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Review Article

Implications of Inducible Nitric Oxide Synthase Expression and Enzyme Activity

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ABSTRACT

We summarize here our current knowledge about inducible nitric oxide synthase (NOS) activity in human diseases and disorders. As basic research discovers more and more effects of low or high concentrations of NO toward molecular and cellular targets, successful therapies involving inhibition of NO synthesis or application of NO to treat human diseases are still lacking. This is in part due to the fact that the impact of NO on cell function or death are complex and often even appear to be contradictory. NO may be cytotoxic but may also protect cells from a toxic insult; it is apoptosis-inducing but also exhibits prominent anti-apoptotic activity. NO is an antioxidant but may also compromise the cellular redox state via oxidation of thiols like glutathione. NO may activate specific signal transduction pathways but is also reported to inhibit exactly these, and NO may activate or inhibit gene transcription. The situation may even be more complicated, because NO, depending on its concentration, may react with oxygen or the superoxide anion radical to yield reactive species with a much broader chemical reaction spectrum than NO itself. Thus, the action of NO during inflammatory reactions has to be considered in the context of timing and duration of its synthesis as well as stages and specific events in inflammation. Antiox. Redox Signal. 2, 585–605.

INTRODUCTION

In 1986, Louis Ignarro identified nitric oxide (NO) synthesized by mammalian cells as the long searched "endothelium-derived relaxing factor" (Nathan, 1992) and in 1998 the Nobel Prize for medicine was awarded for "NO as a signaling molecule in the cardiovascular system." The enzymes that synthesize the signal molecule NO are the so-called constitutively expressed neuronal and endothelial NO synthases (ncNOS and ecNOS). After binding of Ca^{2+}-calmodulin complexes, both of these enzymes synthesize NO as short pulses or after specific phosphorylation for extended periods of time in a tightly regulated fashion. In contrast, a third NO-synthesizing enzyme is expressed after activation in most nucleated mammalian cells, only by inflammatory mediators like bacterial products and/or proinflammatory cytokines. All three types of NOSs are active only as homodimers and synthesize NO via a five-electron oxidation of a nitrogen atom from the L-arginine guanidinium group and O_2. It is one of the most complicated enzymatic activities currently known and the exact mechanism of NO synthesis is still not completely understood.

This review will focus on the role of the inducible NOS (iNOS), which was first discovered in macrophages around 1987/88 by John Hibbs, Dennis Stuehr, and Michael Marletta

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(Nathan, 1992). The iNOS produces NO as long as the protein is functionally intact, substrate (L-arginine) and cofactors are available, and the effector cell does not undergo apoptosis or necrosis. Except for a possible feedback inhibition by NO, *in vivo*-acting and specific regulatory mechanisms that will turn down iNOS enzyme activity are not known to date. *In vitro*, iNOS-expressing cells can produce up to 5 μM steady-state NO concentrations for 24 h or even longer (Laurent *et al.*, 1996). Thus, our current understanding is that the concentration of NO in conjunction with the duration of its synthesis determines whether NO acts as essential signal molecule or whether it may cause nitrosative stress (Fig. 1).

CHEMISTRY OF NITRIC OXIDE

NO is an inorganic gas that is soluble in aqueous solutions at concentrations of up to 2 mM. Therefore, in biological systems NO should not be regarded as a gas, because NO concentrations do not exceed the low micromolar range under physiological or under pathophysiological conditions. NO has an unpaired electron allowing for interactions with metals, *e.g.*, the iron of the heme group of guanylate cyclase. Although being a radical, the reactivity of NO in biological systems is relatively low, thus allowing for diffusion. Due to its small size and its lipophilicity, it easily crosses membranes. However, NO will react with oxygen, thus explaining its oxidation under aerobic conditions (Fig. 2). This reaction is of third order, with two molecules of NO reacting with one molecule of O_2. Thus, the half-life of NO depends almost exclusively on the concentration of NO. In other words, the higher the concentrations of NO, the more likely its reactions with oxygen. In addition, hydrophobic milieus like the cell membrane can accelerate the reaction of NO with O_2 several hundredfold (Liu *et al.*, 1998a). Products of this reaction are the so-called reactive nitrogen oxide intermediates (RNOI), also termed higher nitrogen oxides (NO_x) like NO_2, N_2O_3, and N_2O_4 (Fig. 2). These RNOI are a highly reactive and short-lived species and exhibit a much broader reaction spectrum toward biomolecules than NO itself. Thus, it is primarily the concentration of NO and the resulting concentrations of RNOI that will determine the chemical properties of NO. This allows for explaining the signaling functions of NO produced by the cNOSs in a tightly regulated fashion, resulting in low local concentrations of NO for short periods of time. On the other hand, the unregulated iNOS activity results in highly increased local NO concentrations (probably in the low micromolar range) and thus elevated levels of RNOI for ex-

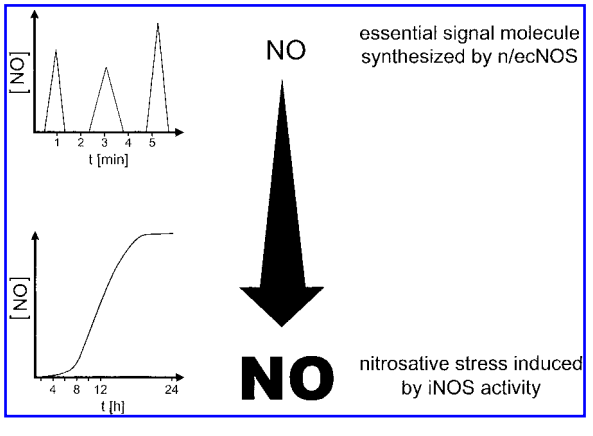

FIG. 1. NO acts as an essential signal molecule or causes "nitrosative stress." Depending on its concentration, the duration and the context of its synthesis, effects of NO are totally different. Thus, the pulsative Ca^{2+}-regulated mode of NO formation results in low local NO concentrations serving messenger functions, whereas the constant enzyme activity of iNOS, once expressed, will produce a situation best termed as "nitrosative stress."

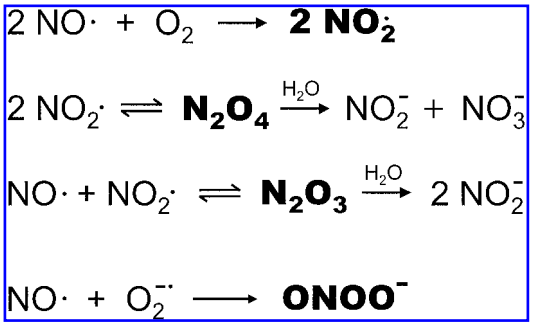

FIG. 2. Reaction products of NO with oxygen and the superoxide anion radical. The multiple chemical reactions are highly dependent on the local NO concentrations. Stable oxidation products of NO are nitrite and nitrate. NO may also react with $O^{\bullet}{}_2^-$ yielding peroxynitrite. Unstable and highly reactive intermediate products are shown in bold letters.

tended periods of time). In addition, NO may also react with the superoxide anion radical ($O^{\bullet}{}_2^-$) yielding the strong oxidant peroxynitrite (Fig. 2). It is this complex chemistry of reactive oxygen and reactive NO_x species that plays key roles in the redox regulation of cellular activation, transcription, proliferation, and cell death.

EXPRESSION OF iNOS IN HUMAN DISEASES

iNOS is now thought to be inducible in all mammalian nucleated cell types. Main inducers are lipopolysaccharide (LPS) and/or proinflammatory cytokines (Fig. 3), and other stimuli. After binding to respective cell receptors, phosphorylation signaling cascades like Janus kinases (JAK), p38 mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK 1/2), and protein-tyrosine kinases lead to activation of specific transcription factors like nuclear factor κB (NF-κB), interferon regulatory factor (IRF), and signal transducer and activator of transcription 1α (Stat 1α). Together with other proteins such as high-mobility-group I(Y) protein [HMG]I(Y)], the activated transcription factors translocate into the nucleus and bind to the iNOS promoter and enhancer region, thereby inducing iNOS transcription and subsequent translation (for reviews, see Kröncke *et al.*, 1998; Murphy, 1999). However, considerable species-, cell type-, and stimuli-specific differences do exist.

iNOS protein expression during infections has been discovered in humans infected with human immunodeficiency virus (HIV), *Helicobacter pylori, Mycobacterium tuberculosis, Plasmodium falciparum* (malaria), and *Mycobac-*

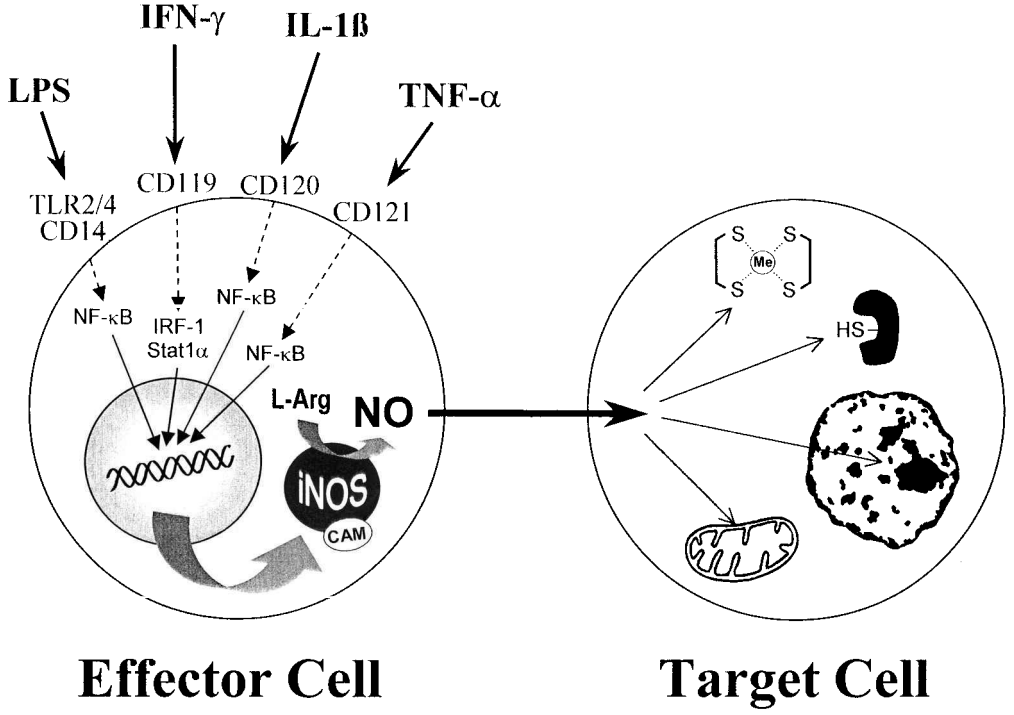

FIG. 3. Pathways for iNOS induction and cellular targets of iNOS activity. Bacterial products like lipopolysaccharides (LPS) and proinflammatory cytokines like interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor-α (TNF-α) bind to respective cell-surface receptors leading to the activation of signaling cascades, which, in turn, lead to activation of transcription factors in the cytosol. These transcription factors than translocate into the nucleus, bind to their respective consensus sequences in the promoter and/or enhancer region of the iNOS gene, and transcription as well as expression of the iNOS protein will start. The synthesized NO is a small lipophilic molecule that leaves the effector cell and may induce nitrosative stress in neighboring target cells. Predominant molecular targets are heme moieties and proteins with metal–sulfur clusters or thiol groups; predominant organelle targets are the nucleus and mitochondia. NO may thus act on several cellular targets simultaneously.

terium leprae (leprosy) as well as in respiratory, urinary tract, and intra-amniotic infections (for review, see Kröncke *et al.*, 1998). In these cases, iNOS was localized predominantly to inflammatory immune cells like macrophages, neutrophils, or other polymorphonuclear leukocytes.

However, iNOS protein has also been found in the absence of infections in a variety of human immune-mediated or autoimmune diseases like rheumatoid arthritis and multiple sclerosis, as well as in a variety of chronically inflammatory diseases of the airways, bowel, skin, blood vessels, heart, kidney, apex of teeth, and other organs of the body, and additionally in a variety of disorders like neurodegenerative diseases, acute ischemic conditions, during cancer development, after transplantation, *etc.* (Table 1). Again, in many cases iNOS protein is found in inflammatory cells such as macrophages, macrophage-like cells, or polymorphonuclear leukocytes, but here iNOS expression often is observed in epithelial cells around inflammatory foci. No data exist concerning iNOS expression in one of the most prevalent human immune-mediated diseases, *i.e.*, type 1 diabetes, where studies on animal strains spontaneously developing this disease show iNOS expression in islet endothelial cells and islet-infiltrating macrophages, and in islet β-cells as well. Thus, it is conceivable that iNOS expression will occur during development of human type 1 diabetes.

iNOS ACTIVITY IN HUMAN DISEASES

Although numerous studies have shown iNOS protein expression in human diseases, as depicted in the previous chapter, much less is known about high-output NO production actually occurring at the sites of iNOS expression. For reasons not yet known, and in contrast to murine cells, *in vitro* production of high levels of NO via iNOS activity in human cells is notoriously difficult to achieve. However, cells isolated from infected or inflammatory sites of patients do show high-output NO production *in vitro* either without further activation (De Groot *et al.*, 1997) or after addition *in vitro* challenge (Takeichi *et al.*, 1998b). These studies suggest that human cells can indeed produce high NO concentrations. In addition, the presence of nitrotyrosine, originally described as a marker for the generation of peroxynitrite (Ischiropoulos *et al.*, 1992), but currently regarded as a marker for RNOI formation (Fig. 4) (for review, see Halliwell, 1997), has been found at sites of iNOS expression. In most but not all of these cases, nitrotyrosine and iNOS co-localize (Table 1) as an indirect indication for iNOS activity and high-output NO formation in diseased human organs.

PEROXYNITRITE FORMATION IN HUMAN DISEASES

The attractive hypothesis has been put forward, that NO is the good, $O^{\bullet}{}_2^-$ the bad, and their reaction product peroxynitrite the ugly (Beckman and Koppenol, 1996). NO and $O^{\bullet}{}_2^-$ are both relatively stable radicals that *in vitro* will combine to yield peroxynitrite in a very fast reaction (for review, see Beckman, 1996). A possible biological significance of this reaction was first suggested by Beckman *et al.* in 1990; they pointed out that peroxynitrite may be formed under pathophysiological conditions and that this potent oxidant might contribute to destruction of critical cellular components. Authentic peroxynitrite added as a bolus has been found to nitrate protein-bound tyrosines which in turn may lead to inactivation of enzymes or prevent phosphorylation by tyrosine kinases.

However, it is still not proven, whether peroxynitrite is really formed at inflammatory sites. Equimolar fluxes of NO and $O^{\bullet}{}_2^-$ indeed interact and yield peroxynitrite. However, excess production of either radical inhibits oxidative reactions (Miles *et al.*, 1996). In addition, simultaneous NO plus $O^{\bullet}{}_2^-$ generation, in contrast to addition of authentic peroxynitrite, will not result in marked tyrosine nitration (Pfeiffer and Mayer, 1998), probably due to a decrease of nitration efficiency at low steady-state concentrations of peroxynitrite (Pfeiffer *et al.*, 2000). In addition, continuous NO plus $O^{\bullet}{}_2^-$ generation has been found to surpress strongly tyrosine nitration (Godstein *et al.*, 2000). A prerequisite for significant *in vivo* peroxynitrite

generation is synthesis of both NO and $O^{\bullet}{}_2^-$ at exactly the same location and time as well as in similar rates (see critical comments by Fukuto and Ignarro, 1997). Thus, it is still an unresolved question, whether we find such a situation at inflammatory sites or within cells or organelles. Moreover, additional chemical and enzymatic reactions involving NO or NO oxidation products leading to nitration of tyrosine residues have been identified recently (Fig. 4). However, we can envisage one situation, where peroxynitrite formation is highly likely, *i.e.,* within cells hit by higher NO concentrations. NO has been shown to inhibit cytochrome *c* oxidase reversibly and thus the mitochondrial respiratory chain (see below), and this might lead to increased production of $O^{\bullet}{}_2^-$. Under these conditions NO and $O^{\bullet}{}_2^-$ could combine to yield peroxynitrite within or near mitochondria, which subsequently may lead to irreversible inhibition of the respiratory chain (for review, see Kröncke *et al.*, 1997).

In conclusion, much work has to be done to really understand the chemical reactions occurring *in vivo* between NO, NO_x, O_2, ROI, and other products present during inflammatory processes.

MOLECULAR TARGETS OF NO

Metal–sulfur complexes

With the exception of the soluble guanylate cyclase, which is activated by NO, all enzymes with high-spin ferrous heme intermediates are likely to be inhibited by NO. NO interacts more strongly with ferrous (Fe^{2+}) than with ferric iron (Fe^{3+}), since Fe^{2+} has an additional *d*-electron for back-bonding. In addition, Fe-S and Zn-S clusters in proteins are targets for NO (Fig. 3). Most iron sulfur cluster, *e.g.,* the electron-transferring iron–sulfur centers in the mitochondrial electron transfer chain, are buried deeply within proteins and, therefore, are relatively inaccessible to NO. However, at high NO concentrations, reaction with Fe_4S_4 clusters in proteins inaccessible to solvent were shown to occur (Foster and Cowan, 1999). Aconitase, an enzyme containing Fe-S-clusters with nonredox roles has been implicated in the regulation of the iron metabolism, and this enzyme is sensitive toward NO, an effect that will contribute to altered iron uptake during disease (for review, see Bouton, 1999).

While Fe-S clusters are essential components for many enzyme activities, Zn-S clusters serve as structural elements (zinc fingers) in proteins for specific DNA or RNA binding as well as for protein–protein interactions. NO destroys zinc sulfur clusters via *S*-nitrosylation and subsequent ejection of Zn^{2+}, thereby inhibiting the DNA binding activity of zinc finger dependent transcription factors (Kröncke *et al.*, 1994). In addition, NO mediates Zn^{2+}-release within cells (Berendji *et al.*, 1997).

Protein thiol groups

In biological systems, *S*-nitrosylation of proteins by NO is preferred over *N*- and *C*-nitrosation reactions. A variety of enzymes contain reduced cysteines in their catalytical centers, and *S*-nitrosylation may inhibit SH-dependent enzyme activities (for review, see Kröncke *et al.*, 1997), alter protein structures, lead to oxidation of vicinal thiols, or, after reaction with glutathione, may lead to the formation of mixed disulfides (*S*-glutathiolatione) (Zech *et al.*, 1999). Conversely, SH-dependent redox-regulated proteins may specifically be activated by NO via *S*-nitrosylation, such as the small GTP-binding protein $p21^{ras}$ (Lander *et al.*, 1997) or the bacterial transcription factor OxyR (Hausladen *et al.*, 1996).

DNA

RNOI cause G:C-A:T transitions and mediate DNA strand breaks, both suggested to be the results of *N*-nitrosylation of primary amines in DNA bases ultimately leading to deamination (for review, see Burney *et al.*, 1999). Because DNA damage is a constant hazard in natural environments induced by chemicals, ionizing radiation or UV light, cells have evolved an array of mechanisms for repair. Most forms of DNA alterations are recognized by DNA excision repair pathways catalyzing removal of damaged or modified sites. Thus, strand breaks induced by endonucleases at active repair sites serve to signal the presence of DNA damage, which is then repaired by polymerization and

TABLE 1. LOCALIZATION OF iNOS AND 3-NITROTYROSINE IN HUMAN DISEASES OR DISORDERS

Disease/disorder	*Localization of iNOS*	*Localization of 3-nitrotyrosine*	*Reference*
AIDS dementia complex	Mϕ, Mg, A	+	Boven *et al.*, 1999; Rostasy *et al.*, 1999; Vincent *et al.*, 1999
Alzheimer's disease	N	N, Mg	Good *et al.*, 1998; Smith *et al.*, 1997; Su *et al.*, 1997 and Vodovotz *et al.*, 1996
Amyotrophic lateral sclerosis	−	N, A, vascular cells	Abe *et al.*, 1997; Beal *et al.*, 1997; Wong and Strong, 1998
Anaphylactoid purpura	Neu, E	Neu, E	Banno *et al.*, 1997
Asthma	Ep, Mϕ, Neu, Eo, E, VSMC	Ep, Mϕ, Neu, Eo, E, VSMC	Hamid *et al.*, 1993; Saleh *et al.*, 1997; Kaminsky *et al.*, 1999
Atherosclerosis	Mϕ, foam cells, VSMC	Mϕ, foam cells, E, VSMC	Beckman *et al.*, 1994; Buttery *et al.*, 1996; Wilcox *et al.*, 1997; Luoma *et al.*, 1998; Baker *et al.*, 1999; Cromheeke *et al.*, 1999; Depre *et al.*, 1999
Atopic dermatitis	E, IC	ND	Rowe *et al.*, 1997
Barrett's esophagus	Ep	ND	Wilson *et al.*, 1998
Burns	K, E, F, VSMC, Mϕ	ND	Paulsen *et al.*, 1998
Cancer			
Bile duct	T-Ep	T-Ep	Jaiswal *et al.*, 2000
Bladder	T-Ep, Mϕ, Neu	ND	Klotz *et al.*, 1999; Swana *et al.*, 1999
Brain	TC, vasculature	ND	Cobbs *et al.*, 1995
Breast	T-Ep, E, Mϕ, myoEp, stroma	ND	Thomsen *et al.*, 1995; Duenas-Gonzalez *et al.*, 1997; Reveneau *et al.*, 1999
Colon	T-Ep, MNC, E	MNC, Neu, T-Ep	Ambs *et al.*, 1998; Kolios *et al.*, 1998; Kojima *et al.*, 1999
Esophageal squamous	T-Ep, Mϕ	ND	Wilson *et al.*, 1998; Tanaka *et al.*, 1999
Head and neck squamous	T-Ep	ND	Rosbe *et al.*, 1995
Kaposi's sarcoma	Mϕ	ND	Weninger *et al.*, 1998
Lung	T-Ep, Mϕ, E,	ND	Fujimoto *et al.*, 1997; Ambs *et al.*, 1998; Liu *et al.*, 1998b
Melanoma	melanoma cells	ND	Tschugguel *et al.*, 1999
Ovary	T-Ep, Mϕ	ND	Hamaoka *et al.*, 1999
Pancreas	ductal Ep, acinar cells	Ductal Ep, acinar + islet cells	Vickers *et al.*, 1999
Prostate	T-Ep, Mϕ	ND	Klotz *et al.*, 1998
Stomach	PMNL, MNC, T-Ep	T-Ep, PMNL, MNC	Goto *et al.*, 1999
Uterus	T-Ep	ND	Hamaoka *et al.*, 1999
Celiac disease	Ep, Mϕ	Ep	ter Steege *et al.*, 1997
Cerebral ischemic stroke	N, A, Mg, Neu, E,	Neu	Krupinski *et al.*, 1998; Forster *et al.*, 1999
Chronic heart failure	My, E, VSMC, Mϕ	+	Habib *et al.*, 1996; Haywood *et al.*, 1996; Adams *et al.*, 1997; Satoh *et al.*, 1997; Fukuchi *et al.*, 1998; Levine *et al.*, 1998; Vejlstrup *et al.*, 1998; Hambrecht *et al.*, 1999
Contact dermatitis	+	ND	Ormerod *et al.*, 1997
Crohn's disease	Ep, MNC	Ep, MNC	Singer *et al.*, 1996; Dijkstra *et al.*, 1998
Cutaneous lupus erythematosus	Basal Ep, E, IC	ND	Kuhn *et al.*, 1998
Diverticulitis	Ep, MNC	Ep, MNC	Singer *et al.*, 1996

Glomerulonephritis	Mesangial cells, Ep, Mϕ	ND	Kashem *et al.*, 1996; Furusu *et al.*, 1998
Granulum pyogenicum	E, IC	ND	Shimizu *et al.*, 1998
Heart infarction	Mϕ, My	ND	Wildhirt *et al.*, 1995
Idiopathic pulmonary fibrosis	Mϕ, Neu, alv. + airway Ep	Mϕ, Neu, alv. + airway Ep, E	Saleh *et al.*, 1997
Inclusion-body myositis	Vacuolated muscle fibers, Mϕ	Vacuolated muscle fibers,	Yang *et al.*, 1996
Kikuchi's disease	Histiocytes	ND	Facchetti *et al.*, 1999
Multiple sclerosis	Mϕ, Mg, A	Mϕ, Mg, A	Bagasra *et al.*, 1995; De Groot *et al.*, 1997; Oleszak *et al.*, 1998; Cross *et al.*, 1998
Myelodysplastic syndrome	Bone marrow Mϕ, myeloid cells	ND	Kitagawa *et al.*, 1999
Myopathy	My	ND	Tews and Goebel, 1998
Nasal allergy	Mϕ, Ep, E	ND	Kawamoto *et al.*, 1998
Necrotizing enterocolitis	Ep	Ep, lamina propria	Ford *et al.*, 1997
Obliterative bronchiolitis	PMNL, Mϕ, alv. + airway Ep, E	PMNL, Mϕ, alv. + airway Ep, E	McDermott *et al.*, 1997; Mason *et al.*, 1998
Osteoarthritis	SLC, VSMC, Ch	ND	Grabowski *et al.*, 1997; Melchiorri *et al.*, 1998
Parkinson's disease	Mg in the substantia nigra	N	Hunot *et al.*, 1996; Good *et al.*, 1998
Periapical periodontitis	Ep, E, F, Mϕ, PMNL	ND	Takeichi *et al.*, 1998a; Kabashima *et al.*, 1998
Progressive supranuclear palsy	A	A, N, oligodendrocytes	Komori *et al.*, 1998
Prostheses failure	Mϕ, E, SLC, F, VSMC	Mϕ, SLC, F	Hukkanen *et al.*, 1997; Moilanen *et al.*, 1997; Watkins *et al.*, 1997
Psoriasis	K, E, IC	ND	Bruch-Gerharz *et al.*, 1996; Ormerod *et al.*, 1998
Pulmonary sarcoidosis	Ep, Mϕ, Ly, F	ND	Moodley *et al.*, 1999
Rheumatoid arthritis	SLC, E, Mϕ, F, VSMC, Ch	ND	Sakurai *et al.*, 1995; McInnes *et al.*, 1996; Grabowski *et al.*, 1997; Melchiorri *et al.*, 1998
Sjögren's syndrome	Acinar ductal Ep, MNC	Ductal Ep	Konttinen *et al.*, 1997
Systemic sclerosis	E, F, Mϕ	E	Yamamoto *et al.*, 1998; Cotton *et al.*, 1999
Ulcerative colitis	Ep, Mϕ, Neu, F	Ep, MNC	Godkin *et al.*, 1996; Singer *et al.*, 1996; Ikeda *et al.*, 1997; Kimura *et al.*, 1998; Dijkstra *et al.*, 1998
Toxic megacolon	MC, Mϕ	ND	Mourelle *et al.*, 1995
Transplant coronary artery disease	Mϕ, VSMC	Mϕ, VSMC	Ravalli *et al.*, 1998; Baker *et al.*, 1999
Transplantation/rejection			
Heart	Mϕ, My, VSMC	My	Lewis *et al.*, 1996; Lafond-Walker *et al.*, 1997; Szabolcs *et al.*, 1998
Kidney	Mϕ, VSMC	Tubular Ep	MacMillan-Crow *et al.*, 1996; Romagnani *et al.*, 1999

Abbreviations: +, positive staining; −, not detectable; ND, not determined; A, astrocytes; Ch, chrondrocytes; E, endothelium; Eo, eosinophils; Ep, epithelium; F, fibroblasts; IC, infiltrating cells; K, keratinocytes; Ly, lymphocytes; MC, muscle cells; Mg, microglia; MNC, mononuclear cells; Mϕ, macrophages; My, myocytes; N, neurons; Neu, neutrophils; PMNL, polymorphonuclear leukocytes; SLC, synovial lining cells; TC, tumor cells; T-Ep, tumor epithelial cells; VSMC, vascular smooth muscle cells.

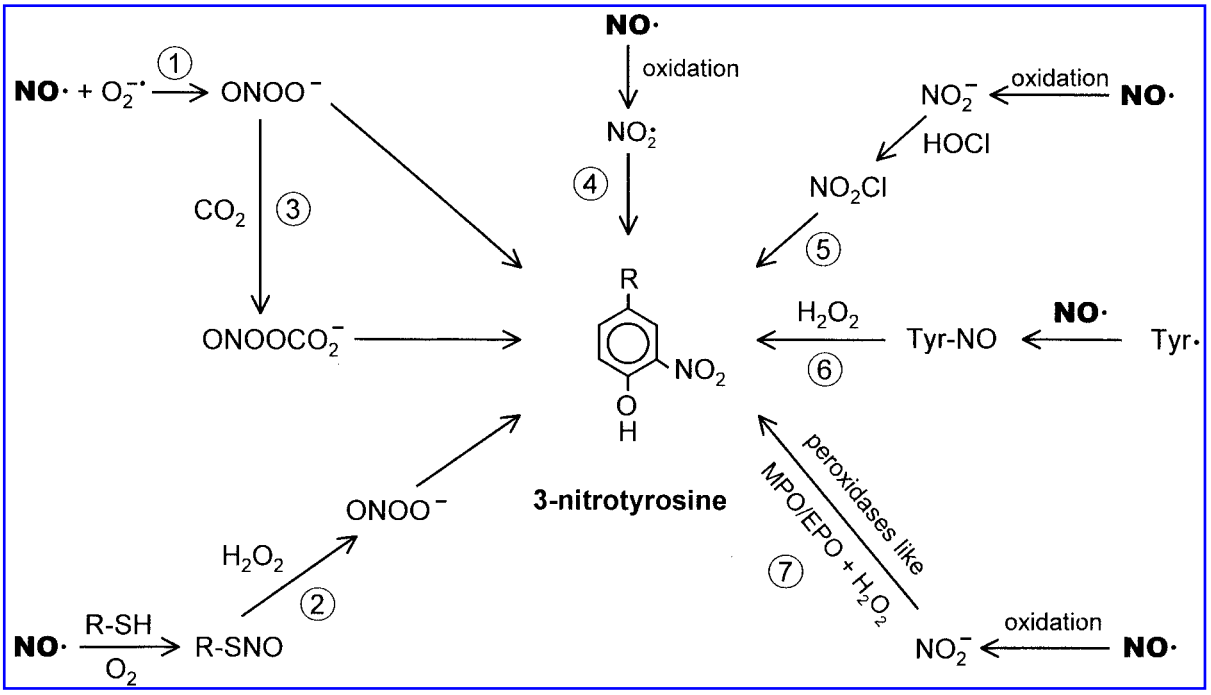

FIG. 4. Reactions yielding nitrotyrosine formation. There are several pathways that may lead to 3-nitrotyrosine formation. Peroxynitrite may be generated via reaction of NO with $O^{\bullet}{}_2^-$ (1) or of *S*-nitrosothiols with excess H_2O_2 (2) (Coupe and Williams, 1999). Besides peroxynitrite (Ischiropoulos *et al.*, 1992) and/or its CO_2-adduct (3) (Uppu *et al.*, 1996), $NO_2^{\bullet}$ (4), and NO_2Cl may also directly nitrate tyrosine residues (Eiserich *et al.*, 1998). Furthermore, NO has been shown to react with tyrosine-radicals which in the presence of oxidants like H_2O_2 or HOCl yields nitrotyrosine also (6) (Eiserich *et al.*, 1995). In addition, peroxidases like the myeloperoxidase (MPO) of neutrophils or the eosinophil oxidase (EPO) in the presence of H_2O_2 and NO_2^- (7) induce nitrotyrosine formation (van der Vliet *et al.*, 1997; Wu *et al.*, 1999). Note, that in all cases NO is a prerequesite for nitrotyrosine formation.

ligation. DNA repair systems recognize base modifications mediated by RNOI, and subsequent repair will lead to transient DNA single-strand breaks. Indirect reactions leading to induction of DNA strand breaks are also feasible, as for instance by intracellular ROI and/or peroxynitrite generation, via *N*-nitrosamine formation and subsequent alkylation reactions, or via activation or inhibition of enzymes necessary for nuclear homeostasis. Poly(ADP-ribose)polymerase (PARP), an abundant nuclear protein activated by DNA nicks has been shown to be activated within nuclei after NO treatment (Radons *et al.*, 1994; Zhang *et al.*, 1994). Following its binding to DNA breaks, PARP automodifies itself as well as histones by adding branched polymer chains of up to 200 ADP-ribose residues. The physiological role of the PARP is not exactly known to date. It may either protect DNA strand breaks during early stages of recombination and repair, or it may transiently block DNA replication, thus inducing a cell-cycle arrest to provide time and/or space for assembly of the DNA repair complex. Whatever the exact role of PARP, activation of this enzyme after NO treatment of cells and subsequent nuclear autopoly(ADP-ribosylation) reactions may lead to severe cellular depletion of NAD^+ and ATP, ultimately leading to cell death (Radons *et al.*, 1994; Zhang *et al.*, 1994; Heller *et al.*, 1995).

Additionally, NO has been found to inhibit ribonucleotide reductase, a rate-limiting enzyme involved in DNA synthesis and repair, and the DNA repair enzyme Fapy-DNA glycosylase (Fpg) (Lepoivre *et al.*, 1991; Wink and Laval, 1994).

IMPACT OF NO ON CELLULAR FUNCTIONS

Intracellular redox state

Treatment of cells with steady-state NO concentrations in the low-micromolar (1–5 μM) range for several hours will exert nitrosative

stress compromising the cellular thiol redox status. The most prevalent cellular nonprotein thiol is reduced glutathione (GSH), which is present in virtually all cells at concentrations ranging from 0.5 to up to 10 m*M* and is thus regarded to be the primary determinant of the cellular redox state. NO has been found to *S*-nitrosylate intracellular GSH (Fig. 5), and most of this *S*-nitrosoglutathione (GSNO) is subsequently converted to oxidized glutathione (GSSG) (for review, see Padgett and Whorton, 1997). In addition, transfer of NO from GSNO to other thiols (transnitrosylation) (Liu *et al.*, 1998c; Tsikas *et al.*, 1999) and *S*-glutathiolation reactions (Ji *et al.*, 1999) have also been described. Depletion of cellular GSH renders cells sensitive to the toxic effects of NO as well as of other compounds. Susceptibility of cells during nitrosative (and oxidative) stress is determined by the GSH concentration, but also by the capacity to recycle intracellular GSH via enzymes of the glutathione redox cycle as well as by the synthesis of new GSH (Berendji *et al.*, 1999a). In addition, the capacity of cells to generate sufficient reductive equivalents like NAD(P)H will decide, whether a cell survives or dies.

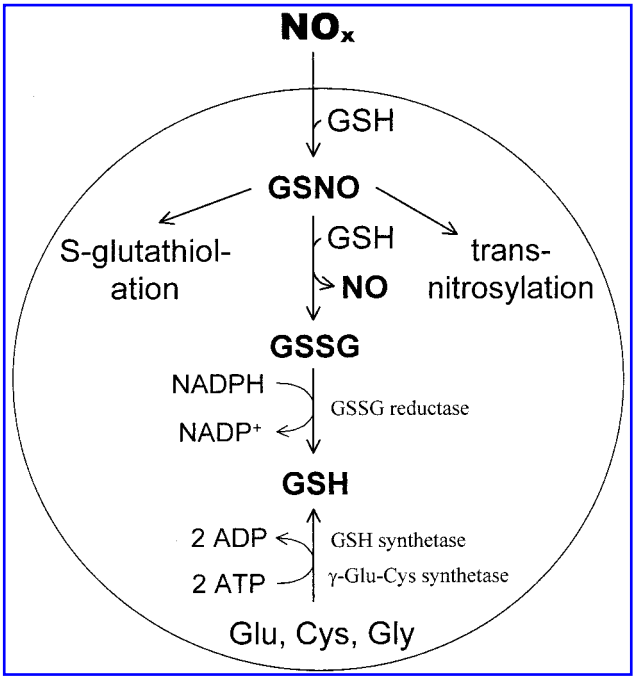

FIG. 5. Effects of NO on the intracellular reduced glutathione pool. NO or, more exactly, NO_x *S*-nitrosylates intracellular reduced glutathione (GSH) to yield *S*-nitroso-glutathione (GSNO), which may react with another GSH molecule to yield oxidized glutathione (GSSG) and NO. Alternative pathways are *S*-transnitrosylation or *S*-glutathiolation reactions of GSNO. All of these pathways lead to GSH depletion. GSH concentrations may be restored either by enzymatic reduction of GSSG, leading to consumption of NADPH, or by enzymatic resynthesis of GSH, leading to ATP consumption. Thus, besides the initial cellular GSH concentration, the capacity to provide reducing and energy equivalents for restoring GSH determines the cellular thiol redox state during nitrosative stress.

Cellular respiration

NO causes a reversible and relatively specific inhibition of mitochondrial cytochrome *c* oxidase via binding to its heme moiety. This may lead to enhanced leakage of electrons from the respiratory chain, yielding increased $O^{\bullet}{}_2^-$ production that may react with NO to form peroxynitrite (Fig. 2). Long-term exposure to NO irreversibly inhibits the complexes I, II, the ATPase and possibly complex III, but not complex IV, probably as a result of peroxynitrite formation. Mitochondrial aconitase is also inhibited under these conditions, resulting in an inhibition of both the citric acid cycle activity and respiration. Peroxynitrite nitrates as well as oxidizes and thereby inhibits mitochondrial MnSOD (MacMillan-Crow *et al.*, 1998), thus increasing the half-life of $O^{\bullet}{}_2^-$ and leading to even enhanced peroxynitrite formation. Peroxynitrite causes oxidation and cross-linking of proteins, inhibition of most of the mitochondrial complexes, nitration of tyrosine residues, oxidation of non-protein thiols and of membrane-lipids, and disruption of membranes, thus representing a potent toxic molecule (for review, see Brown, 1999).

In conclusion, many cytotoxic effects of NO are likely consequences of NO (and/or $ONOO^-$?) interfering with energy metabolism, especially the mitochondrial respiratory chain as well as activation of energy-consuming DNA-repair pathways.

Signal transduction

The picture of how NO influences signal transduction is complex and far from being complete (see review by Beck *et al.*, 1999). NO is able to activate tyrosine kinases of the src protein family like $p56^{lck}$ (Lander *et al.*, 1993a) or c- and v-Src (Akhand *et al.*, 1999). In addition, all three parallel MAPK cascades, *i.e.*, the stress-activated protein kinase/c-Jun N-termi-

nal kinase (SAPK/JNK) cascade, the stress-activated p38 MAPK cascade, and the ERK cascade (Lander *et al.*, 1996) may be activated by NO via modulation of upstream factors like G-proteins (Lander *et al.*, 1993b) or of the small GTP-binding protein $p21^{ras}$ (Lander *et al.*, 1995). The predominant mechanism appears to be *S*-nitrosylation. Furthermore, phosphotyrosine protein phosphatase activity is inhibited by NO via *S*-nitrosylation and subsequent disulfide formation (Caselli *et al.*, 1994), which may result in a prolonged half-life of phosphorylated proteins. In contrast to inducing or prolonging signal transduction, however, NO can inhibit signal flow also. NO has been shown to block the activity of rekombinant JNK2 (So *et al.*, 1998), protein kinase C (Gopalakrishna *et al.*, 1993), and autokinase activities of JAK 2 and 3, respectively (Duhé *et al.*, 1998). It appears, that NO is able to modulate the intracellular phosphorylation-dephosphorylation equilibrium and thus signal transduction pathways, either activating or inhibiting them (Fig. 6), depending on its concentration, on the cell type involved as well as on the cellular redox state, on the stimulus and/or on the signal transduction pathway involved.

Transcription

DNA binding of the transcription factor HSF1 is induced by low concentrations of NO (Xu *et al.*, 1997). In addition, low NO concentrations have been shown to initiate DNA binding of the transcription factors NF-κB and AP-1, respectively, which may be mediated indirectly through activation of MAP kinase pathways (Lander *et al.*, 1993b; von Knethen *et al.*, 1999).

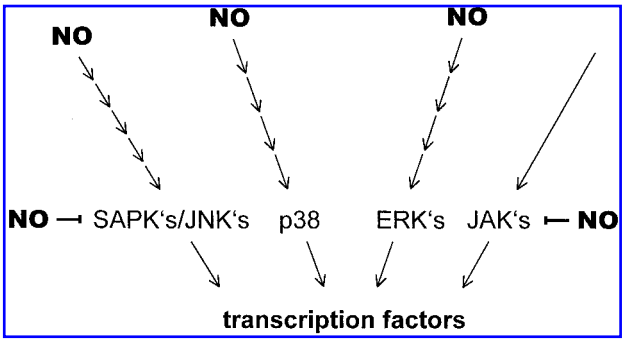

FIG. 6. Modulation of signaling cascades by NO. NO activates mitogen-activated protein kinase (MAPK) pathways (SAPK/JNK, p38, ERK), *e.g.*, via activation of G proteins or of small GTP-binding proteins, but conversely can also directly inhibit individual members of the different protein-tyrosine kinase cascades via *S*-nitrosylation. NO is thus able to modulate intracellular phosphorylation-dephosphorylation balances and signal transduction pathways. SAPK/JNK, Stress-activated protein kinase/c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; JAK, Janus kinase.

However, NO or nitrosative stress may also directly modulate the activities of transcription factors. Members of the NF-κB family are important transcription factors regulating a variety of genes involved in immune and inflammatory processes. IκB proteins functionally retain NF-κB in the cytoplasm and render it inactive. After phosphorylation, IκB proteins are ubiquitinylated and rapidly degraded by proteasomes, thus allowing free NF-κB to translocate into the nucleus, where it can transactivate gene enhancer or promoter elements. In TNF-α-treated endothelial cells, NO has been found to inhibit NF-κB activity via induction of IκB synthesis and its nuclear translocation (Spiecker *et al.*, 1997). In IL-1β-treated vascular smooth muscle cells, NO-mediated inhibition of NF-κB activity correlated with inhibition of IκB phosphorylation and degradation (Katsuyama *et al.*, 1998). In addition, NO has been found to inhibit the DNA-binding activity of NF-κB via *S*-nitrosylation of a cysteine within the DNA binding domain (Matthews *et al.*, 1996; Moormann *et al.*, 1996). Thus, overall effects of NO on NF-κB transactivating activity probably depend on the balance between potential effects on IκB expression/stability and NF-κB activation/nuclear translocation and/or potential effects on the redox state of the cysteine residue involved in DNA binding.

The DNA-binding activity of other redox-sensitive transcription factors containing a cysteine residue within or near its DNA-binding domain, such as AP-1 (Nikitovic *et al.*, 1998) or c-Myb (Brendeford *et al.*, 1998), have also been found to be inhibited by NO via *S*-nitrosylation (Fig. 7). Recently, we described that NO inhibits the DNA binding activity of transcription factors containing zinc fingers, as found in the members of the nuclear receptor superfamily (Kröncke & Carlberg, 2000), Sp1, and EGR-1 (Berendji *et al.*, 1999b). The molecular mechanism involved again is *S*-nitrosylation of one or of several of the cysteines involved in Zn^{2+} complexation, thereby leading to Zn^{2+} ejection and subsequent conformational

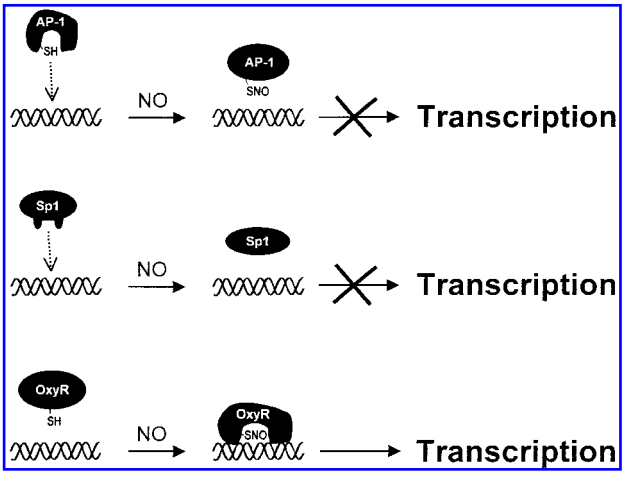

FIG. 7. Direct effects of NO on gene transactivators. NO can act on gene transcription via *S*-nitrosylation of redox sensitive transcription factors like AP-1 or zinc finger transcription factors like Sp1, thus inhibiting transcription. Alternatively, *S*-nitrosylation of transcription factors like the bacterial OxyR leads to induction of transcription. NO may thus induce or inhibit transcription, depending on the individual transcription factors involved.

changes of the DNA-binding domain. If a redox-sensitive transcription factor functions as a transactivator, inhibition of its DNA binding activity leads to inhibition of transcription (Fig. 7), as has been shown for Sp1 and the IL-2 gene (Berendji *et al.*, 1999b). Conversely, if a redox-sensitive transcription factor functions as a gene repressor, inhibition of its DNA-binding activity by NO may upregulate the respective promoter activity, as demonstrated recently for Sp1 and the tumor nerosis factor-α (TNF-α) promoter (Wang *et al.*, 1999).

However, NO will also activate transcription factors directly as shown for the bacterial transcription factor OxyR. *S*-Nitrosylation of a cysteine essential for the DNA-binding activity leads to activation of OxyR (Fig. 7), thus inducing expression of antioxidative enzymes (Hausladen *et al.*, 1996). OxyR may thus be regarded as an SOS signal in bacteria to protect against nitrosative (and oxidative) stress.

In conclusion, NO may induce transcription indirectly via activation of signaling pathways (see chapter above), but NO may also inhibit or activate transcription via direct chemical reactions with transcription factors. The net effect of NO on transcription will thus depend on the NO concentration, on the transcription factor(s) involved, and on the function (activator or repressor) of the NO-sensitive transcription factors.

DESTRUCTIVE VERSUS PROTECTIVE ROLES OF NO IN INFLAMMATION

As pointed out in the previous chapters, NO at high concentrations may be cytotoxic. At present, two types of cell death are known, necrosis and apoptosis. Necrosis is a form of cell death caused by disruption of the cell membrane with concomittant cell swelling and lysis. In contrast, apoptosis (or programmed cell death) is executed by an innate cellular suicide program leading to the disintegration of cells in an orderly fashion via a cascade of specific biochemical and structural events and avoiding pro-inflammatory spill of intracellular components by maintaining the barrier function of the cell membrane. Apoptosis finally leads to orderly packaged cell fragments phagocytosed by neighboring cells or professional phagocytes. Thus, pathway and cellular morphology of apoptosis are distinct from necrotic cell death.

NO may induce cellular necrosis or apoptosis

Higher concentrations of NO induce cell death in a variety of susceptible mammalian cells (for review, see Kröncke *et al.*, 1997). The mode of the cell death, however, may vary. While islet cells after exposure to NO die by necrosis (Kröncke *et al.*, 1993), NO-exposed lymphocytes die via apoptosis (Fehsel *et al.*, 1995). The mode of cell death will have serious consequences, as necrosis *in vivo* correlates with overt inflammatory and activated immune reactions, whereas apoptosis usually does not. Apoptosis may be regarded as the opposite of cell proliferation and thus as a secure mechanism to remove unwanted cells from the organism. However, both massive apoptosis as well as the failure to undergo apoptosis may result in local inflammation.

NO may protect from necrosis or apoptosis

Besides inducing necrosis or apoptosis, NO is now known to also protect cells from necrosis or apoptosis mediated by various insults (Table 2) (for reviews, see Brüne *et al.*, 1998;

Table 2. NO Has Been Shown to Prevent Cell Death Induced by the Compounds, Stimuli, or Treatments Listed

NO protects from cell death induced by:
H_2O_2
Singlet oxygen
Alkyl peroxides
Fe^{2+}
LPS
TNF-α
Fas/Apo-1
Growth factor withdrawal
UV-A irradiation

Kim *et al.*, 1999; Liu and Stamler, 1999). The decision for a cell to undergo apoptosis is the result of a shift in the balance between numerous anti-apoptotic and pro-apoptotic forces within a cell, and NO contributes to this balance. In endothelial cells and NK cells, ecNOS activity is able to inhibit TNF-induced apoptosis (Dimmeler *et al.*, 1997; Furuke *et al.*, 1999), while iNOS activity effectively suppresses LPS- and UV-A-induced apoptosis (Tzeng *et al.*, 1997; Suschek *et al.*, 1999). Multiple mechanisms for the inhibition of apoptosis by NO may exist in a single cell type. For instance, NO blocks apoptosis in hepatocytes via cGMP-mediated interruption of apoptotic signaling and in addition via direct inhibition of caspase activities (Kim *et al.*, 1997).

Studies on the antiapoptotic actions by NO have identified a series of interactions with the ever-growing list of molecular components of the apoptotic machinery. Although several endogenous inhibitors of caspase activation and activity have been described, none has been shown to be more prevalent than NO. One way to start off the apoptotic cascade represents relocation of cytochrome *c* from mitochondria into the cytoplasm, which will then activate proteolytic enzymes of the caspase family. Thus, cytochrome *c* leakage from mitochondria is currently regarded as a key event for the onset of apoptosis and may be mediated by proteins of the Bcl-2 family forming a transition pore, which is open with excess Bax (pro-apoptotic), or closed with excess Bcl-2 (antiapoptotic). Both proteins are located in the outer mitochondrial membrane and in other organelle membranes. This complex life–death rheostat is sensitive to NO by indirect or direct interactions with the apoptotic machinery. Indirect effects of NO may be envisaged via induction of expression of proteins that protect from cell death, among them heat shock proteins, heme oxygenases, stress-activated protein kinases, C-reactive protein, *etc.* (see Brüne *et al.*, 1998; Kim *et al.*, 1999). Direct effects of NO (Table 3) involve suppression of apoptotic signal transduction by inhibition of cytochrome *c* release from mitochondria (Kim *et al.*, 1998), inhibition of proteolytic processing and activation of caspases (Li *et al.*, 1999), inhibition of caspase activity via *S*-nitrosylation (Dimmeler *et al.*, 1997;

Table 3. NO-Mediated Mechanisms Active in Preventing Apoptosis

Anti-apoptotic mechanisms of NO:
Scavenging of peroxyl radicals
Inhibition of lipid peroxidation
Induction of protective proteins
Inhibition of mitochondrial cytochrome c release
Inhibition of proteolytic caspase activation
Inhibition of caspase activity
Inhibition of ceramide accumulation
Increase of Bcl-2 protein expression
Inhibition of proteolytic Bcl-2 cleavage

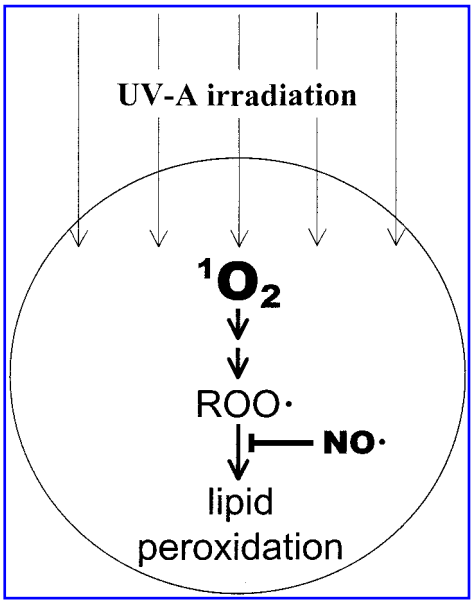

FIG. 8. NO can protect cells from UV-A-induced cell death via inhibition of lipid peroxidation. Studies using irradiation with UV-A light have shown that apoptosis or necrosis occur as a result of singlet oxygen ($^1O_2$) generation, which reacts with unsaturated fatty acids forming peroxyl radicals. These, in turn, induce lipid peroxidation via radical chain reactions. NO can effectively scavenge peroxyl radicals thus protecting from cell death.

Li *et al.*, 1997), and positive modulation of the expression and the activity of proteins from the Bcl-2 family (Kim *et al.*, 1998; Suschek *et al.*, 1999).

In the case of UV-A-induced apoptosis, high-output NO synthesis as well as exogenously applied NO fully protects endothelial cells from apoptosis, and this protection strongly correlates with NO-mediated increases in Bcl-2 protein expression together with inhibition of UV-A-induced increased Bax expression (Suschek *et al.*, 1999). In addition, NO scavenges lipid peroxyl radicals generated after formation of UV-A-induced singlet oxygen (Fig. 8), thus protecting cells from necrosis or apoptosis (Suschek, Kröncke, and Kolb-Bachofen, unpublished observations). The molecular mechanism of this chain-breaking antioxidative activity of NO is shown in Fig. 9.

In conclusion, whether apoptosis occurs in a given cell depends on the balance of pro- and antiapoptotic factors, and this balance will be tipped by NO in either direction, depending on the concentrations of NO, the cell type involved, and on the apoptotic impact upon the cell.

SYNOPSIS

In summary, high-output NO synthesis as produced by iNOS activity shows cytotoxic as well as protective effects. It influences cellular gene expression via modulation of signal transduction pathways and transcription factor activities. NO produced by macrophages but also by epithelial cells will potentially serve to limit bacterial invasion as well as to prevent overshooting local immune reactions, but can also contribute to local tissue damage. Thus, depending on the timing and the degree of activation, iNOS activity will display dual effects serving as a positive modulator of cell responses to inflammatory stimuli, but also amplifies and augments tissue destruction. These Janus-faced effects of iNOS activity result in a complex scenery showing us that our current knowledge is insufficient to predict whether a disease therapy will benefit from using a selective iNOS inhibitor or rather will profit from exogenously added NO due to its protective and antioxidative efficiency. Whereas the discovery of NO as a signaling molecule has already been rewarded with a Nobel Prize, there is still room from a distinguished award, when the precise impact of iNOS activity in specific human diseases will be completely understood and thus taken advantage of cure or prevention of human diseases.

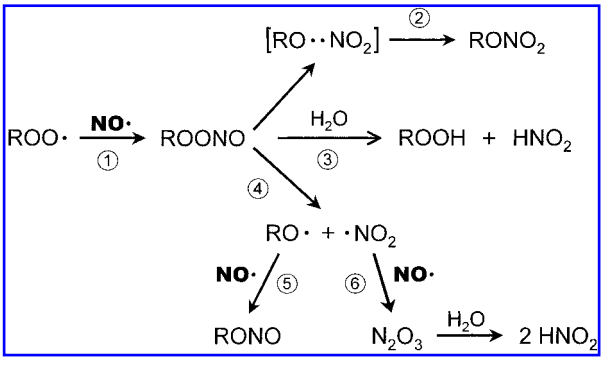

FIG. 9. NO terminates lipid peroxidation reactions. NO reacts with the peroxyl radical $ROO^{\cdot}$ (1), yielding ROONO, which may either isomerize (2) to $RONO_2$, may hydrolyze (3) yielding ROOH and nitrite, or may decompose (4) yielding $RO^{\bullet}$ and $NO_2^{\bullet}$, both of which may subsequently react with NO yielding RONO (5) and nitrite (6), respectively (according to O'Donnell *et al.*, 1997).

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ABBREVIATIONS

ERK, Extracellular signal-regulated kinases; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GSSG, oxidized glutathione; IRF, interferon regulatory factor; JAK, Janus kinases; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NOS, NO synthase; NO_x, nitrogen oxides; PARP, poly(ADP-ribose)-polymerase; RNOI, reactive nitrogen oxide in-

termediates; SAPK, stress-activated protein kinase; Stat, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α.

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