$ can react with glutathione and form $GS\text{-}OA\text{-}NO_2$ and $GS\text{-}LNO_2$. The effects of RNS on ceramide metabolism can modulate cellular apoptosis.

Nrf2 have been reported (334). These findings indicate that LNO_2 could have an important role in the growth of vascular smooth muscle cells and participate in the development of novel therapeutic strategies for cardiovascular diseases.

Conversely, several lines of evidence suggest that RNS are tightly linked to the regulation of the sphingolipid metabolism through regulating the enzymes responsible for their metabolism, including sphingomyelinase (SMase) and ceramidase (346). Sphingolipids are ubiquitous constituents of membrane lipids in mammalian cells. They are involved in proliferation, differentiation, apoptosis, and inflammation (346). Sphingolipid formation of ceramide and its derivatives such as sphingosine is regulated by the action of SMase. Paradoxic effects have been observed between $NO^{\cdot}$ and SMase activity (Fig. 5). Exogenous or endogenous $NO^{\cdot}$ is described as an inhibitor of apoptosis through the inhibition of ceramide generation (22). However, excessive amounts of $NO^{\cdot}$ activate apoptosis *via* the increase of ceramide production (251). Among RNS, $ONOO^-$ induces apoptosis through the modulation of ceramide levels in human airway epithelial cells by activating an acidic SMase (46), whereas $NO^{\cdot}$ donors increase ceramide levels without triggering apoptosis (47). The latter mechanism implicates the increase in the protein–protein interaction between SMase and caspase-3 that prevents its cleavage. Besides, certain sphingolipids, such as ceramide, are able to induce cellular oxidative and nitrosative stresses through the activation of NADPH oxidase, NOS, and mitochondrial dysfunction (for a review, see ref. 346). These data suggest a bidirectional regulation of sphingolipid metabolism and oxidative and nitrosative stresses.

B. DNA and RNA bases

DNA damage induced by RNS may contribute to increased mutation rates, genome instability, apoptosis, and associated tissue regeneration and cell proliferation, all of which can lead to carcinogenesis (281, 313). $ONOO^-$ and nitrogen oxides are the two RNS mainly implicated in DNA and RNA damage by inducing base modifications and strand breaks (281). In particular, on reaction with guanine, $ONOO^-$ is able to form 8-nitroguanine ($8\text{-}NO_2\text{-}G$), which induces G:C to T:A transversions in DNA *in vitro* (356) (Fig. 6). 8-Nitroguanosine ($8\text{-}NO_2\text{-}Guo$) is formed by the reaction of $ONOO^-$ with guanosine (355) (Fig. 6). The formation of $8\text{-}NO_2\text{-}Guo$ from guanosine in RNA from *in vivo* virus-infected lungs from mice suggests that $8\text{-}NO_2\text{-}Guo$ could be involved in the toxicity of $NO^{\cdot}$ during infection and inflammation, at least in murine models (5). The biologic significance of $8\text{-}NO_2\text{-}G$ and $8\text{-}NO_2\text{-}Guo$ is difficult to determine because of their relative instability and the difficulty of quantitative estimation (233). Thus, both 8-nitropurines can be easily oxidized *in vitro* with $ONOO^-$ (171). However, several studies have shown that $8\text{-}NO_2\text{-}Guo$ is a highly redox-active nucleic acid derivative that strongly stimulates $O_2^{\cdot -}$ generation in the presence of cytochrome P450 reductase and all isoforms of NOS (5, 280). In addition, nitrated nucleic acids may be formed in the cytoplasmic nucleotide compartment or in RNA by RNS generated in cell compartments other than the nucleus. Under these conditions, $8\text{-}NO_2\text{-}G$ and related nucleosides and nucleotides may be incorporated into RNA to interfere with RNA function and metabolism (234), and thus, interfere with enzymes that use guanine nucleotides as substrates.

At the end of the cascade mediated by $ONOO^-$, the increase in DNA strand breakage triggers the activation of poly-ADP-ribose polymerase (PARP), a DNA-repair enzyme. However, this mechanism, taking place to remove the lesions induced by $ONOO^-$, becomes deleterious (162) and results in energy depletion (NAD^+ and ATP depletions) and consequently in necrotic cell death (337) (Fig. 6).

C. Proteins

A large number of proteins are cellular targets of RNS. Consequent to RNS exposure, proteins are modified, lead-

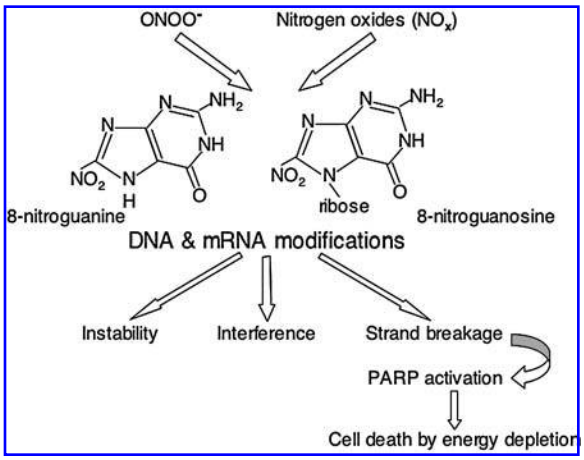

FIG. 6. Damaging effect of RNS on DNA and mRNA. Direct action of RNS [mainly $ONOO^-$ and nitrogen oxides (NO_x)] on DNA and mRNA, *via* the formation of 8-nitroguanine and 8-nitroguanosine, induces changes in cellular behavior that can lead to carcinogenesis, and poly-ADP-ribose polymerase (PARP) activation, inducing cell death by energy depletion.

ing to an activation or inhibition of their activities, as well as altering the protein–protein interactions. In this current review, we concentrate mainly on the molecular targets of RNS, depending on their function.

1. Ion channels. Ion channels are transmembrane proteins that are implicated in a wide variety of cellular functions by selectively controlling the passage of ions across the plasma membrane, as well as the mitochondrial outer and inner membranes, endoplasmic reticulum, and other organelles. They mainly control activity of excitable cells (neurons and muscle cells). However, ion channels may also be expressed in the so-called "nonexcitable" cell types, including lymphocytes, glia, fibroblasts, and endothelial cells (74). Depending on the structure of ion channels, they appear as susceptible targets of RNS, and the subsequent aberrant ion fluxes have been associated with cell death. We focus this part of the review on Ca^{2+} channels mainly expressed in neurons but also in other excitable and nonexcitable cells, and Na^+ and K^+ channels.

a. Ryanodine receptors. The ryanodine receptor (RyR) functions as the principal ion channel that controls the release of Ca^{2+} from the endoplasmic and sarcoplasmic reticulum and, thereby, muscle contractions. RyR contains cysteine residues, which are susceptible to modification by oxidation, *S*-nitrosylation, *S*-glutathionylation, and alkylation. Modifications induced by GSNO or $NO^{\cdot}$ donors significantly enhance RyR activity in the skeletal muscle (309). Eu and co-workers (82) showed that Ca^{2+} flux through RyR of skeletal muscle is activated by *S*-nitrosylation. Moreover, the canine cardiac form of RyR can be *S*-nitrosylated under basal conditions, resulting in an activation of RyR, which could be associated, in isolated rabbit cardiac sarcoplasmic reticulum vesicles, with the inhibition of the Ca^{2+}-ATPase–mediated uptake of Ca^{2+} into the sarcoplasmic reticulum (347, 348). Taken together, RNS through the activation of RyR are potential inotropic agents to increase Ca^{2+} transient and therefore the strength of the contraction (Fig. 7). By contrast, other authors have described the inhibition of both cardiac and skeletal RyRs by RNS (202), depending on their concentrations (114), suggesting that, *in vivo*, initial release of $NO^{\cdot}$ activates RyRs, but greater release inhibits RyR activity and contraction.

b. Inositol 1,4,5-triphosphate (InsP3) receptors. These receptors are a family of Ca^{2+}-release channels localized predominantly in the endoplasmic reticulum and in the nuclear envelope of all cell types. They are able to release Ca^{2+} into the cytoplasm in response to InsP3 produced from the plasma membrane after engagement of extracellular ligands. The InsP3-receptor activation generates complex local and global Ca^{2+} signals that regulate numerous cell physiologic processes (for a review, see ref. 91). To fulfill this function, the InsP3 receptors depend on interaction with accessory subunits and regulatory proteins. Little is known about the interactions of InsP3 receptors and RNS. Chung *et al*. (54) reported that $NO^{\cdot}$ donors inhibit agonist-evoked inward cation current oscillations (see later), and these effects are mediated, in part, by functional modulation of the InsP3 receptors. Other authors demonstrated that $NO^{\cdot}$ donors and $ONOO^-$ induce Ca^{2+}-mobilization from InsP3-dependent internal stores through the activation of cGMP pathways in bovine chromaffin cells (333) (Fig. 7). Under hypoxic conditions, the InsP3-receptor characteristics [number of receptor sites (B_{max}) and dissociation constant (K_d)] were modified by $NO^{\cdot}$ in neuronal nuclei of piglets (209). These $NO^{\cdot}$-mediated modifications of the InsP3 receptors may lead to the increase of intranuclear Ca^{2+}, resulting in altered transcription of apoptotic genes and activation of cascades of hypoxia-induced programmed neuronal death. However, further studies are needed to understand the effects of RNS on InsP3 receptors.

c. Sarco/endoplasmic Ca^{2+} pumps (SERCA). SERCAs function to maintain the pools of Ca^{2+} in either the sarcoplasmic or endoplasmic reticulum by using the energy of hydrolysis of ATP as opposed to the nonspecific leakage of Ca^{2+} from a high concentration in these organelles (277). The SERCA family includes the products of three genes, called *SERCA1, SERCA2*, and *SERCA3*. They are especially expressed in skeletal, cardiac and smooth muscles (mainly *SERCA1* and *SERCA2*), regulating the contraction/relaxation coupling, but also in nonmuscle tissues (*SERCA3*). These proteins present several tyrosine residues susceptible to nitration and also, cysteines able to be *S*-glutathionylated. Indeed, dual effects of $ONOO^-$ on SERCA have been described, depending on the residue target. $ONOO^-$ treatment of pig smooth muscle or endothelial cells inhibited the SERCA pump more severely in the former (281). This effect may be mediated by nitration of SERCA, as described in other models associated with different pathologies (2, 315, 350) (Fig. 7). Whereas the effects of SERCA nitration may have deleterious consequences, *S*-glutathionylation may be beneficial. It has been demonstrated that, under physiologic

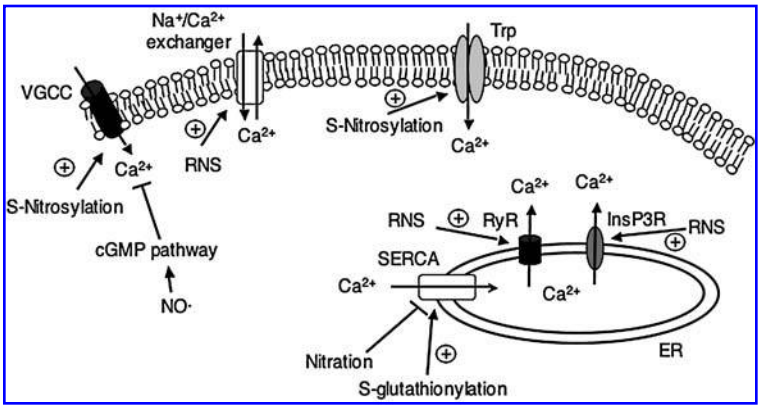

FIG. 7. Selected effects of RNS on calcium channels and pumps regulating calcium currents in living cells.

conditions, $NO^{\cdot}$ forms *S*-glutathione adducts with SERCA, increasing its activity, accelerating Ca^{2+} uptake by the endoplasmic reticulum, and decreasing intracellular Ca^{2+} (3). In more detail, physiologic levels of $NO^{\cdot}$ react with $O_2^{\cdot -}$ to form $ONOO^-$, which, together with GSH, reacts with cysteine residues and increases SERCA activity (Fig. 7). *S*-Glutathionylation may be implicated in the arterial relaxation independent of cGMP, and its alteration could be related to failure processes. In pathologic states, such as atherosclerosis, an increase in ROS/RNS irreversibly oxidizes thiols of SERCA and decreases their activity (3).

Recently, it was been suggested that capsaicin-sensitive sensory neurons in isolated rat hearts could regulate myocardial function through the maintaining of *S*-nitrosylation of SERCA induced by $ONOO^-$ (24).

d. Store-operated Ca^{2+} channels. Once InsP3 receptors are activated and Ca^{2+} is released from the endoplasmic reticulum, the resulting depletion of Ca^{2+} within this organelle serves as a message, which is returned to the plasma membrane, resulting in the slow activation of "store-operated" Ca^{2+} channels. This Ca^{2+} entry phase sustains cytosolic Ca^{2+} increase and, at the same time, replenishes intracellular stores (173).

It has been reported that Ca^{2+} entry can be directly activated by *S*-nitrosylation (86), but the relation between this process and the "store-operated" Ca^{2+} entry is complex. Whereas activation of "store-operated" Ca^{2+} channels appears to require InsP3 receptor as a coupling intermediary, $NO^{\cdot}$ donors act directly on the channel (329) (Fig. 7). The nature of these channels remains to be elucidated, although a transient receptor potential channel (TRP) has been proposed. Very recently, evidence that the *S*-nitrosylation of TRPC5 elicits Ca^{2+} entry into endothelial cells has been proposed, suggesting that members of the TRP family can be considered $NO^{\cdot}$ sensors (357).

e. Na^+/Ca^{2+} exchangers. These proteins are members of a transporter family whose main role is to provide control of Ca^{2+} flux across the plasma membranes or intracellular compartments by extruding Ca^{2+} from the cytoplasm. Curiously, Na^+/Ca^{2+} exchanger is reversible and, under certain conditions, may allow Ca^{2+} to enter the cytoplasm (for a review, see ref. 190). It was recently reported that $NO^{\cdot}$ activates Na^+/Ca^{2+} exchanger activity and that Na^+/Ca^{2+} exchanger is involved in $NO^{\cdot}$-induced depletion of Ca^{2+} in the endoplasmic reticulum from cultured microglia, leading to endoplasmic reticulum stress (198) (Fig. 7). Maczewski and Beresewicz (192) also showed that the inhibition of Na^+/Ca^{2+} exchanger attenuates postischemic myocardial formation of $NO^{\cdot}$ and ROS in rat heart, suggesting that prevention of calcium overload is cardioprotective *via* the $NO^{\cdot}$ pathway.

f. Voltage-dependent Ca^{2+} channels. Voltage-dependent Ca^{2+} channels (VGCCs) mediate Ca^{2+} entry into excitable cells in response to membrane depolarization. They are heterodimeric multiprotein complexes consisting of a pore-forming $\alpha 1$ subunit and other modulatory subunits (β, γ, and $\alpha 2\delta$) (352).

Although RNS exert pronounced effects on VGCC activity, no clear conclusion can be reached, inasmuch as contradictory data have been reported. Indeed, depending on the subunit of VGCC affected by RNS, the observed effects can be diverse (Fig. 7). Regarding the stimulation of Ca^{2+} currents through these channels, Wang *et al*. (340) showed that $NO^{\cdot}$ released by agonists stimulates L-type Ca^{2+} current in cat atrial myocytes. The same group showed that $NO^{\cdot}$ can act *via* an *S*-nitrosylation mechanism to prevent the β_2-adrenoceptor agonist-induced inhibition of L-type Ca^{2+} current in the same model (65). The latter results indicate that $NO^{\cdot}$ may increase the L-type Ca^{2+} current in this system and exert cardioprotective effects. In neurons, $NO^{\cdot}$ donors enhance Ca^{2+} current amplitude and block adrenoceptor-induced Ca^{2+} current inhibition *via* stimulation of cGMP formation, suggesting that $NO^{\cdot}$ donors can interfere with neuromodulation of Ca^{2+} channels (48). In smooth muscle cells, NO donors inhibit L-type Ca^{2+} channels through a cGMP signaling pathway, thereby suppressing the sustained increase in intracellular Ca^{2+} concentration, suggesting that these channels are important targets for nitrovasodilators (208).

S-Nitrosothiols, in particular GSNO, exert a potent inhibitory effect on L-type Ca^{2+} channels by affecting central functions (gating and ion permeation) of the pore-forming α subunit of these channels (253) by a mechanism independent of cGMP (128). $NO^{\cdot}$ donors also inhibit L-type Ca^{2+} in rabbit carotid body sensory activity, through a direct action on the channel or an associated channel protein or both, rather than through a cGMP-dependent mechanism (307). This may explain the fact that $NO^{\cdot}$ can modulate neurotransmitter release in these cells to maintain homeostasis during hypoxemia. Abi-Gerges and co-workers (1) proposed the hypothesis that the inhibitory effects of $NO^{\cdot}$ donors on L-type Ca^{2+} channels in rat cardiomyocytes is dependent on the redox complex environment in cells, taking place directly on the channel itself or on other auxiliary proteins or lipids, or both.

g. K^+ channels. K^+ channels are a diverse and ubiquitous family of membrane proteins present in both excitable and nonexcitable cells, playing key roles in cellular signaling processes such as neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and volume regulation (for review, see ref. 293). These channels selectively conduct K^+ ions across the cell membrane along its electrochemical gradient. A very large number of K^+-channel genes have already been identified and, consequently, deduced amino acid sequences and secondary structure information can be used to generate structural classification. Three groups of K^+ channels have been characterized: (a) K^+ channels with six or seven transmembrane segments and one pore region (voltage-activated and Ca^{2+}-activated K^+ channels); (b) large-conductance voltage-gated K^+ channels with seven transmembrane segments; and (c) finally, the inward rectifiers K^+ channels with two transmembrane segments and one pore, including ATP-sensitive and ATP-regulated K^+ channels. It is obvious that many different K^+ channels with diverse kinetics and functions exist, increasing the possibility of interaction with RNS. Although the most important posttranslational modifications of K^+ channels are palmitoylation, glycosylation, and phosphorylation (27), RNS can affect these channels by activating or inhibiting their activities.

During the 1990s, several studies performed in vascular smooth muscle cells showed that $NO^{\cdot}$ induces nitrosylation of sulfhydryl groups of large conductance Ca^{2+}-activated K^+ channels (4, 28). With inside-out patch recording, Lu and coworkers (189) showed that $NO^{\cdot}$ increases whole colonic smooth muscle cell outward K^+ current by activating Ca^{2+}-activated K^+ channels through a cGMP pathway (210); this phenomenon could play an important functional role in the control of the vascular peripheral resistance induced by $NO^{\cdot}$. Recently, a similar effect was described in human dermal fibroblasts (179). $NO^{\cdot}$ may interact with the α-subunit of the channels and activate them directly (140) without any involvement of covalent modification, such as *S*-nitrosylation proposed by other authors.

Regarding the effects of RNS on ATP-dependent K^+ channels in pancreatic β cells, $NO^{\cdot}$ donors inhibit glucose-induced insulin secretion by acting directly on the activity of these channels. These effects result in the opening of the channels (321). $NO^{\cdot}$ itself and $NO^{\cdot}$ donors affect the activity of ATP-dependent K^+ channels by different mechanisms (78). On the one hand, ATP-dependent K^+ current is stimulated *via* a reduction in the ATP/ADP ratio, and on the other hand, it is inhibited by direct interference with the channel or a regulatory protein in its close vicinity (78).

Finally, it has been suggested that $NO^{\cdot}$ donors may exert an inhibitory effect on outward K^+ currents *via* an interaction with ROS in a cGMP-independent way (314). These results may have considerable relevance for the understanding of the early events leading to ischemia-related toxicity in excitable tissues associated with alterations in the balance between ROS and RNS.

h. Voltage-gated Na^+ channels. Voltage-dependent Na^+ currents are responsible for the rapid upstroke of the action potential in neurons and striated muscle and determine the membrane excitability. Taking in consideration their importance in action-potential generation, modulation of Na^+ currents causes significant changes in membrane excitability (26). Acting on these channels by *S*-nitrosylation, $NO^{\cdot}$ donors decrease Na^+ currents in baroreceptor neurons (174), which play a crucial role in acute regulation of the arterial pressure. $NO^{\cdot}$ donors also reduced the three types of Na^+ currents described in C-type dorsal root ganglion neurons (268). In contrast, other authors showed that $NO^{\cdot}$ donors increase the amplitude of the Na^+ currents in rat hippocampal neurons involving *S*-nitrosylation (111). Altogether, these data suggest that $NO^{\cdot}$ donors may modulate Na^+ currents differently, depending on cell type.

i. NMDA. *N*-methyl-D-aspartate receptors (NMDA) are glutamate-gated ion channels that are essential for the regulation of synaptic function in the central nervous system. They are highly permeable to Ca^{2+}, and this Ca^{2+} influx is necessary for synaptogenesis, experience-dependent synaptic remodeling, and long-lasting changes in synaptic efficacy (for a review, see ref. 170). An excessive activation of NMDA receptors is implicated in neuronal damage, resulting in exaggerated Ca^{2+} influx through the channel; this process has been called "excitotoxic activation." Lipton's group (180) was the first to show that *S*-nitrosylation of NMDA receptors may play a neuroprotective role. Among the five cysteine residues of the NMDA receptors, Cys399 of the NR2A subunit mediates the predominant inhibitory effect of *S*-nitrosylation, decreasing NMDA-receptor function (52). Recent data indicate that *S*-nitrosylation of other proteins involved in cell death (see later) can act in synergy with NMDA *S*-nitrosylation and promote neuronal survival (222).

j. Other ion channels. Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride channel present in the apical membrane of epithelia in many organs. The maturation defect of CFTR presenting the δF508 mutation is responsible for cystic fibrosis disease. Recent data open promising new therapies, because GSNO can normalize expression, maturation, and activity of CFTR (49, 291). In contrast, other studies showed that, although GSNO effectively affects CFTR activity, this effect is not mediated by *S*-nitrosylation but rather by induction of the expression of cysteine string proteins (358). Moreover, higher doses of $NO^{\cdot}$ can augment CFTR breakdown by causing tyrosine nitration (23, 142) or alteration of CFTR trafficking (299).

2. Cytoskeletal and structural proteins. One of the major structural components of the cells is the cytoskeleton, a filamentous network of F-actin, microtubules, and intermediate filaments. In addition, the filamentous cytoskeletal network provides a scaffolding on which other proteins such as kinesin, dynein, and myosin can translocate to move organelles or generate internal stress (138). It is formed by a large number of proteins that play a crucial role in dynamic cellular processes, including the maintenance of the structural and mechanical integrity of cells and tissues and, more specifically, cell migration and adhesion, cytokinesis, membrane trafficking, and morphogenesis. Because a large number of cytoskeletal proteins are affected by RNS, we focus in this review on proteins with well-established functions.

a. Actin. Actin, one of the most abundant proteins in eukaryotic cells, constitutes 5% or more of cell protein and, with other cytoskeletal proteins, is a critical target for nitration-induced functional impairment because of its high percentage of tyrosine residues. The functional consequences of tyrosine nitration on actin dynamics observed in several pathologies, such as human inflammatory bowel disease or sickle cell disease, can be the loss of control of filament formation, with subsequent alterations in cell motility, attachment, and intracellular transport (10, 58, 148). $ONOO^-$ is able to induce tyrosine nitration of β-actin and dysfunction of the membrane barriers *via* the disruption of the cycling of the actin monomers and actin polymerization; however, direct evidence of nitration of β-actin and dysfunction of the membrane barriers has not been demonstrated (228).

b. α-Tubulin. α-Tubulin has also been identified as a target of nitration associated with the more stable fraction of the cytoskeleton in neuron-like differentiating PC12 cells (41). Concerning this protein, it has been proposed that nitration could play a novel physiologic role in the stabilization of neurites occurring in differentiated neuronal cells (41). Moreover, disruption of microtubule assembly and architecture in intestinal epithelium as a consequence of the nitration of α-tubulin has been described *in vitro*, suggesting novel effective strategies to suppress structural damage in the intestinal epithelium (18).

c. Calpains. In skeletal muscle, calpains are thought to initiate cytoskeletal degradation by cleaving important proteins involved in linking components of the cytoskeleton together and to the cell membrane. Among these elements, talin is colocalized with calpain at the cell membrane. When talin is cleaved by calpain, a breakdown of the muscle cytoskeleton takes place (129). The inhibition of calpain activity through *S*-nitrosylation impairs talin proteolysis (159), suggesting that $NO^{\cdot}$ protects against cytoskeletal degradation.

d. N-Ethylmaleimide sensitive factor (NSF). Endogenous $NO^{\cdot}$ and $NO^{\cdot}$ donors are able to regulate exocytosis by *S*-nitrosylating NSF, a cytosolic protein necessary for intracellular vesicular transport, thereby inhibiting NSF disassembly activity (199). This mechanism, which could regulate a variety of physiologic processes mediated by granule exocytosis, has been proposed as a plausible explanation for the antiinflammatory effects of $NO^{\cdot}$.

3. Matrix metalloproteinases (MMPs). This family of extracellular soluble or membrane-bound proteases is involved in remodeling the extracellular matrix. Through *S*-nitrosylation, $NO^{\cdot}$ donors induce MMP-9 activation, which can be relevant under certain pathophysiologic conditions, such as cerebral ischemia (108). Thus, extracellular proteolysis cascades triggered by MMPs can disrupt the extracellular matrix, contribute to cell detachment, and provoke apoptosis (108). MMP-2 produced by cultured vascular smooth muscle cells also is nitrated and activated by $ONOO^{-}$; this mechanism may be important for modulation of MMP activity in cardiovascular pathologies in which extracellular matrix plays a key role, as described later (262).

4. Proteins involved in cell cycle (proliferation). Interactions of RNS with signaling molecules involved in essential pathways related to the cell cycle may affect important biologic processes such as proliferation and differentiation. RNS affect proliferation by direct or indirect interactions with different targets, such as cyclins (the elements involved directly in the cell cycle), the kinases regulating cyclins, and the growth factors or their receptors or both.

Few data concerning the specific effects of RNS on growth factors are available. Among the growth factors playing a critical role in cell growth and development, neuregulin-1 (NRG-1), a growth factor strongly expressed in lung, can be nitrated at three tyrosine residues. This posttranslational modification is critical for the binding of NGR-1 to its receptor, resulting in a loss of ligand-induced proliferation, at least in human breast adenocarcinoma *in vitro* (227). An alteration of the epidermal growth factor (EGF) by nitration has also been observed in the intestinal epithelium, in particular in Caco-2 cells. $ONOO^{-}$ inhibits EGF-induced cell proliferation by abolishing EGF-stimulated receptor autophosphorylation and by inducing the nitration of the EGF receptor on tyrosine residues (322). Nerve growth factor (NGF), a growth factor necessary for the differentiation and survival of specific neuronal populations during development, is tyrosine nitrated by $ONOO^{-}$, which transforms NGF into an apoptotic agent for rat motoneuron cultures (244). This gain of apoptotic function of NGF is the result of the formation of high-molecular-weight oligomers, because nitrated tyrosine facilitates covalent cross-links between NGF molecules (244). Nitrated NGF could be formed during pathologic and inflammatory conditions under which NGF upregulation coincides with increased production of $ONOO^{-}$ or other RNS, as observed in mice models of amyotrophic lateral sclerosis (ALS) (188). Increased *S*-nitrosylation of cysteines of insulin-like growth factor-1 (IGF) after *in vitro* exposure to RNS suggests that the formation of *S*-nitrosothiols may result in functional modifications of IGF, possibly leading to the chondrocyte insensitivity to IGF-1 observed in articular diseases (304). Finally, fibroblast growth factor (FGF-1), an inductor of angiogenesis, is nitrated *in vitro* on tyrosine residues critical for its activity, by low levels of $ONOO^{-}$, leading to a nonreversible inactivation of FGF-1. Because FGF-1 regulates angiogenesis, these modifications may alter the ability of tissue repair of this factor (14).

Another target of RNS is the Src family of nonreceptor tyrosine kinases, which also regulate cell proliferation and are activated mainly by phosphorylation at key tyrosine residues. However, $ONOO^{-}$-induced nitration of tyrosine residues of this family of kinases has been also described (160, 254). Nitration of Src prevents phosphorylation of these residues, leading to the decrease of Src activity in intestinal epithelial cell lines (25, 41) and *in vitro* studies (160). These results reinforce the notion that nitration may modify cascades that control signal-transduction processes and regulate cell cycles.

RNS can indirectly modify the signaling pathway of mitogen-activated protein kinases (MAP kinases). In particular, $p21^{ras}$, an upstream element in the MAP kinase cascade, is nitrosylated by RNS. By using human transfected T Jurkat cells, Lander's group (167) showed that RNS stimulate $p21^{ras}$ and MAP kinase activities, suggesting that *S*-nitrosylation of $p21^{ras}$ may be responsible for triggering downstream signal transduction. These effects are associated with the increase in Raf-1 kinase activity, a key effector of $p21^{ras}$ function (70).

In parallel, RNS act directly on MAP kinase cascades. MAP kinase pathways activate ERK, JNK, and p38 kinases playing an important role in the regulation of a variety of cell functions (164). For example, angiotensin II nitrates tyrosine residues of ERK1/2 by inducing $ONOO^{-}$ production and facilitates their activation in vascular smooth muscle cells (252). ERK proteins are also phosphorylated by $ONOO^{-}$ in rat cardiomyocytes, but, in this case, $ONOO^{-}$ acts through oxidative but not nitrosative reactions (249). ERK-1 also can be endogenously *S*-nitrosylated in endothelial cells and, under hyperglycemic conditions *in vitro*, a reduction of endogenous *S*-nitrosylation has been described, suggesting that alteration in protein *S*-nitrosylation may underlie the adverse effect of hyperglycemia on the cardiovascular system (338). Like ERK, c-Jun N-terminal kinases (JNKs) are also affected by *S*-nitrosylation. $NO^{\cdot}$ donors inhibit the interaction between JNK1 and its molecular target, the transcription factor c-Jun through *S*-nitrosylation (242). Some contradictions concerning the direct effects of $ONOO^{-}$ on JNK cascade have been reported. Whereas in rat hepatocytes, $ONOO^{-}$ has no effect on JNK activity activated by Fas ligand (267), in murine alveolar epithelial cells, $ONOO^{-}$-mediated JNK activation depends on Fas (295).

Finally, p38 MAPK can be nitrated in endothelial cells from preeclamptic pregnancies, and this was associated with reduced catalytic activity of p38 MAPK (344).

Previous studies have shown that the p85 regulatory subunit of PI3-kinase is a target for $ONOO^-$-induced protein nitration on tyrosine (117). More recently, it was suggested that the increased tyrosine nitration on the p85 subunit of PI3-kinase blocks both PI3-kinase and Akt kinase activities, and this effect is accompanied by a disruption of vascular endothelial growth factor (VEGF)-induced prosurvival function in endothelial cells (80). By contrast, $ONOO^-$ has been shown to promote the nitration of regulatory sites at tyrosine kinase receptors coupled to the PI3-kinase/Akt pathway, to induce its activation and thereby to prevent cell death in fibroblasts and neurons (68, 154). Some RNS can be relevant second messengers for the activation of the survival kinase Akt through a mechanism involving the tumor-suppressor phosphatase and tensin homologue (PTEN) by *S*-nitrosylation in bovine pulmonary endothelial cells (43). PTEN, as a phosphoinositide 3-phosphatase that antagonizes PI3-kinase action, negatively regulates cell proliferation and survival signals (193).

Not only is tyrosine nitration responsible for modifications induced by RNS on proteins involved in cell proliferation, but also, *S*-nitrosylation of proteins regulating cell survival has been described. Several studies have demonstrated that $NO^\cdot$ donors can inhibit tyrosine phosphatase activity by *S*-nitrosylation, leading to their reversible inactivation (21, 177). Because tyrosine phosphatases are important in controlling the VEGF-induced endothelial-proliferation response necessary for tumor progression (36), RNS may provide possible new strategies for the development of anticancer therapies.

Erwin *et al*. (81) were the first to describe receptor-modulated reversible *S*-nitrosylation of eNOS in bovine aortic endothelial cells. eNOS is constitutively *S*-nitrosylated in resting endothelial cells; after addition of the eNOS agonist VEGF, eNOS is denitrosylated and then progressively renitrosylated. The renitrosylation of eNOS after VEGF treatment is markedly attenuated by the addition of NOS antagonists, indicating that eNOS *S*-nitrosylation depends acutely on eNOS activity. This elegant study suggests that changes in cellular redox state might influence the denitrosylation of eNOS and provides a partial explanation for the attenuation of eNOS activity by oxidative stress.

5. Proteins involved in cell death. Programmed cell death, or apoptosis, is a regulated, energy-dependent process of cell shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation. Caspases represent the central effectors of cell death. They are a family of cysteine proteases that propagate death signals by cleaving a number of cellular protein substrates. $NO^\cdot$ can prevent or induce apoptosis, depending on its concentration, cell type, or the oxidative environment. $NO^\cdot$ inhibits apoptosis by *S*-nitrosylation of the active site of caspases (see the excellent review, ref. 147). The ability of $NO^\cdot$ to *S*-nitrosylate caspases depends, as for a large number of other proteins, on multiple factors including the presence of free iron and intracellular redox potential. Bcl-2, an antiapoptotic protein that regulates the mitochondrial death pathway, can be *S*-nitrosylated by endogenous $NO^\cdot$ in a model of human lung epithelial cancer cells (13). By using transfection approaches, these authors show that *S*-nitrosylation of Bcl-2 prevents its ubiquitin-proteasomal degradation, suggesting a novel antiapoptotic mechanism that may represent a potential way to control cancer progression.

In contrast, in other models, $NO^\cdot$ promotes apoptotic pathways in numerous cell types through indirect activation of caspases. $NO^\cdot$ donors induce cytotoxic effects in bovine chromaffin cells by upregulation of p53 tumor suppressor, by increasing the ratio of proapoptotic to antiapoptotic mediators (Bax and Bcl-Xs *vs.* Bcl-2 and Bcl-XL), cytochrome *c* release, and caspase activation (247). The *S*-nitrosylation of cytochrome *c* during Fas-induced apoptosis of human mononuclear cell lines enhances caspase activation, resulting in the induction of apoptosis (285). Cytochrome *c* also can be nitrated (220), and in this case, inhibition of apoptosis has been observed. In rat glioma cells, $ONOO^-$ is able to nitrate several tyrosine residues of cytochrome *c* and thereby decreases its effects on caspase cascade activation (220).

Apoptosis can also be influenced by tyrosine nitration. In rat and human hepatocytes, it has been shown that $ONOO^-$ induces CD95-tyrosine nitration, which is associated with the prevention of CD95-tyrosine phosphorylation and apoptosis (267). *In vivo* CD95-tyrosine nitration has been described in livers from endotoxin-treated rats (267). Altogether, these results suggest that tyrosine nitration and phosphorylation of CD95 are mutually exclusive and that both mechanisms can be observed *in vivo*.

In parallel, the status of intracellular redox elements can affect the ability of the cell to become apoptotic. Several mechanisms have been proposed to explain the effects of *S*-nitrosylation on the antiapoptotic functions of thioredoxin, a redox regulator implicated in the control of cell growth and apoptosis inhibition, which is *S*-nitrosylated under basal conditions (110). First, overexpression of *S*-nitrosylable thioredoxin reduces ROS formation in endothelial cells (110); and second, in *in vitro* T cells, thioredoxin may act by transnitrosylation of proteins such as caspases, through the formation of a protein–protein complex, and inhibit their activity (211). Thus, the basal *S*-nitrosylation of thioredoxin is a requirement for preserving its redox regulatory activity and for scavenging ROS in endothelial cells. Moreover, $NO^\cdot$ donor exposure results in thioredoxin nitration and abolishes thioredoxin activity *in vitro* (316). Thus, inactivation *via* nitration of thioredoxin might play a proapoptotic role in pathologic states in which production of RNS is increased.

6. Proteins implicated in cell metabolism. Mitochondria are the central organelles producing cellular energy. $NO^\cdot$ donors and $ONOO^-$, in *in vitro* tests, inhibited NADH:cytochrome *c* reductase activity by nitration, whereas succinate:cytochrome *c* reductase activity remained unaffected (275); thus, one can advance the hypothesis that RNS may limit the energy supply in tissues. Other enzymes implicated in the metabolism, such as succinyl-CoA:3-ketoacid coenzyme A transferase (the mitochondrial enzyme involved in the breakdown of ketone bodies in the extrahepatic tissues) in rat heart mitochondria (264), the purified pyruvate dehydrogenase complex (the mitochondrial matrix enzyme catalyzing the oxidative decarboxylation of pyruvate) (274), and cytosolic aconitase from murine macrophages (103) can be nitrated or *S*-nitrosylated by RNS. Creatine kinases are critical energy controllers that regulate cardiac myocyte contractility in both physiologic and pathologic states. An impairment of this system during cardiac failure may be the consequence of

the nitration and inactivation of creatine kinases in isolated cardiac trabeculae from mice (205). Furthermore, *S*-glutathionylation of creatine kinases has been proposed as a parallel mechanism explaining the temporary reversible loss in activity of this system during ischemic injury (265).

a. Enzymes playing a key role in insulin-associated cascades. Glucokinase activity is a major determinant of β-cell glucose metabolism and insulin secretion. An increase of glucokinase activity by insulin through the *S*-nitrosylation of this enzyme has been described in cultured β cells, as well as its association with nNOS, suggesting that this mechanism represents a sensitive means for modulating glucokinase activity and insulin secretion (276).

Much evidence indicates that *S*-nitrosylation as a key mechanism in the regulation of insulin secretion and resistance. In brief, when insulin binds to its receptor, dimerization and autophosphorylation of insulin receptor tyrosine kinase take place. Then, tyrosine phosphorylation of insulin-receptor substrates leads to activation of the Akt/PKB pathway. This pathway plays a central role in glucose transport, protein and glycogen synthesis, and inhibition of hepatic gluconeogenesis, and all of these effects regulate insulin actions (for a review, see ref. 145). In addition to enzymes implicated in glucose metabolism (see earlier), the Akt/PKB pathway is reversibly inactivated by *S*-nitrosylation *in vitro* and in intact cells (354). In this study, it has been shown that $NO^{\cdot}$ donors inhibit Akt/PKB at the cysteine 224, and this process is increased in skeletal muscle of diabetic mice, suggesting that *S*-nitrosylation–mediated inactivation of Akt/PKB could contribute to the pathogenesis of $NO^{\cdot}$-mediated insulin resistance, at least in mice. Moreover, $NO^{\cdot}$ donors may directly inhibit insulin secretion partly by *S*-nitrosylation of key proteins in the stimulus–secretion coupling (298). Furthermore, insulin may be a potential target of $ONOO^-$. The $ONOO^-$-mediated nitration of insulin partially affects its receptor binding and hypoglycemic capacities in a mouse model (51). At least four nitrated tyrosine residues in insulin receptor substrate-1 have been detected (230). Interestingly, among these residues, Tyr939, which is critical for association of insulin-receptor substrate-1 with the p85 subunit of PI-3 kinase, is nitrated (230). Very recently, a novel molecular mechanism resulting in insulin resistance was proposed. Thus, Clavreul *et al*. (57) showed that *S*-glutathionylation of $p21^{ras}$ by $ONOO^-$ is involved in the alterations in the insulin-signaling pathway induced by LDL, at least in bovine aortic endothelial cells. Altogether, these findings indicate that RNS may modulate insulin resistance and type 2 diabetes at different levels, leading to a sustained activation of inflammatory pathways.

7. Nuclear factors. NF-κB family members are heterodimers consisting of p65/RelA and p50/NF-κB1, but only the p65 subunit has transactivation domains capable of initiating transcription (69). NF-κB is complexed in the cell cytoplasm with the inhibitory proteins, IκBα and IκBβ. On cell stimulation, IκB is phosphorylated, removed, and degraded, allowing NF-κB to induce transcription. *S*-Nitrosylation of p65 by GSNO, which consequently inhibits NF-κB activation, has been shown in endothelial cells (Fig. 8). In addition, *S*-nitrosylation of p65 is associated with an inhibition of monocyte adhesion to the activated endothelial cells *via* the downregulation of endothelial cell adhesion molecules (256). These authors suggest that GSNO could have a protective effect in several pathophysiologic conditions by inhibition of cellular infiltration of monocytes (256). In contrast, the inhibition of NF-κB by $NO^{\cdot}$ donors could represent one mechanism by which *S*-nitrosylation can initiate apoptosis in A549 cells (191) (Fig. 8). By using mutant versions of inhibitory κB kinase (IKKβ), the prerequisite enzyme complex necessary to induce NF-κB, Reynaert *et al*. (269) demonstrated that cysteine-179 is the main target of IKKβ by *S*-nitrosothiols (*S*-nitroso-*N*-acetyl-$_{D,L}$-penicillamine (SNAP) and GSNO), leading to inhibition of its enzymatic activity. Importantly, when NOS was inhibited, IKKβ was activated and denitrosylated, illustrating the importance of endogenous $NO^{\cdot}$ in regulating the extent of NF-κB activation.

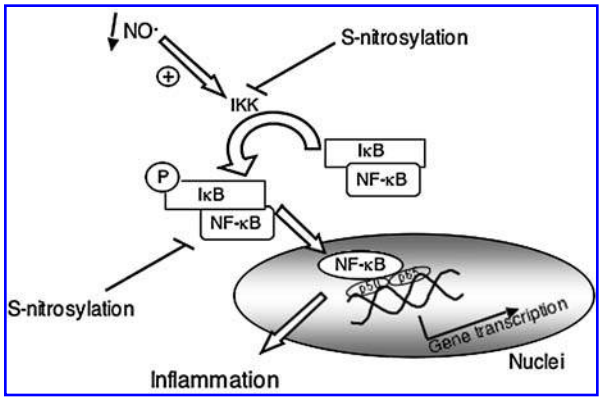

FIG. 8. Alterations of the NF-κB pathway by RNS. The decrease in $NO^{\cdot}$ production, after NOS inhibition, is able to activate IKK, which is necessary to phosphorylate IκB and the subsequent activation of the NF-κB pathway. *S*-Nitrosothiols are implicated both in the direct and indirect inhibition of NF-κB. *Arrows without heads*, inhibition.

8. Proteins transporting oxygen. Under physiologic conditions, reactions of endothelium-derived $NO^{\cdot}$ with hemoglobin are considered to be the most important pathway for limiting $NO^{\cdot}$ bioactivity (102). This reaction involves the iron-containing heme groups of oxy- and deoxyhemoglobin with $NO^{\cdot}$ and results in production of methemoglobin and NO_3^- ions and iron-nitrosyl-hemoglobin, respectively (100). Much evidence has shown that $NO^{\cdot}$ reacts with hemoglobin to form stable metabolites, which may be considered means of transportation and subsequent release of $NO^{\cdot}$. Cannon *et al*. (40) showed that, in humans, the inhalation of NO gas is associated with increases in plasma NO_2^- levels, as well as iron-nitrosyl-hemoglobin, and they suggest that NO_2^- may be reduced to $NO^{\cdot}$ in the circulation and contribute to endocrine $NO^{\cdot}$ delivery. Hemoglobin possesses an allosteric regulated NO_2^- reductase activity that reduces NO_2^- to $NO^{\cdot}$ along with the physiologic oxygen gradient, and this potentially contributes to hypoxic vasodilatation (101). In addition, $NO^{\cdot}$ may be stabilized, transported, and released by intramolecular $NO^{\cdot}$ group transfers between the heme iron and cysteine residues to form *S*-nitroso-hemoglobin, which, under hypoxic conditions, delivers *S*-nitrosothiol and induces relaxation (151). Therefore, hemoglobin derived from red cells may regulate the vascular tone, depending on tissue

oxygen tension. However, this hypothesis has been argued recently (102, 141). Thus, direct measurement by electron paramagnetic resonance of *S*-nitroso-hemoglobin has shown that, although its formation in either venous or arterial blood depends inversely on oxygen concentration, *S*-nitroso-hemoglobin gradients across the circulation are not described (141).

More recently, by using new mouse models that exclusively express either the human wild-type of hemoglobin or human hemoglobin in which the βCys93 has been replaced with alanine, Isbell *et al*. (136) showed that *S*-nitroso-hemoglobin is not required for red cells to stimulate hypoxic vasodilatation. Altogether, these data suggest that *S*-nitroso-hemoglobin might not be critical for the coupling of red cell deoxygenation with increased $NO^{\cdot}$ bioactivity.

It has been reported that myoglobin, an important intracellular oxygen binding hemoprotein in heart and skeletal muscle, can be nitrated and probably *S*-nitrosylated (229, 288). In both cases, myoglobin acts as a scavenger of $NO^{\cdot}$ and may limit the deleterious effects of RNS.

9. Albumin. Albumin is the most abundant transport and depot protein in the mammalian vasculature. Taking into consideration that albumin is present in plasma at elevated concentrations (>0.5 m*M*), it is plausible to imagine that it can transport free $NO^{\cdot}$. Indeed, Rafikova and colleagues (261) showed that albumin accelerates formation of low-molecular-weight *S*-nitrosothiols by transferring NO^{+}. These authors suggest that albumin could represent a major reservoir of $NO^{\cdot}$ and its reactive oxides controlling the dynamic of $NO^{\cdot}$-dependent processes in the vascular system.

In addition, $ONOO^{-}$, which can lead to protein-bound tyrosine nitration, is able to induce mild nitration on albumin and low-density lipoprotein (LDL)-albumin complex. Interestingly, human serum albumin decreases tyrosine nitration in this complex *in vitro*, raising the question of the pathophysiologic significance of these nitrations and their interactions, which may potentially prevent actions of LDL proteins on endothelial cells (320). Finally, a protective effect of albumin treatment has been reported in experimental endotoxic shock by its capacity to reduce the inflammatory process leading to oxidative and nitrosative stresses and vascular hyporeactivity in mice (204).

10. Enzymes involved in prostaglandin generation. Cyclooxygenase and prostacyclin synthase. Cyclooxygenase (COX) catalyzes the first step in the synthesis of prostaglandins, which are critically implicated in a variety of physiologic and pathophysiologic processes, including inflammation, vascular homeostasis, and immunity (61). Nitration of the proinflammatory isoform COX-2 and the increase in COX-2 expression induced by $ONOO^{-}$ after treatment with arsenic have been detected in bovine aortic endothelial cells (34), suggesting that COX-2 may exacerbate the inflammatory state in the cardiovascular system under situations in which $ONOO^{-}$ production is increased (Fig. 9). In contrast, $NO^{\cdot}$ inhibits COX-2–derived prostaglandin E2 production in mouse macrophages, and this effect is associated with both decreased expression and nitration of COX-2, indicating that the regulation of COX-2 by $NO^{\cdot}$ is complex and may depend on the local environment of the cell (56) (Fig. 9). In human platelets, $ONOO^{-}$ is able to induce nitration in tyrosine residues of both COX-1 and COX-2 and the subsequent inhibition of their activities (30). *S*-Nitrosylation of cysteine residues of COX by RNS has been described in murine macrophage cell line (RAW264.7) (325). *S*-Nitrosylation of COX-2 may have a cardioprotective effect, because it has been shown that, in cardiomyocytes, atorvastatin (an agent that improves cardiovascular function) is able to increase the activity of iNOS and activate COX-2 by *S*-nitrosylation (11) (Fig. 9).

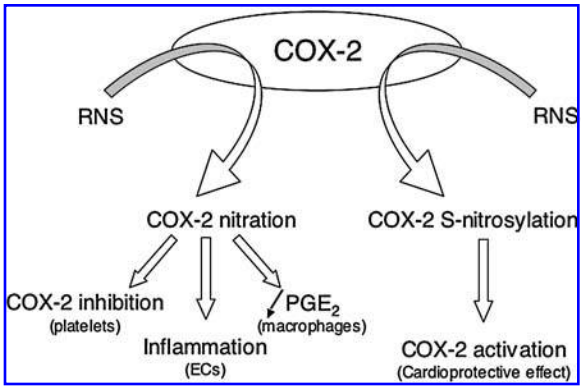

FIG. 9. Effects of RNS on cyclooxygenase (COX)-2. RNS affect COX-2 activity differently, depending on the cell type. Nitration of COX-2 leads to COX-2 inhibition in platelets, to a decrease of prostaglandin E_2 (PGE_2) production in macrophages, and to an increase of inflammatory responses in endothelial cells (ECs). By contrast, *S*-nitrosylation of COX-2 may have a cardioprotective effect by increasing its activity.

It has been shown that prostacyclin synthase is inhibited by $ONOO^{-}$-dependent nitration in human aortic endothelial cells (64), indicating that this mechanism may be involved in several pathologies associated with endothelial dysfunction, such as atherosclerosis or diabetes. Prostacyclin synthase in smooth muscle cells, in contrast to endothelial cells, is resistant to nitration and inhibition by $ONOO^{-}$ (155).

11. Mn superoxide dismutase (MnSOD). SOD catalyzes the dismutation of $O_2^{\cdot -}$ to hydrogen peroxide and molecular oxygen. Among SODs, MnSOD, the first line of defense against $O_2^{\cdot -}$ generated in mitochondria, is the only one to be modified by RNS. Indeed, MnSOD is nitrated and inactivated in neurodegenerative diseases (ALS, Parkinson, and Alzheimer diseases) and atherosclerosis (9, 350). This nitration and the subsequent inactivation of MnSOD could lead to the exacerbation of oxidative stress and could explain the mitochondrial dysfunction described under these pathologic situations. Other authors suggest that the interaction of $NO^{\cdot}$ with MnSOD in *Escherichia coli* may represent a novel mechanism by which MnSOD protects the cell from deleterious effects associated with overproduction of $NO^{\cdot}$ (89).

V. RNS in Physiologic and Pathophysiologic Conditions

A. Physiology

Shear stress is one of the most important stimuli for the synthesis and release of $NO^{\cdot}$ (35). Agonist-dependent recep-

tor activation also is able to release NO·, mainly from eNOS (35) but also from nNOS (290). Whereas in the cardiovascular system, the effects of NO· released from eNOS are reflected by vasorelaxation, inhibition of platelet aggregation, and inhibition of vascular smooth muscle cell proliferation, NO· generated from nNOS, acting as a neurotransmitter in nonadrenergic, noncholinergic nerves, is involved in synaptic transmission and plasticity in the cerebral cortex and hippocampus (127). Furthermore, NO· from nNOS is also implicated in the regulation of basal and β-adrenergic myocardial function (44) and, in association with eNOS, in the regulation of penile erection (130). The main physiologic effect of NO· is the activation of GC and the subsequent cGMP production (Fig. 10). NO· reacts directly with the heme group of GC; thus, we can consider that the receptor for NO· is GC. At least seven different isoforms of GC have been identified. The soluble isoform of GC is a heterodimer with an α subunit and a β subunit. When NO· binds to the ferrous heme iron, the inhibition of the catalytic activity of GC by the heme is removed (195). The levels of intracellular cGMP are regulated by the ratio between its formation and the rate of its degradation and inactivation by phosphodiesterases. The latter represents a way to regulate the activity of NO·. Once cGMP is produced, it interacts with cGMP-dependent protein kinase, which is present at high concentrations in all smooth muscle cells, cardiomyocytes, platelets, and nervous and juxtaglomerular cells (125). Although several substrates of cGMP-dependent protein kinases have been described, this enzyme inhibits receptor-induced vascular smooth muscle contraction mainly by three mechanisms involving inositol-1,4,5-trisphosphate receptor–associated cGMP kinase substrate (IRAG), the regulator of G protein signaling (RGS2) and the myosin light-chain phosphatase. All these mechanisms account for reducing the intracellular Ca^{2+} concentration or for decreasing the Ca^{2+} sensitivity of contraction (or both), leading to the vasorelaxant effects of NO· and NO· donors. In addition, NO· increases cGMP levels in platelets and inhibits platelet activation by direct interaction of cGMP with the cGMP-dependent protein kinase that phosphorylates vasodilator-stimulated phosphoprotein (VASP) and IRAG (185). Interestingly, it has also been proposed that cyclic ADP ribose (cADPR), an endogenous modulator of RyR, is involved in the regulation of endothelial function, by participating in endothelial NO·-dependent vasodilator response (121). Thus, in bovine coronary arterial endothelium, bradykinin-induced intracellular Ca^{2+} increase and NO· response are associated mainly with cADPR levels, suggesting that cADPR-mediated Ca^{2+} signaling plays an important role in the regulation of endothelial NO· production and could play a critical role in endothelium-dependent vasodilatation (360).

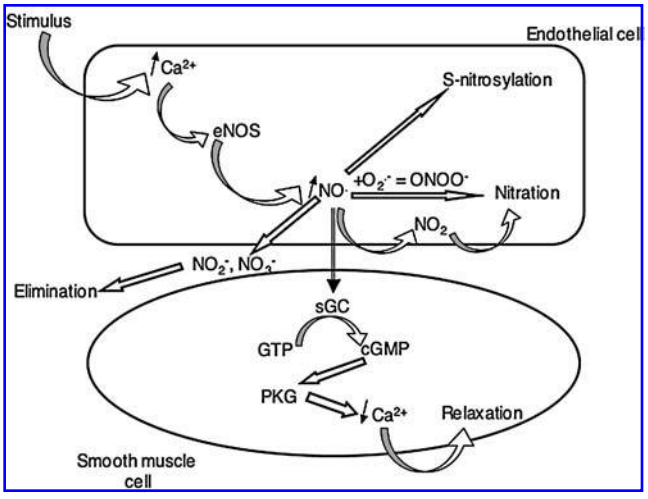

FIG. 10. Different effects of NO· in vascular cells. The increase of NO· after activation of calcium-dependent eNOS in endothelial cells is followed by activation of soluble guanylyl cyclase (sGC) in smooth muscle cells, which produces cGMP as second messenger. Through the activation of protein kinase G (PKG), cGMP is able to decrease intracellular Ca^{2+} concentration and Ca^{2+} sensitivity in smooth muscle cells, leading to vascular relaxation. NO· can react with $O_2^{\cdot -}$ in endothelial cells and generate $ONOO^-$, accounting for nitration, or NO· can induce *S*-nitrosylation of proteins.

NO· and NO· donors might use pathways independent of cGMP-dependent protein kinase, such as cross-activation of cAMP-associated pathways. This has been described in smooth muscle cells and platelets, although the concentrations of cGMP needed to act on cAMP-dependent protein kinases are 1,000-fold higher than those for cGMP-dependent protein kinase (29).

Some evidence shows that alternative pathways that are cGMP independent exist. Indeed, the selective inhibitor of sGC 1H-[1,2,4]oxadiazolol [4,3-α]quinoxalin-1-one, ODQ, is not able to inhibit several effects mediated by NO· and NO· donors, at high concentrations (micromolar to millimolar range) in blood vessels and platelets (341). NO· and NO· donors can elicit vasorelaxation through a cGMP-independent mechanism, probably *via* GSNO formation and, in this way, protect cells from further oxidative and nitrosative modifications (308). Other posttranslational modifications such as protein nitration are independent of enzyme involvement. Whether protein nitration is important in normal physiologic mechanisms is unclear, but it is probable that alterations in nitration levels may contribute to different biologic outcomes (158) (see later).

$ONOO^-$ generated under physiologic conditions can modulate cell signaling. In particular, tyrosine nitration by $ONOO^-$ could modify phosphotyrosine-dependent cell signaling. $ONOO^-$ has been reported to promote inhibition or activation of tyrosine phosphorylation in several cell types (238). Thus, $ONOO^-$-mediated nitration of critical tyrosine residues in proteins could block downstream signaling, affecting essential cellular functions, as has been observed in bovine pulmonary artery endothelial cells and rat macrophages (104, 117). Conversely, in PC12 cells, $ONOO^-$ activates members of MAP kinases, resulting in the gain of function in the upstream signaling of these proteins (144).

B. Pathophysiology

Under pathologic conditions, RNS production increases and exceeds the cell defenses, inducing deleterious effects in a large number of pathways implicated in cell life. Here, we review the effects of RNS on four categories of pathologies. Taking into consideration that the brain is, with the cardiovascular system, the highest target of damage from nitrosative stress, it seems obvious to start this part of the re-

view by the link between RNS and neurodegenerative diseases, and then to continue with the effects of RNS on the cardiovascular system. Third, we review the role of nitrosative stress in immunologic diseases and cancer, and finally, we summarize the involvement of RNS in the development of metabolic diseases. However, other deleterious effects of RNS on other systems cannot be excluded.

1. Neurodegenerative diseases. Neurodegeneration is associated with selective neuron loss. Among the regions of the brain, hippocampus and the substantia nigra are the most susceptible to cell damage; thus, in Alzheimer's disease, hippocampal degeneration has been mainly described, whereas substantia nigra is affected in Parkinson's disease (236). Many studies indicate the participation of apoptosis in neuronal cell death and in neurodegenerative diseases (for a review, see ref. 236). In general, the excessive production of RNS (and ROS), in addition to the deficiency of antioxidant defense mechanisms, is responsible, at least in part, for the functional deterioration in aged brain and neurodegenerative disorders.

Although the three isoforms of NOS are expressed in the nervous system, $NO^{\cdot}$ released from iNOS possesses both neuroprotective and neurotoxic effects. At low concentrations, $NO^{\cdot}$ elicits vasodilatation and is involved in neurotransmission; by contrast, at higher concentrations, it is potently neurotoxic (240). This is of particular significance in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, ALS, and multiple sclerosis. A common aspect of many neurodegenerative disorders is the accumulation of misfolded proteins critical for neuron life, which triggers cell death and loss (216). Indeed, Alzheimer's and Parkinson's diseases are characterized by accumulations of aggregated amyloid β peptides and tau, and α-synuclein and synphilin-1, respectively (83, 156, 197, 214). With regard to Alzheimer's disease, deposition of the long form of amyloid β, amyloid β 42, in the brain is an early and invariant feature of all forms of the disease, even before a diagnosis of Alzheimer's disease, and before the deposition of the shorter primary form of amyloid β, amyloid β 40, occurs (for a review, see ref. 90). Also, the ubiquitin–proteasome system plays an essential role by acting on the removal of aberrant proteins, but in these pathologies, this system is altered, and the accumulation of misfolded proteins can evoke neurodegeneration (216).

a. Alzheimer's disease. Amyloid β peptide can stimulate production of $NO^{\cdot}$ through the activation of iNOS, as demonstrated by the use of specific inhibitors of iNOS and iNOS knockout mice (200). The large amount of $NO^{\cdot}$ generated leads to $ONOO^{-}$ production, which mediates neuronal damage. Indeed, protein tyrosine nitration can be detected in postmortem samples from Alzheimer patients (118). Specific nitrated proteins in the Alzheimer's disease hippocampus were identified immunochemically by Sultana *et al*. (306) by using a redox proteomics approach. These proteins were related to the control of energy production (α-enolase, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase α chain, voltage-dependent anion channel protein 1, and carbonic anhydrase II). In addition to those in brain, peripheral lymphocytes and plasma from these patients present high levels of markers linked to nitrosative stress that are associated with increased expression of iNOS (*i.e*., heme oxygenase-1, heat-shock protein 60, heat-shock protein 72, and thioredoxin) (37). Nitration of tau protein, which promotes tubulin polymerization and maintains microtubule stability, has been detected in Alzheimer's disease (271). This may inhibit the ability of tau to self-associate and stabilize microtubules, as demonstrated by using an *in vitro* purified protein model (270, 359) (Fig. 11). In addition, amyloid β peptide could increase the activity of NMDA-type glutamate receptor with a subsequent increase of intracellular Ca^{2+} (see earlier). This increase in intracellular Ca^{2+} could elicit the release of RNS from NOS. RNS could induce *S*-nitrosylation of NMDA receptors, decrease their activity, and favor neuronal survival; in this way, RNS could be neuroprotective in Alzheimer's disease (180) (Fig. 11). In contrast, *S*-nitrosylation of endoplasmic reticulum chaperones, such as protein-disulfide isomerase, can be deleterious by inducing accumulation of misfolded proteins or contributing to excitotoxic neuronal damage (323) (Fig. 11).

b. Parkinson's disease. Parkinson's disease, the second most common neurodegenerative disease after Alzheimer's disorder, is characterized by a prevalent degeneration of dopaminergic neurons localized in the substantia nigra. RNS-dependent mechanisms contribute to neurodegeneration in Parkinson's disease, as shown by the increased expression of iNOS in Parkinson experimental models associated with dopaminergic neuronal loss (178, 237). Intraneuronal aggregates of α-synuclein, a protein that reg-

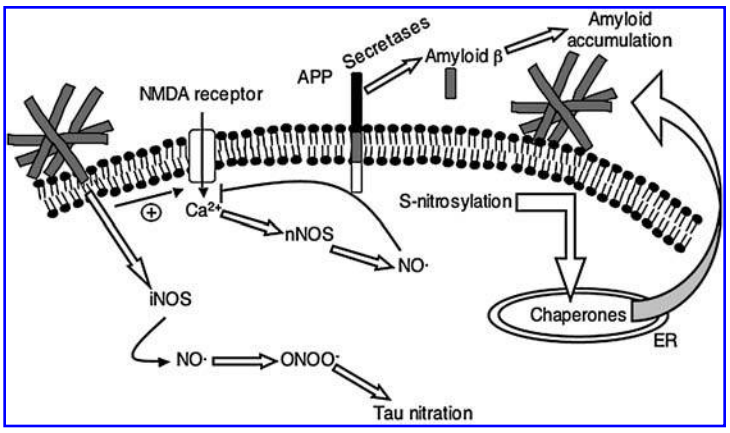

FIG. 11. Changes induced by RNS on critical proteins in Alzheimer's disease. Amyloid-β comes from the transmembrane helix of the amyloid precursor protein (APP), through the action of secretases. In neurons, excess generation of $NO^{\cdot}$ by iNOS activated by amyloid-β peptide is responsible for the nitration of proteins such as tau. Amyloid-β increases the activity of the NMDA channel, with the subsequent increase of intracellular Ca^{2+} concentration, activation of Ca^{2+}-dependent nNOS, and generation of $NO^{\cdot}$ and other RNS that could inhibit NMDA activity by *S*-nitrosylation. In addition, *S*-nitrosylation of chaperones can elicit accumulation of misfolded proteins.

ulates the trafficking of lipid secretory vesicles, have been described in Parkinson's disease. α-Synuclein possesses several tyrosine residues, which can be nitrated, as described in postmortem human brain and in mouse models (98, 258). With structural and conformational analysis of purified human α-synuclein, it has been reported that such modifications induce the formation of α-synuclein oligomers and the inhibition of filament assembly, decrease the ability of α-synuclein to bind lipid membranes, and reduce proteolysis, favoring aggregate formation (124, 326, 351). Furthermore, other proteins can be affected by RNS in Parkinson's disease. Thus, parkin, a member of the family of E3 ubiquitin ligases that stabilizes microtubules, can be *S*-nitrosylated, forming SNO-parkin; this has been detected by the biotin-switch assay in Parkinson's brain from either mice or humans (353). However, contradictory data have been described. *S*-Nitrosylation may stimulate or inhibit E3 ubiquitin ligase activity, depending on the models (55, 353). As in Alzheimer's disease, *S*-nitrosylation of endoplasmic reticulum chaperones such as protein-disulfide isomerase can be involved in the protein misfolding or neurotoxicity (323).

c. Amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disorder characterized by motoneuron death. In this pathology, increased expression of iNOS enhances the production of $NO^{\cdot}$ and $ONOO^{-}$ and triggers apoptosis in motor neurons (19). Other enzymes involved in the ROS pathways, such as Cu,Zn SOD and Mn SOD, have also been associated with the pathogenesis of ALS. Whereas Cu,Zn SOD catalyzes nitration by $ONOO^{-}$, Mn SOD is selectively nitrated, leading, at least in part, to the neuronal damage in ALS (272). Finally, neurofilament aggregates colocalize with nitrotyrosine proteins in upper and lower motoneurons, suggesting that nitration occurs in these deteriorating cells (53). In a transgenic model of mice overexpressing the mutant Cu,Zn SOD from humans with familial ALS, an elevated protein nitration and $NO^{\cdot}$ synthesis in brain tissues and cerebrospinal fluid were reported (45, 181).

d. Prion diseases. The transmissible neurodegenerative disorders, also known as prion diseases, include Creutzfeldt–Jakob disease, bovine spongiform encephalopathy, and scrapie. These pathologies are characterized by enhanced apoptosis of neurons associated with cerebral accumulation of misfolded prion protein. In scrapie and Creutzfeldt–Jakob disease, increased expression of iNOS as well as immunoreaction for nitrotyrosine have been described during the disease progression (88, 93).

To summarize, RNS can have either beneficial or deleterious effects, depending on the species and cellular target, on the neuronal signaling pathways involved in the pathophysiology of the neurodegenerative disorders. Although many of the data concerning the effects of RNS on neurodegeneration described in the literature were obtained in animal models, one can hypothesize that RNS may play an important role in human neurodegenerative diseases. However, it remains to be elucidated whether RNS are the cause or the consequence of neuronal degeneration.

2. Cardiovascular diseases. Although the involvement of RNS in cardiac signaling has been clearly demonstrated (112), many important questions still remain unresolved. A large number of targets for RNS exist in cardiomyocytes, mainly the ion channels and Ca^{2+} pumps implicated in myocyte contraction (1, 65, 192, 202, 340, 347, 348). Besides, $NO^{\cdot}$ from eNOS inhibits L-type Ca^{2+} channels through the activation of GC (201) and reduces β-adrenergic myocardial contraction (20). In contrast, $NO^{\cdot}$ from nNOS favors Ca^{2+} movements between sarcoplasmic reticulum and cytosol and increases myocardial contractility (20). Among cardiac pathologies associated with an increase in nitrosative stress, congestive heart failure and myocardial infarction are the most representative. In these diseases, nitrosative stress generated by inflammatory changes that occur in the cardiomyocytes is accompanied by contractile dysfunction (161, 186, 267).

a. Heart failure. Heart failure occurs progressively and affects the heart muscle, resulting in a loss of cardiac myocytes or in a disruption of the ability of the myocardium to generate force and a reduction of effectiveness of the pump (187). In general, it occurs after damage or disorder in the heart, as, for example, coronary endothelial dysfunction as the consequence of myocardial ischemia and infarction, but also after cardiac hypertrophy or cardiomyopathies (187). The nitrosative–oxidative imbalance could contribute to reduction of cardiac performance (112). In failing human heart, reduced $NO^{\cdot}$ production from the endothelium associated with reduced eNOS activity and eNOS uncoupling have been described (75). A correlation between increased nitrotyrosine formation in myocardial specimens from patients with heart failure and increased iNOS expression has been observed (328). Besides, the production and delivery of $NO^{\cdot}$ in the form of *S*-nitrosohemoglobin are impaired in the setting of heart failure, suggesting that $NO^{\cdot}$ donors could have beneficial effects in this pathology (63). Conversely, iNOS expression is increased in the myocardium after infarction. Thus, in addition to the severe oxidative stress, the increased $NO^{\cdot}$ production from iNOS increases infarct size, myocardial dysfunction, and mortality in mice (87).

b. Hypertension. Accumulating evidence suggests that alterations in $NO^{\cdot}$ synthesis and pathways or a reduction in the bioavailability of $NO^{\cdot}$ plays a critical role in the pathogenesis of hypertension (241). Interestingly, extensive endothelial dysfunction is observed simultaneously with an increase in eNOS expression and $O_2^{\cdot -}$ production associated with enhanced nitrotyrosine formation (99). Thus, $ONOO^{-}$ formation and protein nitration have been found to be elevated in the serum, thoracic aorta, and kidneys of different hypertensive animal models and in the human plasma (38, 191, 278).

c. Pulmonary arterial hypertension. Pulmonary arterial hypertension is characterized by increased pressure in the pulmonary arteries, which is associated with right ventricular hypertrophy and remodeling of small pulmonary arteries. Pulmonary arterial hypertension can be caused by hypoxia, and the subsequent hypoxia-regulated genes in the pulmonary endothelium could play an important role (85). Very recently, it was shown that mice treated with the antioxidant *N*-acetylcysteine develop pulmonary arterial hypertension mimicking the effects of chronic hypoxia (increase of right ventricle pressure, right ventricle hypertrophy, and vascular

remodeling), whereas eNOS-deficient mice were protected from these effects (239). These authors demonstrated that *N*-acetylcysteine is converted to *S*-nitro-*N*-acetylcysteine. They suggest that this conversion during blood deoxygenation is necessary for the development of pulmonary arterial hypertension in this animal model and underline the potential toxicity of *N*-acetylcysteine administration.

d. Atherosclerosis. All of these diseases are the consequences of vascular atherosclerosis. In atherosclerosis and hypercholesterolemia, the $NO^{\cdot}$ pathway regulating the vascular tone is altered. A decrease in either expression or activity of both eNOS and iNOS and a local increased scavenging of $NO^{\cdot}$ through the reaction with ROS have been suggested. An impaired interaction of $NO^{\cdot}$ with GC, and thereby the reduction of cGMP production, also occurs (224). Thus, strategies targeting the increase of $NO^{\cdot}$ synthesis or activity or both may be useful in the treatment of atherosclerosis and related diseases (279). In addition to the effects on $NO^{\cdot}$ synthesis, prostaglandin synthases located at the human atheroma plaques are inhibited by iNOS-mediated nitration (67), leading to a reduction in prostaglandin production. Furthermore, an increase of circulating nitrated HDL in plasma is correlated with an increased risk of coronary artery disease (246), whereas nitrated LDL has been detected in human thoracic aorta atheroma plaques (172), suggesting that nitrated LDL is retained within the atheroma plaque. In this pathology, MMP-2 is activated by $ONOO^{-}$, underlining the effects of RNS on the regulation of the atherosclerotic plaque rupture (262).

e. Preeclampsia. In another type of inflammatory vascular disease such as preeclampsia, evidence exists of endothelial cell activation and dysfunction. Preeclamptic women are characterized by pregnancies associated with hypertension linked to generalized endothelial dysfunction, proteinuria, and fetal growth delay (113). Although several markers of endothelial dysfunction (reduced endothelium-dependent relaxation and $NO^{\cdot}$ synthesis) have been detected, a novel mechanism was recently suggested. Levels of microparticles, small fragments of plasma membrane released from apoptotic or activated circulating cells or cells of the vascular wall (196), are elevated in preeclamptic women. More particularly, shed membrane particles of leukocytes and platelets have the ability to induce vascular inflammation and hyporeactivity, which is associated with increased $NO^{\cdot}$ production and upregulation of iNOS (203). Most interestingly, whereas microparticles from both platelets and leukocytes account for nitrosative stress, only leukocyte-derived microparticles induce COX-2 metabolite generation (Fig. 12). These findings demonstrate the pathophysiologic relevance of preeclamptic platelet microparticles, which are pro-inflammatory and lead to significant signs of nitrosative stress in the vascular wall.

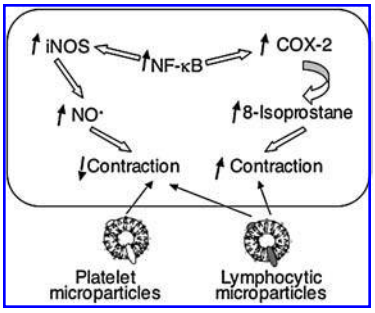

FIG. 12. Microparticles as new mediators of nitrosative stress. In preeclampsia, circulating levels of microparticles from platelets and lymphocytes are elevated. Both types of microparticles induce NF-κB activation, leading to upregulation of pro-inflammatory proteins, such as iNOS, accounting for overproduction of $NO^{\cdot}$ and vascular hyporeactivity. In contrast, only lymphocytic microparticles are able to upregulate COX-2 and generate 8-isoprostane, which evoke hyperreactivity. The balance between both vasorelaxant and vasoconstrictor factors released by the vessel wall results in hyporeactivity.

3. Inflammation

a. Airways diseases. In inflammation states, $NO^{\cdot}$ is produced by iNOS induced by microorganisms, environmental products, and cytokines (for a review, see ref. 84). Together with inflammation and subsequent remodeling of airways (273), RNS are generated during the development of numerous airways diseases such as asthma, chronic obstructive disease, and cystic fibrosis. For example, although all three NOS isoforms are expressed within the respiratory tract, iNOS is strongly induced in the airway epithelium in asthmatic patients (109), and subsequent alterations in the pathways dependent on $NO^{\cdot}$ have been described, including airway hyperresponsiveness (273). Thus, elevated concentrations of $NO^{\cdot}$ have been observed in the exhaled air of asthmatics (149). In patients with asthma and cystic fibrosis, the levels of *S*-nitrosothiols in the bronchoalveolar lavage fluids are extremely and paradoxically reduced (96, 105), suggesting a disorder of *S*-nitrosothiol metabolism. In addition, an exacerbated nitration of proteins in airway epithelial cells from asthma patients, in particular, the antioxidant enzyme catalase, was recently described (97). Altogether, these results suggest that, in asthma, RNS affect $NO^{\cdot}$-dependent pathways and amplify oxidative stress by decreasing *S*-nitrosothiol bioavailability and catalase activity. These effects probably contribute to the chronic inflammatory state.

Airways inflammation plays an important role in the pathogenesis of chronic obstructive pulmonary disease. In this pathology, markers of nitrosative stress, such as exhaled $NO^{\cdot}$ and tyrosine nitration of proteins, have been detected in the sputum and lung specimens from asthma patients (133). In addition, inhibition of iNOS is associated with a reduction of nitrotyrosine, and most important, this is correlated with the improvement in forced expiratory volume and airway responsiveness to histamine, suggesting that RNS are involved in the reversible component of inflammation in chronic obstructive pulmonary disease. Therefore, specific inhibition of RNS actions could represent a novel therapeutic approach for this pathology (305) (Fig. 13). Similar results have been shown during *Candida albicans*–induced acute lung injury. Selective inhibition of iNOS suppresses the production of $ONOO^{-}$, as well as nitrotyrosine and expression of iNOS (235).

b. Sepsis. Sepsis represents a pathology associated with the dysregulation of the $NO^{\cdot}$ pathway. Sepsis is the result of

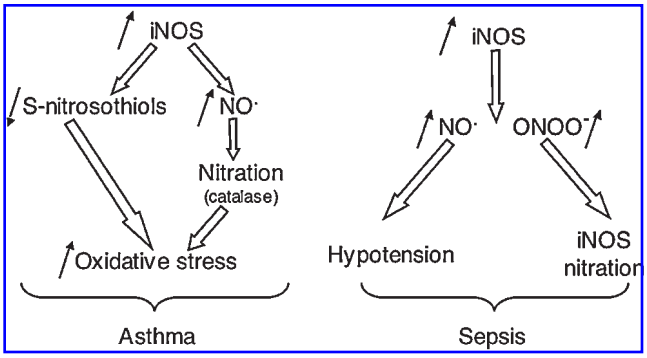

FIG. 13. RNS in inflammatory states. In asthma or sepsis, the increase of iNOS is accompanied by an augmentation of RNS production that induces nitration of proteins implicated in the defenses against oxidative stress, such as catalase or iNOS in asthma or sepsis, respectively. In addition, in asthma, oxidative stress can be increased by the reduction of *S*-nitrosothiols. Moreover, the excess of $NO^{\cdot}$ generated from iNOS accounts for the hypotension in septic patients.

an acute and systemic immune response to a variety of noxious insults, in particular to bacterial infections. This response leads to the activation of a number of host mediator systems, including the cytokine, leukocyte, and hemostatic networks. Sepsis is associated with evidence of organ dysfunction characterized by tissue hypoperfusion and hypoxia, lactic acidosis, and oliguria (17). Large amounts of $NO^{\cdot}$ and $ONOO^{-}$, generated by enhanced expression of the iNOS, as detected in human vessels from septic patients (302), are implicated in the observed symptoms (Fig. 13). Although it is reasonable to imagine that inhibition of $NO^{\cdot}$ synthesis could represent a potential therapeutic approach in sepsis, $NO^{\cdot}$ inhibitors cause a progressive decrease in cardiac output, amplify organ dysfunction, and increase mortality, probably because of the lack of specificity against the NOS isoform inhibited (177, 250). Independent of the direct effects of $NO^{\cdot}$ on GC and the subsequent exaggerated vasorelaxation accounting for hypotension in sepsis, it has been shown that accumulation of *S*-nitrosothiols in GSNO reductase$^{-/-}$ mice is linked to tissue damage and mortality after endotoxemia (184). These findings indicate that GSNO reductase protects mice from nitrosative stress leading to excessive vasorelaxation and tissue injury after endotoxemia and suggest a central role of *S*-nitrosothiols in $NO^{\cdot}$ biology and disease. Tyrosine nitration of iNOS by $ONOO^{-}$ has been described in rectus abdominis muscle of septic patients (169), suggesting a modulation of activity of this enzyme by RNS. Furthermore, albumin is able to decrease nitrosative stress in a mouse endotoxemia shock model. A recent study shows that albumin prevented vascular hyporeactivity as well as endothelial dysfunction induced by endotoxin in a mouse endotoxic model (204). The effects of albumin are associated with a decreased upregulation of NF-κB, iNOS, and $ONOO^{-}$ in the vascular wall. In addition, albumin prevents the increases in both $NO^{\cdot}$ and $O_2^{\cdot -}$. Although this study in the mouse endotoxic model does not provide evidence of the mechanism(s) involved in the effects of albumin, it suggests that albumin displays a protective effect in experimental endotoxic shock by reducing the inflammatory process leading to oxidative and nitrosative stresses. Conversely, evidence suggests that RNS may play a role in sepsis; in mice, deletion of myosin light-chain kinase-210 (MLCK210) isoform, which plays a central role in the control of endothelial barrier function, decreases susceptibility to endotoxin-induced mortality, and this is associated with a decrease in nitrosative stress, as demonstrated by reduction in iNOS expression and in $NO^{\cdot}$ production in MLCK210-null mice (263). These findings may help to design new therapeutic strategies for the treatment of septic shock, based on pharmacologic inhibition of MLCK210.

c. Immune responses. Nitrosative stress is associated with the innate immune response toward infection, in which $NO^{\cdot}$ is produced at high levels within phagocytes to assure the death of internalized bacteria, virus, and parasites (311). Several studies have shown that rat macrophages, but not mast cells, are able to produce and release $NO^{\cdot}$ under pro-inflammatory stimulation (71, 310). In addition, $NO^{\cdot}$, as well as hydrogen peroxide, from macrophages inhibits mast cell degranulation mediated by immunoglobulin E (71, 310).

RNS possess antimicrobial actions through several mechanisms. $NO^{\cdot}$ and GSNO inhibit the growth of bacteria by inducing double-stranded chromosomal breaks *via* DNA-binding zinc metalloproteins (283). $NO^{\cdot}$ donors and $ONOO^{-}$ have been described as powerful inhibitors of hantavirus and virion replication, respectively (153). Inhibition of bacterial respiratory enzymes by $NO^{\cdot}$ has been reported (319). Although all these results suggest that RNS can act as antimicrobial agents, it is interesting to note that microorganisms have at least partially developed mechanisms to remove or neutralize RNS, generating resistance against these compounds (84).

4. Cancer. RNS can induce DNA damage and structural and functional modifications of proteins, and thereby may play a role in carcinogenesis. Tumor cells express all isoforms of NOS, depending on the tumor type and progression, and in this way, the effect of $NO^{\cdot}$ on tumor progression is determined by the source of cells and the type of NOS (94). Incubation of calf thymus DNA with $ONOO^{-}$ caused DNA cleavage (135). In addition, RNS modify proteins that are essential to cell function, including enzymes involved in cell cycle, apoptosis, and DNA repair (for example, DNA ligase *in vitro*) (106). A positive correlation between iNOS activity and p53 mutations has been observed in human colorectal cancer (7) and in cancer-prone chronic inflammatory disease (132), suggesting that RNS cause mutations of cancer-related genes. In addition, studies performed by using iNOS$^{-/-}$ mice suggested the involvement of $NO^{\cdot}$ generated from iNOS in the initiation or promotion of tumorigenesis in melanoma or both (126). In contrast, $NO^{\cdot}$ from iNOS inhibits transformation and tumor phenotype of mouse epidermal cells (72). These differences might be related to the different activity and localization of NOS isoforms, concentration and duration of $NO^{\cdot}$ exposure, and cellular sensitivity to $NO^{\cdot}$ (94). Nevertheless, $NO^{\cdot}$ has been shown to enhance tumor cell migration and invasion *via* a cGMP-dependent pathway and to increase tumor cell proliferation in a mice mammary model and in a human colon adenocarcinoma cell line DLD-1 (137, 139), all these processes being linked to angiogenesis. Angiogenesis is a process necessary for the formation of new

vessels able to deliver oxygen and nutrients to tumor cells and to favor its growth. $NO^{\cdot}$ from iNOS has been implicated in the angiogenic role of $NO^{\cdot}$ through the ability of iNOS to regulate VEGF expression (296) (Fig. 14). Wang's group (339) described that, depending on the expression level of iNOS in murine pancreatic adenocarcinoma cells *in vitro*, the released $NO^{\cdot}$ could have a metastatic potential and participate in tumor growth. These effects of $NO^{\cdot}$ could be explained by the fact that, *in vitro*, $NO^{\cdot}$ stimulates proliferation and migration of endothelial cells by *S*-nitrosylation of protein tyrosine phosphatases and reduction of their activity (34) or by activation of GC (259). Furthermore, $NO^{\cdot}$ is not only a modulator of the expression of endogenous angiogenic factors, but also mediates their functions (79), suggesting that the regulation of the production and release of $NO^{\cdot}$ might be a novel approach to overcoming tumor cell resistance to conventional therapeutics. Among the suggestions, strategies to increase or to decrease $NO^{\cdot}$ pathway have been described. Thus, in rat medullary thyroid cancer, modification of *iNOS* gene delivery has been tested by using iNOS-expressing vectors and, in this model, a volume reduction of tumors has been observed (301). Generation of RNS at high concentrations by using $NO^{\cdot}$ donors is directly cytotoxic to rat and human cultured glioma cells or mouse melanoma, and sensitizes to radiation and to some chemotherapeutic agents (*e.g.*, cisplatin) (163, 248). In other cases, inhibition of the $NO^{\cdot}$ pathway is an antiangiogenic therapeutic strategy and, as described in murine plasmacytoma (324), the selective inhibition of $NO^{\cdot}$ production from iNOS inhibits tumor growth, probably by reducing blood flow and creating a hypoxic environment that reduces the supply of nutrients and oxygen into tumors (123).

Taken together, these data suggest that $NO^{\cdot}$ may play a key role in the regulation of cell tumor growth and progression; thus, manipulation of the $NO^{\cdot}$ pathway in cancer could represent the potential novel opportunities in the treatment of this disease, at least for some types of cancer.

5. Metabolic diseases. Among the metabolic diseases in which the $NO^{\cdot}$ pathway is implicated, diabetes is one of the most studied. Diabetes-associated vascular complications are the most common major clinical problem that patients with diabetes experience, contributing to the significant morbidity and mortality rate. Thus, patients with diabetes have an elevated incidence of macrovascular complications, such as atherosclerosis, which increase the risk for myocardial infarction, stroke, and peripheral artery disease (often leading to limb amputation), and microvascular complications such as retinopathy and nephropathy, which cause blindness and renal failure. In addition, it is essential to differentiate between type 1 and type 2 diabetes. The former, also called insulin-dependent, is characterized by autoimmune destruction of pancreatic β cells producing insulin; the latter, also called insulin-independent diabetes, is often complicated by other cardiovascular risk factors (345).

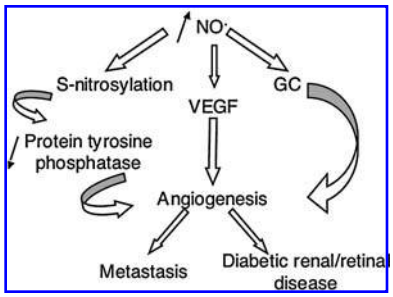

FIG. 14. RNS and angiogenesis. RNS can modulate angiogenesis through several pathways. First, $NO^{\cdot}$ favors angiogenesis *via* the vascular endothelial growth factor (VEGF). Second, reduction of the activity of protein tyrosine phosphatase by *S*-nitrosylation is proangiogenic. Finally, $NO^{\cdot}$ can promote angiogenesis through the activation of guanylyl cyclase (GC). Taken together, RNS may play a significant role in the development of metastasis and in certain symptoms of diabetic diseases associated with alteration of the angiogenesis process, such as retinopathy or nephropathy.

Under physiologic conditions, insulin stimulates the production of $NO^{\cdot}$ from the endothelium, leading to regulation of vascular tonus (115). However, high levels of insulin elicit an excessive generation of $ONOO^{-}$, which triggers an impairment of endothelium-dependent relaxation *via* a decrease in SERCA function, as shown in diabetic rats (157). Endothelial dysfunction is a consistent feature of insulin-resistant states, including diabetes, obesity, and the metabolic syndrome (for review, see ref. 218). Signaling pathways involved in the coupling between insulin and the insulin receptor display similarities with the cascade regulating $NO^{\cdot}$ production. Thus, both insulin resistance and endothelial dysfunction are related to inhibition of the PI3-kinase pathway and overstimulation of the MAP kinase pathway. Recent studies have implicated dysfunctional eNOS as a common pathogenic pathway in diabetic vascular complications (12, 213). Thus, as described earlier, almost all insulin-activated pathways (Akt/PKB, insulin-receptor substrate-1 or p21ras) can be altered by *S*-nitrosylation, *S*-glutathionylation, or nitration by RNS (57, 230, 354) (Fig. 15). Insulin secretion and receptor activation and the downstream intracellular transduction signaling can be potentially affected by $NO^{\cdot}$ or $NO^{\cdot}$ donors, suggesting that RNS could be regulators of the progression of this pathology, in particular of cardiovascular events associated with diabetes. Nevertheless, it should be noted that the effects of RNS in diabetes may be dependent on their source; for example, in contrast to iNOS, gene disruption of eNOS or nNOS in mice leads to insulin resistance (292).

Generation of $O_2^{\cdot -}$ *via* the uncoupling of eNOS has been reported in blood vessels of streptozotocin-induced diabetic rats (122). Recent data obtained in diabetic mice suggest that angiotensin II could be implicated in this process, because angiotensin II–targeted therapy improves endothelial function in diabetic mice *via* the recoupling of eNOS (231).

In bovine microvascular retinal endothelial cells, elevated glucose decreases eNOS expression and activity, as reflected by a significant dose-dependent decrease in nitrate levels in conditioned medium, as well as both laminar shear stress and pulsatile flow–induced activity (59). These effects may account for endothelial dysfunction and impaired autoregulation in diabetic retinopathy. By using a knockout mouse model, Camici and colleagues (39) showed that deletion of the $p66^{Shc}$ adaptor protein prevents not only hyperglycemia-induced endothelial dysfunction but also the generation of $ONOO^{-}$ and tyrosine nitration. These results suggest that $p66^{Shc}$ adaptor protein could be part of a signal-transduction

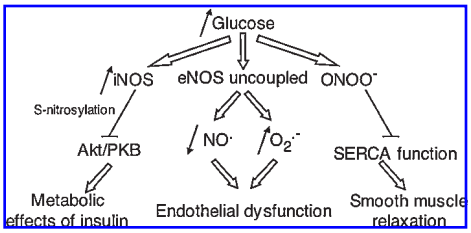

FIG. 15. Effects of RNS on insulin signaling. Elevated glycemia increases iNOS expression and activity, as well as $ONOO^-$ production. iNOS inhibits the Akt/PKB pathway, contributing to reduction of metabolic effects of insulin. In parallel, glucose decreases $NO^\cdot$ production and increases $O_2^{\cdot -}$ release through the uncoupling of eNOS, leading to the endothelial dysfunction observed in diabetes. Finally, SERCA is nitrated by $ONOO^-$, leading to an impairment of endothelium-dependent relaxation. *Arrows without heads*, inhibition.

pathway relevant to hyperglycemia vascular damage and may represent a potential novel therapeutic target against diabetic vascular complications.

Furthermore, the $NO^\cdot$/cGMP pathway has been described as participating in the contractility of cardiomyocytes in streptozotocin-diabetic rats (33, 300). An increased production of $NO^\cdot$, probably by both iNOS and constitutive NOS, and the subsequent increased production of cGMP within the myocytes from streptozotocin-diabetic rats suppressed the shortening response to β-adrenergic stimulation without affecting the Ca^{2+} current through L-type Ca^{2+} channels (300). Moreover, small coronary arteries from diabetic rats exhibit enhanced $ONOO^-$ formation and concurrent impairment of voltage-dependent K^+ channel function by direct nitration of the channels (33).

Another aspect of diabetes is the neuropathy that affects at least 30–50% of diabetic patients, independent of the type of the diabetes. Recent data show that nitrosative stress plays an important role in early experimental neuropathy in streptozotocin-induced diabetic rats (232), by inducing motor and sensory nerve-conduction velocity deficits, associated with increased nitrotyrosine in the sciatic nerve and dorsal root ganglia. The same group reported that, in leptin-deficient *ob/ob* mice, a model with type 2 diabetes with mild hyperglycemia and obesity, $ONOO^-$ decomposition catalysts restore motor and sensory nerve-conduction velocity, improving nerve dysfunction induced by $ONOO^-$, underlining the deleterious effects of RNS in neuropathy in type 2 diabetes, at least in mice (330).

Nephropathy also is implicated in morbidity and mortality in patients with diabetes. Kanetsuna and colleagues (146) proposed a vital role for eNOS-derived $NO^\cdot$ in the prevention of diabetic nephropathy in mice. By using diabetic eNOS-knockout mice, these authors have demonstrated that deletion of eNOS leads to the development of albuminuria, hypertension, and glomerular mesangiolysis, linked to glomerular hyperfiltration and injured endothelial morphology. This has been corroborated by another group, showing that diabetic eNOS knockout mice developed nephropathy, which is associated with increased endothelial proliferation and VEGF expression. In addition, all these effects are improved with insulin therapy (220). Altogether, these findings suggest that a relative deficiency in eNOS-derived $NO^\cdot$ levels may participate in the increased susceptibility to nephropathy in diabetic subjects (146, 221).

Patients with diabetes display impairment of re-endothelialization after vascular injury, associated with a dysfunction in the recruitment of endothelial progenitor cells (EPCs) in the affected zones (134). Recently, it was shown that EPC levels in patients with diabetes are reduced compared with those of control subjects. In addition, EPCs from patients with diabetes are able to produce excessive $O_2^{\cdot -}$, which is reduced when eNOS is inhibited (318). These authors propose an uncoupling of eNOS in EPCs from patients with diabetes, resulting in $O_2^{\cdot -}$ production. This may be responsible for the impaired neovascularization described in diabetes. Besides, a potentially deleterious role of VEGF in diabetic renal disease has been proposed, because new extra vessel formation in the glomerulus is correlated with increased VEGF expression in biopsies from human diabetic kidney (147) (Fig. 14).

Dermal application of GSNO is able to promote local vasodilatation in streptozotocin-induced diabetic rats, without inducing protein nitration or alterations in blood pressure or heart rate, suggesting that GSNO may be an effective treatment that prevents or reverses the consequences of endothelial dysfunction in patients with diabetes (289).

VI. Future Directions

During the 1980s, RNS were considered to be molecules with complex properties, at least within the cardiovascular system. However, in the last decade, the numbers of identified RNS and of potential targets of RNS have proportionately increased. In addition, RNS possess different effects (beneficial or deleterious) in relation to their cellular origin, the enzymatic source, their concentration and, finally, the cell target. The use of $NO^\cdot$ or $NO^\cdot$ donors (sodium nitroprusside or nitroglycerine, for example) as pulmonary or coronary vasodilators in different pathologies is now routine (317). These effects are based on the $NO^\cdot$ signaling with cGMP as second messenger. In this case, modulation of expression or activity or both of eNOS, as well as modification of cGMP degradation by phosphodiesterases, remains the main axis of therapy. However, as shown in this review, much information about other intracellular transduction signaling pathways that mediate $NO^\cdot$ effects is now available, and the modulation of these "new" pathways might open novel potential therapeutic strategies by neutralization of RNS, or inhibition or activation of RNS generation, depending on the pathologic situation.

Different molecules acting on a variety of targets are able to reduce RNS-induced cellular dysfunction or to inhibit enzymes on which RNS induce an excess of function. Many of the examples reported in the other sections of this review illustrate this point. As an example, acting on the angiotensin-converting enzyme by using enalapril (an inhibitor of this enzyme) reduces dysfunction of vascular smooth muscle SERCA and enhancement of tyrosine nitration in diabetic rats (315). It is plausible to consider that, in humans with diabetes, inhibition of the angiotensin II pathway could ameliorate cardiovascular complications.

Beneficial effects of PPAR agonists in the human insulin-resistance syndrome are well documented (for a review, see

ref. 302). PPARs act as central transcriptional mediators of metabolic processes. Thus, PPAR agonists are used as insulin sensitizers for their ability to correct the abnormal blood flow in patients with diabetes. Although PPARα and PPARγ agonists can contribute to amelioration of the macrovascular dysfunctions, the combinations of PPARα, PPARβ, and PPARγ may better serve to improve the microvascular defect in diabetes (335). PPAR agonists could act by reducing $NO^{\cdot}$ production from iNOS and the subsequent nitrosative stress (336). It remains to be determined whether the reduction of nitrosative stress by PPAR agonists correlates with an improvement of neurovascular function in the long term in diabetes.

Not many therapies exist for the microvascular complications observed in patients with diabetes. In particular, improvement of the impaired angiogenesis and vasculogenesis could represent a potential field for individuals with diabetes. Gallagher's group (95) recently showed that hyperbaric oxygen therapy, by increasing NOS-derived $NO^{\cdot}$ levels in perivascular tissues, enhances EPC mobilization from bone marrow in streptozotocin-treated mice. In addition, stromal cell–derived factor-1α (SDF-1α) mediates EPC homing, and both treatments have synergistic effects. Although the clinical relevance of this study remains to be established, it could represent a new hope for wound healing in diabetes.

As discussed earlier, *S*-nitrosylation of NMDA receptor could have a protective effect in neurodegenerative diseases by decreasing NMDA activity. New combinatorial drugs, nitromemantines, second-generation memantine antagonists of NMDA receptors, plus a nitro group, are able to target $NO^{\cdot}$ to the *S*-nitrosylation sites of the NMDA receptors. Preliminary studies performed by the Lipton's group have shown that nitromemantines are more effective than memantine as neuroprotectants, both *in vitro* and *in vivo* in animal models, without affecting blood pressure (for a review, see ref. 223). With these molecules, a promising research field is now opened: the modulation of *S*-nitrosylation sites.

In diseases such as cancer, both inhibition and overproduction of $NO^{\cdot}$ have been proposed as strategies for therapy. Whereas the administration of NOS inhibitors, such as N^{G}-nitro-L-arginine methyl ester (L-NAME), can slow the growth of tumors in animal models (322), $NO^{\cdot}$ donors can act directly on cells to inhibit their growth (163, 248). In this way, dietary components targeting the iNOS–VEGF axis could be used as a practical approach for cancer prevention and intervention (245).

Conversely, the formation of *S*-nitrosothiols, which are local $NO^{\cdot}$ stores from which biologically active $NO^{\cdot}$ can be released, offers a novel and promising field in several pathologies in which $NO^{\cdot}$ levels can be compromised (217).

In conclusion, although the $NO^{\cdot}$ pathway continues to be an important target for treatment of different pathologies, application to humans of data obtained from cell or animal models requires more preclinical and clinical studies to confirm the effects of RNS in human diseases. In addition, RNS have both positive and negative effects that contribute to the physiology and pathophysiology of many disorders. Thus, measurements of new biomarkers of nitrosative stress could help us to understand the role of RNS in the regulation of homeostasis.

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Abbreviations

3-NO_2-Tyr, 3-nitrotyrosine; 8-NO_2-G, 8-nitroguanine; 8-NO_2-Guo, 8-nitroguanosine; ALS, amyotrophic lateral sclerosis; BH_4, tetrahydrobiopterin; cADPR, cyclic ADP ribose; CFTR, cystic fibrosis transmembrane conductance regulator; cGMP, cyclic guanosine 3′,5′-monophosphate; COX, cyclooxygenase; CuZn-SOD, copper-zinc superoxide dismutase; EC-SOD, extracellular superoxide dismutase; EGF, epidermal growth factor; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; FGF-1, fibroblast growth factor; GC, guanylyl cyclase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; IGF, insulin-like growth factor-1; IKKβ, inhibitory κB kinase β; iNOS, inducible nitric oxide synthase; InsP3, inositol 1,4,5-triphosphate; IRAG, inositol-1,4,5-trisphosphate receptor–associated cGMP kinase substrate; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LNO_2, nitrolinoleic acid; MAP kinase, mitogen-activated protein kinase; MLCK210, myosin light-chain kinase-210; MMPs, matrix metalloproteinases; Mn-SOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate receptors; nNOS, neuronal nitric oxide synthase; NO^{-}, nitroxyl; $NO^{\cdot}$, nitric oxide; NO^{+}, nitrosonium; NOS, nitric oxide synthase; NSF, *N*-ethylmaleimide sensitive factor; $O_2^{\cdot -}$, superoxide; OA-NO_2, oleic acid; ODQ, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one]; $ONOO^{-}$, peroxynitrite; PARP, poly ADP-ribose polymerase; PETN, phosphatase and tensin homologue; PKG, protein kinase G; PPARs, peroxisome proliferators-activated receptors; RGS2, regulator of G protein signaling; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNO, *S*-nitrosothiol derivative; RSNOs, *S*-nitrosothiols; RyR, ryanodine receptor; SDF-1α, stromal cell–derived factor-1 α; SERCA, sarco/endoplasmic calcium pumps; SMase, sphingomyelinase; SOD, superoxide dismutase; TRP, transient receptor potential channel; VASP, vasodilator-stimulated phosphoprotein; VEGF, vascular endothelial growth factor; VGCCs, voltage-dependent calcium channels.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Ramaroson Andriantsitohaina, Ph.D.
INSERM, U771
Faculté de Médecine
Rue Haute de Reculée
Angers
F-49045 France

E-mail: ramaroson.andriantsitohaina@univ-angers.fr

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